Metabolomics of marbling and residual feed intake in crossbred Wagyu steers

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Declaration of authorship

This thesis has been written in publication style. Chapters 2 to 5 are hence stand-alone manuscripts, each with its own Abstract, Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, and References. Chapter 2 and 3 have been published in Scientific Reports, with the published versions included in this thesis. Chapters 4 and 5 have been submitted to a peer reviewed journal and under review and presented as per journal guidelines. SK Connolly is the first author on all chapters and publications. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged, either in the author list at the beginning of each chapter and publication or in the acknowledgments section. The work presented in this thesis is, to the best of my knowledge and belief, original, except as mentioned in the text. I declare that I have not submitted this material, either in full or in part, for another degree at this or any other university or institution of tertiary education.

Samantha Connolly (27 May 2022)

Research work and Authorship

The inclusion of co-authors in all Chapters (Chapter 2-5) reflects the collaboration between researchers and acknowledges input into team-based research. Feedlot crew and other assistance is recognised in the acknowledgment section of the respective Chapters.

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As the primary supervisor for the candidature, I can confirm that the authorship attribution statement above is correct.

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Publications

Peer-reviewed Publications in scientific journals

Connolly, S., Dona, A., Wilkinson-White, L., Hamblin, D., D'Occhio, M. J & González, L. A. Relationship of the blood metabolome to subsequent carcase traits at slaughter in feedlot Wagyu crossbred steers. *Scientific Reports* **9**, 15139, doi:10.1038/s41598-019-51655-2 (2019).

Connolly, S., Dona, A., Hamblin, D., D'Occhio, M. J. & González, L. A. Changes in the blood metabolome of Wagyu crossbred steers with time in the feedlot and relationships with marbling. *Scientific Reports* **10**, 18987, doi:10.1038/s41598-020-76101-6 (2020).

Connolly, S., Dona, A., Hamblin, D., D'Occhio, M. J. & González, L. A (2021). Predicting intramuscular fat (marbling) in longissimus muscle of crossbred Wagyu cattle with animal and feedlot performance data, and the plasma metabolome using machine learning. (Submitted).

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Conference Publications

2020 – Machine learning techniques to predict marbling of Wagyu crossbred steers from on farm data and metabolomics, Australian Association of Animal Sciences, online.

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- Teys Australia Scholarship to attend the Australian Society of Animal Production conference in Wagga Wagga, Australia (2018)

- Australian Wagyu Association Fellowship (2017)

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Abbreviations

Average daily gain (ADG)

Beef Marble Score (BMS)

Best Linear Unbiased Prediction (BLUP)

Body Weight (BW)

Classification and regression trees (CART)

Days on Feed (DOF)

Electro Ionisation (EI)

Electrospray ionization (ESI)

Estimated Breeding Values (EBV)

Eye muscle area (EMA)

Gas chromatography (GCMS)

Gas chromatography-mass spectrometry (GC-MS)

Genome wide association studies (GWAS)

Hot Standard Carcase Weight (HSCW)

Intramuscular fat (IMF)

Liquid chromatography (LCMS)

Marker assisted selection (MAS)

Mass spectrometry (MS)

Mass to charge ratio (m/z)

Meat standards Australia (MSA)

Messenger ribonucleic acid (mRNA)

Monounsaturated fatty acids (MUFA)

Nuclear overhauser effect spectroscopy (NOESY)

One hydrogen nuclear magnetic resonance (^{1}H-NMR)

Orthogonal partial least squared-discriminant analysis (OPLS-DA)

Principal Component Analysis (PCA)

Proton nuclear magnetic resonance (^{1}H-NMR)

Residual feed intake (RFI)

Quantitative trait loci (QTLs)

Rib eye area (REA)

Single nucleotide polymorphism (SNP)

Time of flight (TOF)

Triacyl glyceride (TG)

Tricarboxylic acid cycle (TCA)

Ultra-pressure liquid chromatography mass spectrometry (UPLC/MS)

Volatile fatty acids (VFA)

Summary

Wagyu beef cattle are recognised for their intrinsic ability to produce high-quality meat product due to high intramuscular fat. Wagyu beef is targeted to premium markets and the amount of intramuscular fat largely determines the value of the carcase. Achieving high intramuscular fat requires the animals to spend a relatively long period in a feedlot (350-600 days).Therefore, animals that do not produce a highly marbled carcase may have a cost of production above the value of the carcase. The ability to identify animals that will produce a superior product early in the feedlot process would allow Wagyu production to become more efficient. Another important aspect of the production system is the amount of feed consumed and feed efficiency of individual animals. The ability to identify animals that are efficient converters of nutrients into high quality beef would also improve the efficiency of the system. Early identification of Wagyu steers that will produce a high value carcase would allow their selection for finishing in a feedlot. The metabolic mechanisms that allow Wagyu cattle to achieve carcases with high marbling and efficiently are poorly understood. A metabolomics approach could help understand the biology of muscle and fat deposition and lead to the discovery of biomarkers to identify individuals that will produce high value carcases. For the reasons above, this thesis explored the relationships between plasma metabolite profiles and carcase attributes (mainly marbling) and residual feed intake (RFI) in Wagyu crossbred steers. The term blood metabolome has been used in the first two chapters of the thesis however it is commonly used interchangeably with the term plasma metabolome.

The objective of the present thesis was to understand the relationships between plasma metabolites and important carcase traits to potentially enable the early selection of animals that will produce a superior product in an efficient manner. The thesis consists of 4 studies using data sourced from 3 experimental groups of Wagyu crossbred steers at a commercial feedlot in Queensland. Chapter 2 investigated the relationship between the plasma metabolome measured at 65, 119 or 163 days on feed (DOF) and carcase traits at slaughter. Chapter 3 examined changes in the plasma metabolome from 196 to 432 DOF. Chapter 4 developed prediction models of marbling using animal farm data and plasma metabolomics with machine learning. Chapter 5 explored the effect of adjusting feed efficiency for carcase fat on the relationship with the plasma metabolome in Wagyu crossbred steers.

The aim of Chapter 2 was to (1) examine the relationships between the plasma metabolome and carcase traits with a focus on marbling, and (2) determine the effect of the length of time

cattle were in a feedlot on the plasma metabolome measured relatively early in the feedlot (65, 119 or 163 DOF). Blood samples were obtained from 181 Wagyu crossbred steers between 300 and 400 days before slaughter. The samples were analysed using ^{1}H-NMR spectroscopy and 35 metabolites were identified. The results showed 7 metabolites positively correlated with marbling (3-hydroxybutyrate, propionate, acetate, creatine, histidine, valine, and isoleucine; $P \leq 0.05$). Carcase weight and growth rate were negatively associated with 3-hydroxybutyrate and growth rate was negatively associated with creatine ($P \leq 0.05$) and positively associated with aspartate ($P \leq 0.05$). Glucose, anserine and arginine showed a significant interaction between marbling and DOF ($P \leq 0.05$). Sire had the greatest influence on the relative concentrations of metabolites and carcase and production traits. These findings suggested the plasma metabolome has the potential to help in the understanding of fat and muscle metabolism in Wagyu steers. The plasma metabolome may also help in the identification and selection of Wagyu steers that will produce a high value carcase.

The objectives of Chapter 3 were to (1) compare the metabolome at two distant time points (196 and 432 DOF) and (2) determine the relationship between the metabolome and marbling at those two sampling points in Wagyu crossbred steers (n = 167). There was a positive relationship between the relative concentration at 196 and 432 DOF for 35 of the 38 metabolites. From 196 to 432 DOF, there was an increase in the relative concentrations of 21 metabolites involved in muscle, energy and glucose metabolism whereas 13 metabolites involved in lipid metabolism decreased ($P < 0.05$). There were 14 metabolites that had a significant relationship with marbling. Of these, glucose, propionate, 3-hydroxybutyrate and lipids are involved in energy and fat metabolism. The metabolites 3-hydroxybutyrate and acetate were positively associated with marbling at 432 DOF but not at 196 DOF. The findings in Chapter 3 indicated that the plasma metabolome of Wagyu crossbred steers can change with time in a feedlot. Results confirmed that the metabolome has the potential to be applied to prediction of marbling in Wagyu cattle. However, the relationship between marbling and the plasma metabolome appears to be affected by sampling time, presumably because of the developmental stage and maturity of the animals.

Chapter 4 combined metabolomics and farm-collected data with machine learning to predict which Wagyu crossbred steers will produce a high value carcase. This involved the use of Naïve Bayes, classification and decision trees, and random forest predictive modelling. Five datasets were used which included routinely recorded animal farm data such as sire, Wagyu percentage, weaning weight and feedlot body weight, together with metabolomics data. The

prediction models that used farm or feedlot data produced accuracies of 73% and 63% to identify animals with high marbling, respectively. The datasets that included both sampling time points with either identified metabolites or all metabolic features (peaks) yielded accuracies of up to 67.4%. The model that included animal farm data, feedlot weight data, and two metabolomic sampling points produced accuracy of 69.6%. These findings demonstrated the potential of machine learning to identify high or low marbling animals. The greatest accuracy was achieved with information on sire, Wagyu percentage and weaning weight. The combination of animal farm data, feedlot data, and metabolomic data in machine learning has shown potential to improve the efficiency of Wagyu production.

Animals with high feed efficiency tend to have leaner carcases with reduced marbling. The objective in Chapter 5 was to evaluate the relationship between the plasma metabolome and residual feed intake (RFI) corrected for carcase fat as a measure of feed efficiency. Blood samples were obtained from 140 crossbred Wagyu steers early (78 DOF) and late (313 DOF) in a feedlot and ^{1}H-NMR spectroscopy identified 36 metabolites. Alternative measurements of RFI were calculated to account for important carcase traits such as marbling and subcutaneous fat. The metabolites methionine, phenylalanine, serine, and histidine had a negative relationship ($P < 0.05$) with all measures of RFI. Metabolites involved in lipid metabolism such as choline, glycoprotein acetyls and lipids all had a positive correlation with RFI ($P < 0.05$) at 313 DOF. Alternative feed efficiency measurements, such as residual gain and gain to feed, showed more significant relationships between metabolites than the adjusted RFI traits. It was concluded that the relationship between the relative abundance of plasma metabolites and RFI was not influenced significantly after adjusting for carcase fat. Chapter 5 showed earlier in the feedlot (78 DOF), feed efficiency was more correlated with metabolites involved in protein than lipid metabolism. However, the opposite was observed later in the feedlot process when steers were more mature the metabolites that were related to lipid metabolism were more correlated. The findings in Chapter 5 suggested that a metabolomics approach could help to understand the biology of feed efficiency and marbling in Wagyu steers. The approach might also assist in the identification and selection of animals that efficiently convert feed resources to tissue deposition including marbling.

The information contained in this thesis shows important relationships exist between the plasma metabolome and carcase traits and feed efficiency in Wagyu crossbred steers. This information has potential to be used to identify and select Wagyu steers that will produce a high value carcase with long-term feeding in a feedlot. The adjustment of RFI for carcase fat

traits has also shown potential in the selection of Wagyu steers with high feed efficiency and which still produce a marbled carcase. This is highly important when addressing the social, economic, and environmental imperatives of sustainable Wagyu beef production.

Chapter 1: Literature review

1.1 Introduction

The production of Wagyu cattle in Australia is becoming more common and the input requirements for this system are extremely large due to the amount of time spent on feed and the amount of nutrients the animals consume. The demand for high quality beef is increasing and the consumer is beginning to require a higher quality product, which is what Wagyu beef produces. The intramuscular fat deposition within the muscle is one of the key traits in regard to meat quality. The increased intramuscular fat takes longer to be deposited into the muscle, which requires larger inputs; some of the animals that are in the Wagyu production system do not meet the quality requirements to ensure the production system is economically viable. There are many new technologies (genomics, metabolomics, lipidomics, proteomics) developed and data being reordered which is moving toward the prediction of marbling early in the animal's life; however, there is a large research gap in the scientific literature as to the relationships between these technologies. The ability to utilise one of the technologies to improve the current selection methods would be incredibly beneficial to Wagyu production in Australia.

The Australian beef industry is vast and encompasses a significant part of the agricultural production in terms of farm-gate production, land use, export, and total profit [1]. There are many beef cattle production methods in Australia such as grass-fed and grain-fed (feedlot) which will be discussed further throughout the review. There are also multiple markets available to producers, and each has specific criteria for the cattle that are supplied to them. The feedlot industry contributes to approximately half of the annual cattle slaughter and can be separated into the supermarket (60-80 days on feed; DOF), short-fed (90-120 DOF), mid-fed (120-150 DOF) or long fed (over 200 DOF) [2]. Wagyu feedlot cattle most often are fed for 350 to 650 days to achieve high marbling and, therefore, the cost of production is high [1]. The value of the animals is influenced by many factors including carcase weight, marbling, subcutaneous fat thickness, and fat cover whilst complying with other factors such as sex, muscling and frame size [3].

The increased demand for animal protein has required the development of new technologies and methods to identify specific breed attributes and how to manage beef cattle efficiently. New technologies including genomics and sensor technologies are widely researched and

adopted in the beef industry [2]. There are other new technologies and approaches being investigated for their potential to improve cattle breeding and management such as metabolomics [4,5]. In addition, metabolomics can improve the understanding of the interaction between metabolites and body tissues, which reflect on the phenotype including growth rate, feed efficiency, and marbling in beef cattle [4,6]. Metabolomics is at technique that encompasses a variety of methods and technologies to examine the metabolites present in a range of biological samples including blood, urine and extracts from meat and faeces. Therefore, metabolomic data could allow selection criteria to be placed on desired traits as currently carcase traits such as marbling are not measured until the animal is slaughtered, and it is difficult to select animals early in the feedlot process. Identification using metabolomics could enable selection of superior animals earlier [7]. The metabolome refers to many small molecules that are present in a biological sample such as sugar phosphates, amino acids, nucleotides, organic acids, small hormones and lipids [8]. Metabolomics is a continually developing field and has been applied to many fields from biomedical research through to product quality and traceability services [8]. The application of metabolomics to livestock production is becoming more evident as the technologies that are used continue to evolve [4,6].

Metabolomics and genomics could be used in a systems biology approach to identify animals that will produce a more desirable phenotype, which may allow beef production systems to become more efficient. The Metabolomics Society (https://metabolomicssociety.org) explains that each individual component of a biological system influences the phenotype of the animal as summarized in Figure 1.1. Thus, genes encode for the production of proteins, which then drive the production and utilization of metabolites, so the final metabolome is a reflection of the interaction between the genome and external factors such as the environment and microbiome. Therefore, examining the effect of metabolites on the overall system is an important aspect. For example, it has been theorized that a single base change in the genome can result in a tenfold increase in the concentration of a small molecule, thus resulting in a phenotypic change in the animal [9].

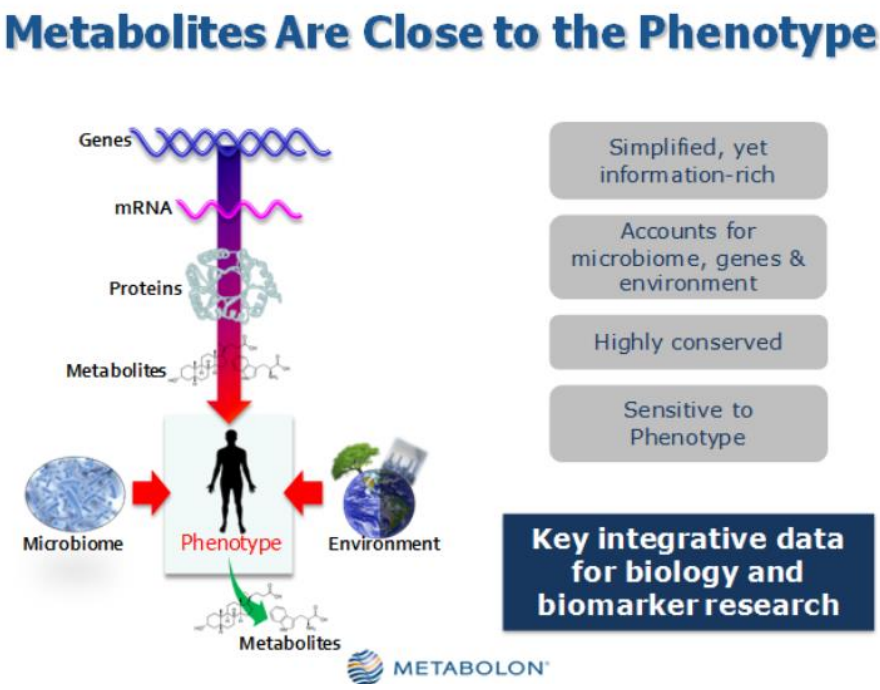


Figure 1.1: Illustration of the interactions that influence phenotype, which then translates to the metabolites (Metabolomics Society, 2013)

The present literature review provides an overview of the beef cattle industry, the potential role and applications of metabolomics, and machine learning in beef cattle. The review also describes the analytical techniques used in metabolomics and applications to multiple fields such as human health, food, toxicology, and beef cattle. The final section of the review includes information on metabolomics of marbling, feed efficiency and the potential of machine learning in beef cattle prediction models.

1.2 The Wagyu Cattle industry

Wagyu beef cattle are native to Japan and were imported into Australia for their intramuscular fat (IMF) content (marbling) and eating quality [10,11]. The assessment of carcase quality is conducted using a method referred to as "chiller assessment'. The chiller assessment describes the meat characteristics in terms of meat colour, fat colour, marbling, eye muscle area (EMA) and rib fat thickness [12]. A trained assessor grades individual carcases for these and other specific targeted markets. Aus-meat™ [13] and MSA [14] are the prevailing grading systems in Australia. The latter predicts eating quality assessed by consumer tests, which is then predicted from carcase grading traits and other factors such as dentition, use of hormonal growth

promoters, and marketing pathway (direct consignment from farm or via auction markets). The IMF is measured by chemical analysis [15] or by marbling scores measured subjectively using scoring cards (reference images) with scores ranging from 0 to 9+ in Aus-Meat and 100 to 1100 in the MSA grading systems. Wagyu cattle can often achieve marbling above the higher threshold of these grading systems and the Wagyu industry has been using other objective measurements such as hyperspectral cameras to measure the proportion of the EMA covered by fat flecks [16].

The marketing of Wagyu beef is based on IMF content (marbling) [10,11]. Currently, there is limited knowledge on the biology and metabolic processes that allow Wagyu animals to achieve high marbling. Nguyen, et al. [17] highlighted multiple factors that influence the ability of the animal to deposit IMF within the muscle, these factors include genetic, sexual, nutritional and management factors. Diet and animal metabolism is one of the most important factors determining IMF. Therefore, the examination of the metabolic profile of animals may allow an enhanced understanding of IMF deposition. The metabolome of cattle may provide additional insight into the biology of marbling. This review examines current literature on biomarkers that are indicative of marbling and could potentially be used to select Wagyu cattle for marbling.

1.2.1 Global Beef Production and Markets

Beef consumption varies across the world and is dependent on multiple factors including economic growth and consumer purchasing power, population growth, dietary preferences and cultural and religion background of consumers, competition from other proteins, trade policies and market access and the resilience of supply chains. The global per capita beef consumption has decreased since 2007, and is expected to further decline by 5% by 2030 but the overall demand for beef continues to increase [18]. However, the demand for high value meat cuts is predicted to increase due to population growth and changes in consumer preferences. Asia is the only region where the consumption of beef is projected to increase [18]. The global beef price has been increasing at a greater rate than both chicken and pork, relative index beef prices have more than doubled while poultry and pork have increased at a rate of 51% and 19 %, respectively. These trends suggest that for beef production to grow it will be required to become more efficient to help consumers make the choice for beef rather than other protein meats such as chicken or pork.

There is a wide range of beef markets accessible from Australia with all cattle breeds being accepted. Figure 1.2 illustrates the main country Australian beef is exported to was Japan in the 2018/2019 year, with a total of 302,756 tonnes tones [19].

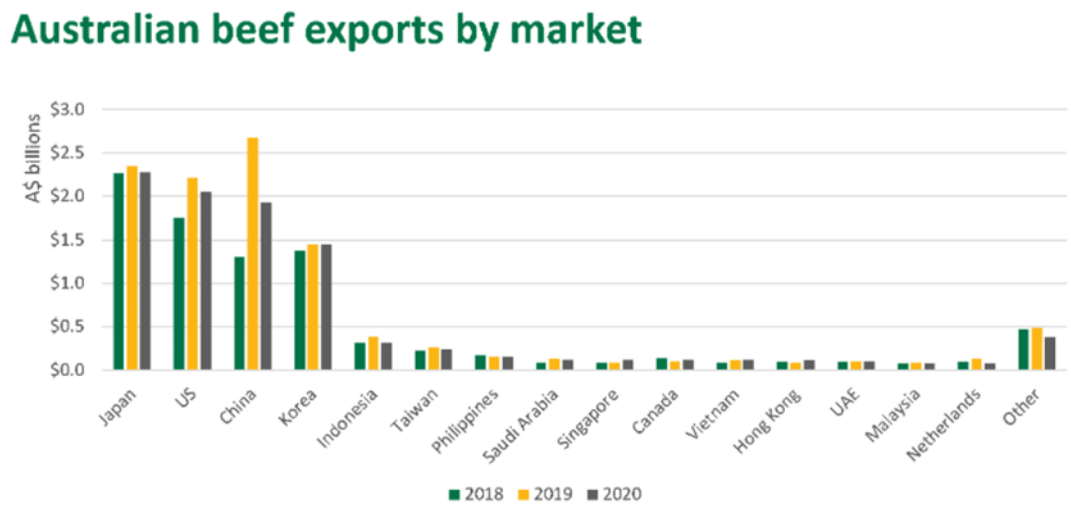


Figure 1.2: Australian top beef exports for the period of 2018-2020 [20]

1.2.2 Australian Grading and Marketing

Multiple factors determine which markets feedlot beef can be sold into, including marbling score measured at the 12/13th rib in Wagyu, hot standard carcase weight (HSCW), P8 fat, age, eye muscle area, rib fat measurement, and carcase maturity measured through ossification. The P8 is a site where the fat is measured on the carcase as can be seen in Figure 1.3.

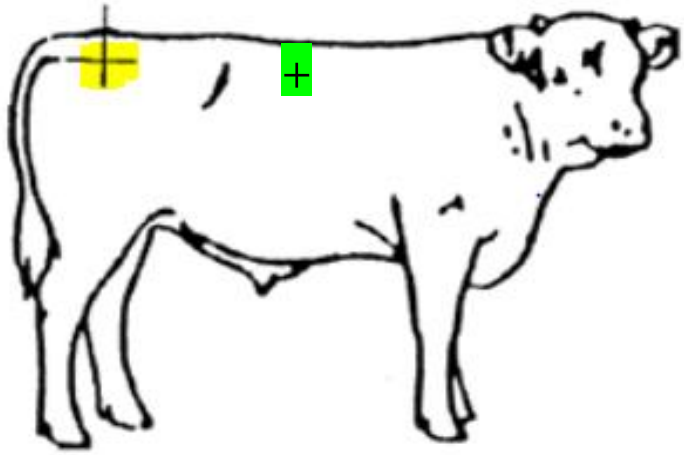


Figure 1.3: The yellow point is where the P8 site is assessed on cattle and the green point is where the 12/13th Rib measurement is taken from (Adapted from (Victoria [21]))

The MSA system is used to ensure the eating quality and paddock to plate traceability of beef. Individual carcases are ticketed and graded by carcase weight, sex, tropical breed content, hanging method, ossification, marbling, rib fat (3mm minimum), pH, and meat colour [14]. Each of these traits influence the tenderness and overall liking of the consumer. Marbling is an integral part of all marketed beef and is a determining factor in the value received for the animal, as it influences which markets can be targeted.

The demand for high quality, tender, flavoursome beef is increasing. The quality of beef generally refers to the attractiveness, tastiness, and quality of the product to the consumer. There are multiple aspects that influence the quality of meat including intra-muscular fat, taste or flavour, texture, juiciness and tenderness [22]. Higher IMF in beef is proven to increase the palatability of the meat to consumers. Smith [23] illustrated that IMF and oleic acid content had a positive correlation with palatability. Oleic acid content (on a g/g of meat basis) is also important as it can reduce the risk of cardiovascular disease in humans due to it being a monounsaturated fatty acid (MUFA). The deposition of MUFA occurs in the eye muscle which results in a more fluid mouth feel that is perceived as desirable [24].

1.2.3 Wagyu Cattle

Japanese cattle in Australia are known as either Black or Red Wagyu. This breed is native to Japan and has very high-quality meat characteristics. Wagyu animals can survive in tropical areas; however, they need to be managed for parasitic infestations such as ticks and their performance is lower than in temperature regions [10]. There has been exponential growth in the Wagyu breed in recent years. Since 2015 the sire and dam registrations have increased from 4,704 sires and 47,264 dams through to 12,223 sires and 104,222 sires registered indicating there are huge increases in the registrations of Wagyu cattle in 5 years [25]. The increased market growth can be attributed to several factors such as increased demand for Australian Wagyu and increased store market value for F1 animals. This has enticed a large portion of beef producers to include Wagyu genetics in their beef herd as part of a crossbreeding strategy [26].

The Wagyu cattle of today were developed in Japan by crossing the native Japanese cattle with European cattle such as Brown Swiss, Devon, Ayrshire, Simmental, Shorthorn and Korean cattle in the 1860's through until 1910. The breed was then closed off to genetic infusion from outside sources. With all the registered animals only crossed with other registered animals, generating the lines of Wagyu present today [27]. The first Wagyu animal imported into Australia in 1990 was a female cow. Further embryos and semen become available later. There was then

closure of the importation due to the Japanese government placing a total restriction on genetic exports out of Japan. However, there were animals from the Westholme herd in the US and Canada with genetic material being imported into Australia. In 2005 and 2006 all breeding stock was slaughtered for meat and no further breeding of the Westholme animals was continued in the US [28].

There are three main strains of Wagyu breed in Australia, which include Tajima, Fujiyoshi, and Tottori. These are all from different regions within Japan and have distinguishing traits based on their geographical differences. The Tajima cattle originated from the Hyogo prefecture and were used to pull ploughs and carts through rice paddies. This resulted in a smaller framed animal with lower growth rates but excellent meat quality and larger eye muscle area. The Fujiyoshi strain originated from the Okayama prefecture with medium size frame and good meat quality. The third strain Tottori were developed for pulling grain carts which resulted in larger framed cattle with straight backlines and good growth rates however their meat quality is more variable compared to that of the Tajima or Fujiyoshi. A combination of all three lines is generally used in Australian fullblood Wagyu production to enable a high marbling content along with larger framed animals [29].

The overall production of Wagyu cattle in Australia is mostly crossbred cattle consisting of fullblood Wagyu bulls crossed with predominantly Angus females to produce an F1 animal [10]. These F1 animals can be re-joined to fullblood male bulls to increase the Wagyu content from 50% to 75%, or further over generations. Although this is widely assumed that the increase in Wagyu content will increase the marbling, there is no scientific literature to confirm this resulting in a research gap. This eventually results in either a purebred animal or terminal F1s that are slaughtered for meat consumption. Different companies utilize different marketing strategies; however, it is becoming increasingly popular to have both a strict F1 product with a marble score less than five, and then a marble score 5+ product that targets higher value markets.

The Wagyu industry in Australia experienced rapid growth in 2019-2021 due to the increased demand for the product and the premiums that are available for these animals. The current market is paying approximately $2.50-$3.00/kg live weight more for Wagyu crossbred cattle compared to other *Bos indicus* or *Bos taurus* cattle [30]. There are different markets for Wagyu steers and heifers, which include a store market for animals between 200-250 kg being sold for backgrounding on pastures before entering the feedlot system. Another market is for animals

sold as feeder steers (approximately 350-400 kg) ready to enter the feedlot. Finally, another market involves finished animals being sold to processors or marketers off the hook after the feedlotting process (400 kg HSCW). Each of these markets has specific benefits and downfalls. An example of a drawback to selling young animals for a Wagyu breeder may include the difficulty regarding receiving feedback from carcase data after slaughter. Not receiving carcase feedback can render the process of making breeding decisions difficult for genetic improvement.

The grading system of Wagyu cattle is different to other breeds of cattle as marble score has a heavy weighing on the grid by which prices are generated. The profit drivers specifically for Wagyu are HSCW and a dollar value that is placed on marble score of individual carcases. There are significant differences in carcase value between animals that marble well compared to those that do not (Figure 1.4). The value of the carcase is also dependent on the HSCW, as the whole value of the carcase is based on a grid which encompasses different prices for each marble score bracket ($/kg HSCW), multiplied by the HSCW of the animal. Therefore, it is important to understand the biology of fat deposition in Wagyu cattle, which allows to select superior animals with increased fat deposition while maintaining HSCW.

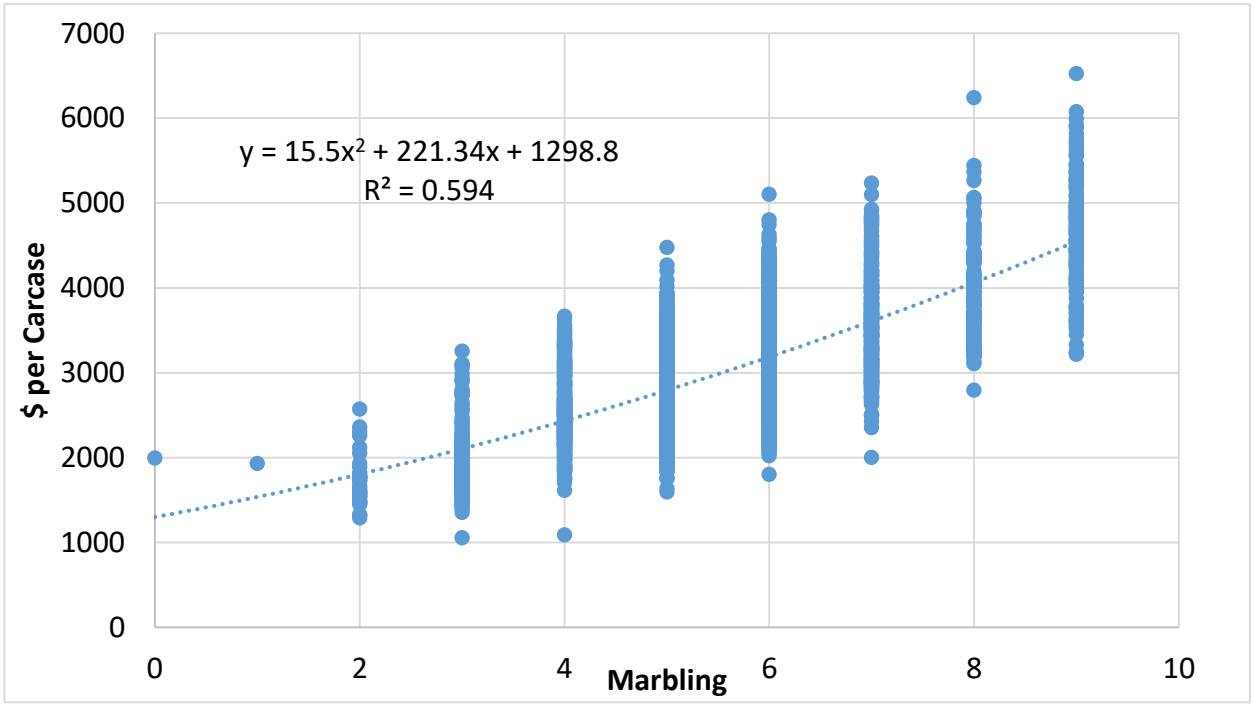


Figure 1.4: Value of a carcase in relation to marble score (Connolly, 2020 unpublished data).

1.3 Metabolomics

Genetics and genomics have been the preferred technologies to select, breed and manage animals for desirable traits that influence productivity and meat quality [31,32]. Gene discovery and genetic associations require the measurements of a phenotype to link with, and estimate heritability or genes associated with such traits. The phenotypic traits such as marbling, HSCW, are then measured once the animal is slaughtered which is difficult to measure early in the animal's life. Metabolomics offers another phenotypic trait which can be integrated with other 'omics' technologies to improve the selection process above traditional phenotypic traits and has the potential to improve animal breeding, selection and management. This section looks at metabolomic techniques, sample preparation, methods of finding metabolites and analysis of the spectrum. Various technologies are utilised to determine or examine the metabolites in samples. The different methods, sampling techniques, technology platforms and applications are discussed in this section.

1.3.1 Overview

Metabolomics is an emerging field that provides a view of an individual's phenotype. Metabolites are the building blocks for many biological components in the body such as regulation and signalling of genes, proteins, and enzymes, and key components of the primary and rapid response to the environmental influences or changes. Despite this, not every individual metabolite is followed by changes at the transcriptional level [33]. Minor invasive procedures such as the extraction of blood, saliva, milk, or urine make the use of metabolomics accessible to a wide range of disciplines. Homeostasis of the body is generally interrupted with pathological diseases, environmental stress or metabolic disorders, thus making metabolomics an efficient process for determination or diagnosis of disease [34].

The metabolome can be studied individually or in conjunction with other functional measures such as genomics, proteomics, lipidomics, transcriptomics or phenomics [4,8,35]. Metabolites are generally characterized as chemical structures with low molecular weight, different in comparison to proteins and nucleic acids that are studied in DNA analysis. The function of metabolites is also different to that of proteins, peptides, transcripts mRNA and genes. Metabolomics encompasses multiple fields with multiple applications.

1.3.2 Metabolomics Technologies

Several technologies can be used to characterize the metabolome. These include proton nuclear magnetic resonance (^{1}H-NMR), mass spectrometry (MS), gas chromatography-mass spectrometry (GC-MS), total ion chromatogram and ultra-pressure liquid chromatography mass spectrometry (UPLC-MS) [8]. Each technology has its own set of capabilities and limitations such as detection limits, sensitivity, costs, and the speed of sample processing. Figure 1.5 illustrates the different spectrums that are generated using different technologies such as ^{1}H-NMR, LC-MS, MS, and the total ion chromatogram.

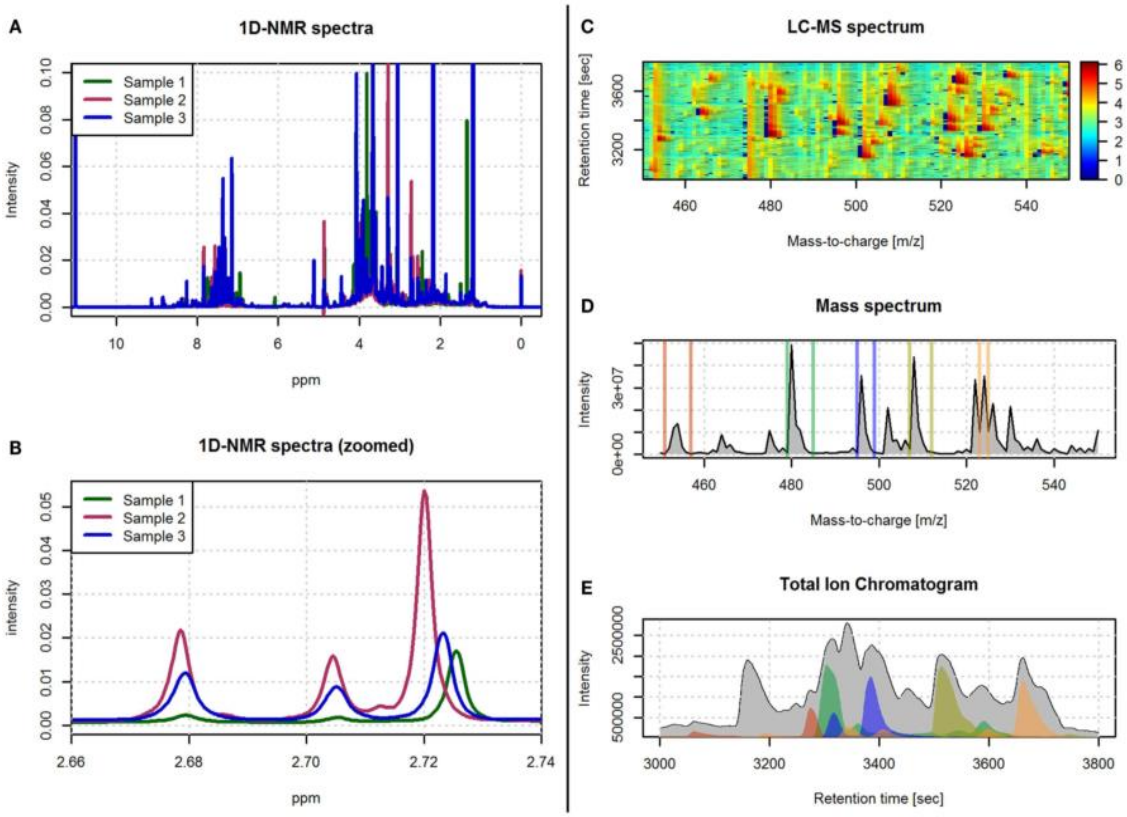


Figure 1.5: Examples of spectra obtained with 1Dimensional ^{1}H-NMR (panel A and B), LC-MS with the colour coded intensity referred by the m/z and retention time axes (C), and the sum of the LC-MS spectrum across the m/z axis (D) and Total Ion Chromatogram which is the sum of the LC (E). The coloured regions in (E) correspond to the sum of the LC-MS spectrum limited to the m/z ranges depicted with the same colour in (D) (Sourced from Alonso, et al. [36]).

1.3.2.1 Nuclear Magnetic Resonance

Nuclear magnetic resonance uses an instrument that can examine the physical phenomenon in which electromagnetic radiation is adsorbed and re-emitted from atomic nuclei that are aligned with a strong magnetic field. It allows for the quantification of magnetic properties of the

atomic nucleus [36]. The ^{1}H-NMR is the main technology that is utilised for the examination of the metabolome. The ^{1}H in the NMR refers to the hydrogen-1 nuclei of the substance, which allows the structure of the molecules to be identified within a sample. The NMR instrument can examine and characterize structures of molecules, screen the composition of liquids, and quantify known and unknown components of a sample. ^{1}H-NMR provides the benefit of ease of quantification, simple sample preparation, high number of molecule measurements per experiment, and relatively simple assignments of features [37]. The NMR can determine the purity of a sample and classify mixtures into known compounds. For unknown compounds, the ^{1}H-NMR spectrum can be matched against spectral libraries to determine the structure directly [38]. NMR is a non-destructive method, which allows the sample to be examined multiple times.

1.3.2.2 Mass Spectrometry

Mass spectrometry is a method that attains spectral data examining mass to charge ratio (m/z) and relative intensity of compound. There are two main techniques, which are gas chromatography (GCMS) and liquid chromatography (LCMS). The spectrometer first ionizes the sample, and the ionized compounds generate different peak patterns. The variants are generally identified by different ionization and mass selection methods [39]. The techniques are coupled with a separation technique of the molecules – either LCMS or GCMS. The two techniques use different columns, which allows for the chromatographic separation of different molecules, as the molecules interact differently with the different properties of the columns [36]. The LC method separates molecules using a liquid mobile phase to pass the sample through the column whereas the GC method involves vaporizing the molecules without decomposition and is commonly used to test the purity of a substance.

The adoption of mass spectrometry (MS) in metabolomics is an ever-increasing approach and provides a higher sensitivity and selectivity than ^{1}H-NMR. The electrospray ionization (ESI) MS approach is able to provide chemical information such as the elemental formula and structural elucidation through the identification of parent and fragment ions [40]. Gu, et al. [41] illustrate a global metabolite profiling method using LC coupled with a triple quadrupole MS, with the key to this method being the global search of the precursor and product ion scan. In MS, the mass to charge ratio (m/z) is examined, with most metabolites having only one charge due to their low molecular weights. This is in contrast to proteins which contain higher molecular weights and charges [42].

1.3.3 Metabolomics Sample Preparation

The sample preparation for metabolomics is dependent on the instrument that is being used and the type of sample that is being examined, and the type of metabolic screening that is being performed. Most samples prepared for ^{1}H-NMR are in a solution state however it is possible to examine intact tissues with High Resolution - Magnetic Angle Spinning NMR. The sample preparation for plasma on the ^{1}H-NMR instrument is straightforward and includes the use of a 1:1 volume ratio of buffer and sample [38]. However, a recent NMR method performs sample pre-processing to filter or eliminate large molecules (3.0 kDa cut-off) that can overlap with the peaks of smaller metabolites, and it also suppresses the water peak. This results in 'cleaner' spectra where small metabolites can be better differentiated with a flat baseline and high peak-to-noise ratio to aid easy identification of metabolites [43].

The preparation of a sample for the mass spectrometer is a diverse process dependent on the sample type and the method in which the sample is being infused and profiled. Gika, et al. [44] indicated the main method to profile metabolites is using LC-MS based on sheer publication and citation statistics. The addition of an organic solvent such as methanol is required to remove proteins from the sample. This can be a difficult step due to human error and evaporation [42].

1.3.4 Methods of Finding Metabolites

Metabolites can be detected within a sample in two main methods; these include non-targeted profiling and targeted profiling methods [45]. These are described in this section.

1.3.4.1 Non-Targeted Profiling

Non- targeted profiling is a method where preselected metabolites are directly screened for as part of a broad set of compounds that can be quantified accurately. This method includes multiple metabolites being detected within an individual sample; some molecules can be uncharacterized (or unassigned) prior to their discovery due to being rarely found in samples. These groupings of metabolites may only be specifically linked to that sample, appear in minute quantities or were not previously characterized [46]. This method is characterized by the large datasets and complexity of the data obtained from ^{1}H-NMR instruments, which in turn requires large and technical bioinformatics tools for data processing.

1.3.4.2 Targeted Profiling

Targeted profiling identifies specific metabolites in a sample. This approach has been adopted largely in the field of medical diagnostics and searches for specific metabolites of known

chemical structure. The use of targeted metabolites as biomarkers has become more proficient with the diagnosis, prognosis and treatment of diseases becoming the focus [47]. For example, Dutta, et al. [48] have identified and characterized a set of biomarkers which allows for the earlier detection of endometriosis without the need of an invasive laparoscopy procedure to diagnose the patient.

1.3.5 Analysis of the ^{1}H-NMR spectrum

There are many different types of ^{1}H-NMR experiments used for screening biofluids. One of the most common sequences uses nuclear overhauser effect spectroscopy (NOESY). The NOESY can be described as the change in overall intensity of resonance that occurs when another resonance is saturated [49]. Once the spectrum is generated from the NMR instrument and processed, there are multiple free access programs available to determine the number of metabolites and the intensities at which they occur.

One program that enables the quantification of metabolites is Chenomx™, which enables baseline correction and removal of distortions that are larger than the background noise, as well as identifying the metabolites from a spectral library. The program allows for a targeted profiling approach based on the metabolites within the Chenomx® software [50]. After the metabolites are identified, a matrix can be formed, and statistical analysis conducted to examine the relationship between the specific trait and metabolites in the sample.

1.4 Metabolomics in Cattle

Goldansaz, et al. [4] suggested seven main classifications for the application of livestock metabolomics including animal health, nutrition, production, reproduction, physiology, and products. There has been a huge increase in the number of publications since 1999 with large focus on animal breeding. The ability to undertake systems biology approach including genomics, transcriptomics and proteomics coupled with metabolomics could allow the biochemical pathways to be further understood [51]. Metabolomics, can also be used for dynamic measurement of metabolic responses, identification of biomarkers relative to production traits or disease, and understanding potential genetic architecture [52]. This section describes some examples of the application of metabolomics in animal production.

1.4.1 Metabolomics for productivity

Many economically important production traits are of interest in animal production including growth rate, milk production, feed efficiency, nutrition, and environmental footprint, amongst others. One that has received significant attention in cattle is feed efficiency because of the

difficulty, labour, and cost of measuring this trait. The application of metabolomics are varied and include identifying biomarkers, metabolic pathways or networks [53-57]. D'Occhio, et al. [58]have examined the relationship between metabolomics and reproductive health. These authors concluded that the relationship between the metabolome and reproductive function and improvement of the technology would only increase our understanding of biological and metabolic health.

Another application of metabolomics in the dairy industry encompasses metabolites being examined for use in prediction of milk production and quality. A study examined the urine, rumen fluid, serum, and milk of two groups of cattle fed two different diets (alfalfa and corn stover) to determine the influence of feed protein on the production and quality of milk. There were biomarkers associated with milk production and quality of the milk. The metabolites involved were glycine, serine, threonine, tyrosine, and phenylalanine (all commonly quantified and identified in an NMR screen). Animals fed a diet of alfalfa hay had increased concentrations of amino acids, peptides, analogues, and carbohydrates in the rumen samples. This suggested that more nutrients could be absorbed when animals are fed alfalfa compared to lower quality stover. The urine profile indicated there were 31 significantly different metabolites including amino acids, carbohydrates, and lipids. Overall, there were higher concentrations of metabolites in the urine of animals fed the CS diet suggesting there were more metabolic waste or inefficiently used nutrients, or that the urine was more concentrated. All results were in agreement with the phenotypic data such as lower milk production and feed efficiency in the animals fed a poorer diet [59].

Another application of metabolomics in the beef industry was a study undertaken by Osorio, et al. [60]. The objective was to identify the type of feed the animals had been fed using samples of muscle and urine to search molecular biomarkers using NMR. There were four groups of 25 heifers each fed different diets at outdoor pasture (A), silage indoors (B), silage outdoors with outdoor pasture and concentrate (C), and barley-based concentrate indoors (D). There were significant differences in the urine and muscle samples of the animals illustrating that diet influences the biochemical makeup of the beef and this can be comprehensively measured using metabolomics. The main metabolites in urine that had significant differences between the control and the barley diets were creatinine, hippurate and glucose. However, the metabolites that differed in the muscle between animals fed different diets included carnosine, methyl histidine, malonate, and glutamine. Therefore, it is important to highlight that sample

used for metabolomics can have a large influence in the processes being measured, and this should be cautiously considered.

1.4.2 Metabolomics for Genetic Improvement

The application of metabolomics in conjunction with genomics is also an emerging application in livestock production. Metabolites can be seen as the 'mid-point' phenotype resulting from the genetic makeup of an animal and the environmental influences [8]. Widmann, et al. [61] examined metabolites and single nucleotide polymorphism (SNP) data together to develop a systems biology approach of the onset of puberty in young heifers. The metabolites were quantified in this study using the electrospray ionization MS/MS approach and Biocrates™ targeted metabolomic technology. The results indicated GnRH signalling is a relevant genetic modulator and that betacellulin and Diacylglycerol kinase eta are two highly connected hubs within the gene network. The use of metabolites together with genome wide association studies (GWAS) enabled information such as prediction of traits and regions of the genome associated with specific genes to be determined [8]. GWAS is the process of examining a set of genome wide genetic variants such as SNPs to determine if there are any specific variants that influence a specific trait of interest. There seems to be a huge potential for metabolomics to improve genetic progress and prediction of important carcase traits. However, the heritability of the metabolome still uncertain due to limited data available and the difficulty to assemble different datasets that use different techniques, instruments, and analytical processes.

A recent study with crossbred beef cattle demonstrated that 11 out of 33 metabolites had a heritability between 0.09 and 0.36 but no heritability was found for the remaining 22 metabolites [62]. Thus, approximately one third of the concentration of metabolites were due to genetic variants and two-third influenced by the environment. Interestingly, these authors also found candidate genes and networks with GWAS that were associated with the concentration of betaine, alanine, and lactic acid. However, the potential of metabolomics to aid in genetic selection and the heritability of the metabolome in cattle are highly unknown and more research in this area is required.

Gemmer, et al. [63] examined if metabolomics could potentially replace the use of genomics in wheat populations to predict the multi-year agronomic traits. The estimated effects of the genomic prediction and metabolomic prediction were highly concordant however, the results indicated that metabolomics could not be used alone in barley, but the authors suggested it

would be able to assist in unravelling physiological pathways associated with agronomically important traits.

1.5 Metabolomics of Food Products

The use of NMR as a technology is not only limited to examining metabolites. The technology is similarly used extensively in food characterization as reviewed by Marcone, et al. [64]. There have been multiple studies showing the ability of ^{1}H-NMR to ensure the traceability and authenticity of food, such as determining origin, composition or molecular structures of the food [37]. ^{1}H-NMR has also been used in food traceability applications to determine the origin of multiple foods and drinks such as meat, honey, salmon, beer, wine, fruit juice and cheese [64]. Another application of ^{1}H-NMR in the food industry is to examine the water, lipid or protein content of the food sample [65].

The geographical origin of beef is important to consumers due to the presence of "mad cow" disease, and the fact that beef quality can be influenced by the country or even region of origin. Jung, et al. [66] examined beef from four countries and reported that the chemical composition (i.e. amino acids and organic acids) of the beef differed amongst countries (Figure 1.6).

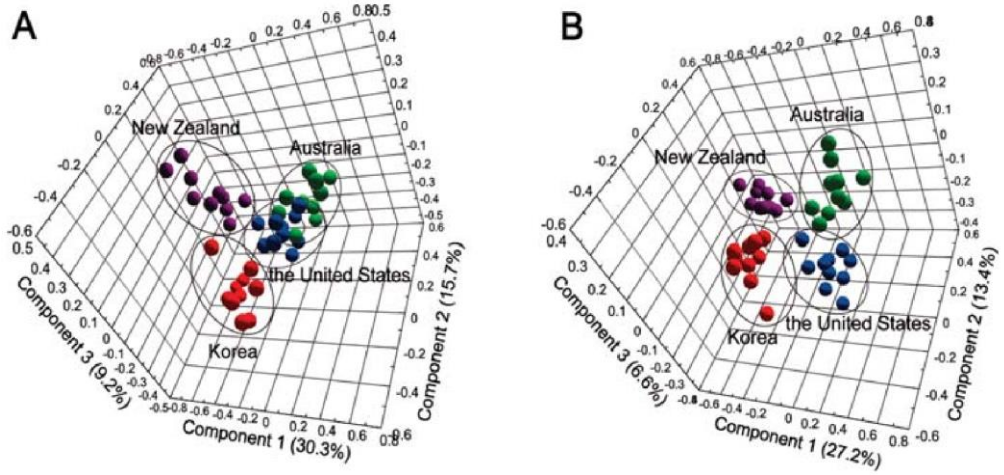


Figure 1.6: PCA(A) and OPLS(B) 3D score plots derived from the ^{1}H-NMR spectra of the beef sirloin extracts obtained from Australia, Korea, New Zealand and United States (Sourced from (Jung, et al. [66]))

The application of metabolomics to food has also been explored by Tomita, et al. [67] where apple juice was examined to determine the difference between different apple cultivars grown in different geographical locations such as Japan and New Zealand. The results indicated this

was feasible and there were significant differences between sugar signals such as sucrose, glucose, and fructose. The minor metabolites such as aspartic acid, 2-methylmalate and one unidentified compound also aided in the process of determining the geographical regions of the apple juice. The application of metabolomics in food science has enabled the quality assurance process to become easier due to the ability of metabolomics to assess the quality and safety of the foods based on the authenticity of the products [68].

The ability to determine the quality of a food product has been made easier using metabolomics. Pinu [69] reported the ability of metabolomics to identify biomarkers that are involved in microbial contamination. The use of both NMR and mass spectrometry could lead to the rapid and early detection of bad pathogens and food spoilage microflora. Xu, et al. [70] utilised volatile organic compounds based GC metabolite profiling to identify 16 spoilage biomarkers in pork. Li, et al. [71] also applied a similar method to detect post-harvest diseases in onions using gas sensors and GC-MS.

1.6 Metabolomics in animal and human diseases

The metabolome is currently being used to search for biomarkers in relation to many diseases of humans. Pathological diseases are shown to disrupt homeostasis of body functions affecting the metabolic profiles [34]. The examination of the metabolic profile of humans is being utilised in medical research including but not limited to cancer, cardiovascular, endocrine, mental, infectious, neonatal, kidney and neurological diseases [72]. Biomarkers have been discovered for the diagnosis of preeclampsia in pregnant woman, which is a condition that leads to a significant amount of maternal and foetal mortalities [73]. There were 40 organic molecules significantly elevated and 5 that were reduced in women who later experienced preeclampsia compared to women who had a normal pregnancy. Bahado-Singh, et al. [74] also reported novel first-trimester biomarkers that were able to determine the chance of early onset of preeclampsia. Cardiovascular disease is one of the largest causes of death in developed countries, with risk factors such as high blood pressure, diabetes and smoking all contributing to the risk. Metabolomics is currently being used to understand the pathophysiological processes associated with the disease [75]. A study was conducted examining the metabolomic profile of 1,627 patients with 1,027 yielding results of diagnosis of Coronary Heart Disease using four specific metabolites which were lysophosphatidylcholine 18:1, lysophosphatidylcholine 18:2, monoglyceride 18:2 and sphingomyelin 28:1. These metabolites provided sufficient evidence for clinical application [76].

The identification of biomarkers in a ^{1}H-NMR spectrum after biofluids have been screened is becoming more of a common practice in metabolomics labs. Imhasly, et al. [77] illustrated the ability of plasma metabolites to be used as potential biomarkers of hepatic lipidosis in transitional dairy cattle. Hepatic lipidosis is a syndrome that occurs in the critical period between calving and early lactation resulting in decreased milk production, reduced health status, reduced fertility, and a shortened lifetime. The results indicated that there were metabolites identified as potential biomarkers, which can aid in the diagnosis of different stages of the disease and potentially aid in prevention [77].

Similar to the application of metabolomics in humans, plasma serum of cattle has been examined to identify metabolites for disease diagnostics. De Buck, et al. [78] examined plasma serum from cattle to identify potential biomarkers for the early detection of the bacteria *Mycobacterium avium subsp. Paratuberculosis* (MAP) that is associated with Johne's disease, a debilitating disease in cattle. The available method in which the disease is diagnosed is inefficient at detecting the disease in sub clinical stages. The study was conducted examining the samples using ^{1}H-NMR spectrometry to examine the concentrations of different metabolites at different stages of infection.

Blakebrough-Hall, et al. [79] used NMR to identify bovine respiratory disease (BRD) in feedlot cattle, there were 85% of animals correctly identified in the validation dataset as having BRD. The ability to identify animals that are pre-disposed to BRD prior to entering or at feedlot induction would enable the industry to be more efficient as the animals that are sick can be treated earlier prior to visual symptoms. Gómez, et al. [80] examined the ability to determine the identification of pregnancy specific biomarkers in blood plasma beef cattle after transfer of in vitro produced embryos. The study identified specific biomarkers that were able to indicate if the recipient would maintain a pregnancy and if there was a difference between the biomarkers required for fresh or vitrified embryos. The results indicated there were metabolite biomarkers that were associated with the ability to identify if the recipient would establish a pregnancy.

Another study conducted in cattle included using metabolic profiling to identify biomarkers associated with Johne's Disease administered at low or high doses [78]. The results indicated that animals that received different doses of MAP showed limited differences of the metabolome compared to the animals that received a lesser does signifying the effects were dose dependent and specific [78].

1.7 Cattle Growth and Fat Deposition

Fat deposition in beef cattle is a significant profit driver as the amount of adipose tissue in muscle (IMF, marbling) can have a large impact on the value of an animal. Other drivers of profit are feed efficiency (kg of beef per kg of feed), and the number of days cattle spend in a feedlot to reach market specifications (weight and marbling). Beef cattle firstly use energy to grow bone and maintain their body, then muscle growth and finally fat deposition occurs later in life when the animal matures physiologically [81]. Bone growth plateaus when the skeletal frame is matured, and muscle growth continues until the animal reaches the mature weight. Figure 1.7 illustrates the increase of the rib eye area (REA) and rib fat depth with increasing days in a feedlot. Fat is deposited at a faster rate as muscle reaches a plateau [82].

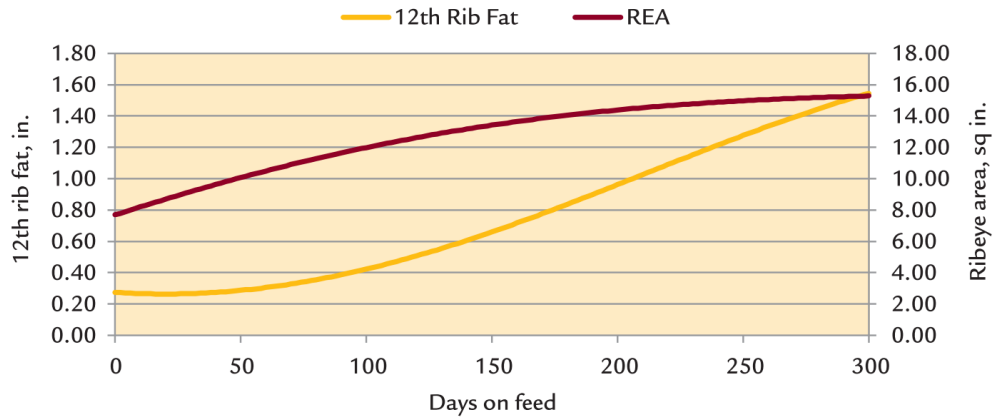


Figure 1.7: Muscle growth relative to fat deposition on the 12th rib in cattle (Sourced from (Maddock [82])).

The first fat to be deposited is thought to be perinephric (internal) fat, then intermuscular, followed by subcutaneous and finally intramuscular [83]. The deposition of subcutaneous and visceral fat on a carcase is costly to the producer as there are penalties associated with excess fat on a carcase, which can be up to 20 cents/kg if there is more than 23 mm in most breeds or 40 mm in Wagyu cattle. Fat requires more kg of feed per unit of fat deposited which adds significantly to cost of production [84]. This highlights the importance of determining which animals will deposit intramuscular fat without excess of internal, intermuscular, or subcutaneous deposits. In addition, accretion rate of different tissues change as the animal matures and it is expected that the relative importance of metabolic processes would also change with degree of maturity. Therefore, the point in time when measurements are taken are of critical importance to achieve different objectives such as identification of high and low

performing animals. Nevertheless, a gap in knowledge seems to exist about the metabolic changes that accompany growth and development of cattle and further research in this space is encouraged.

An animal that is in a feedlot for 450 days can incur costs of up to $1,500 based on contemporary costs of $3.30/head/day for the whole duration in a feedlot (influenced by grain prices). An animal with marble score 3 would be valued at approximately $4.60/kg HSCW or $1,946 (HSCW of 423 kg). This animal would have consumed $1,500 of feed and had a cost of $2300 on feedlot entry. This represents a very small profit. By comparison, an animal with marble score 9 would be valued at $15.00/kg HSCW or $6,423 (HSCW of 423 kg). The return would be $6,423, a significant profit. Identifying animals that marble at an early stage is therefore critical both meat quality and profit for producers. Genetic selection of sires that produce progeny with high value carcases is slow as it can take up to 5 years before carcase feedback is obtained for progeny. Several methods are therefore being explored to accelerate genetic progress and selection including genetic markers, GWAS, metabolomics, lipidomics and phenomics.

1.7.1 Lipid metabolism in cattle

Lipid metabolism in cattle is influenced by many factors. For example, the fat composition of muscle is impacted by age, nutrition, breed, genetics, environmental influence and sex [85]. In ruminants, most of dietary carbohydrates are digested in the rumen by micro-organisms and only 5-20% of dietary carbohydrates consumed are digested in the small intestine. The dietary cellulose, hemicellulose, proteins, and pectins are fermented to volatile fatty acids (VFA) and absorbed from the rumen and other parts of the digestive tract. It has been revealed that adipose tissue is the primary site for fatty acid synthesis in non-lactating ruminants as opposed to the liver in humans [86]. Bovine subcutaneous tissue is mainly synthesized from acetate, which allows glucose to be used in cells such as red blood cells that have an absolute requirement for glucose. Intra-muscular adipose tissue is interesting as it has a high dependency for glucose as the carbon source for fat synthesis, especially in younger cattle [86].

Gluconeogenesis is a process that is partly undertaken in the rumen by the cells that line the inside feed is degraded into substrates which are then processed in the liver into the products of propionate, valerate, amino acids, lactate, and glycerol. In ruminants, the conversion of propionate to glucose occurs in the liver and glucose is transferred to blood in both fasted and fed states, with faster uptake in animals that have a positive energy balance [86]. Steers fed a

corn-based diet had increased propionate production, which enhanced glucose uptake in IMF deposition compared with a hay-based diet (Rhoades, et al. [87]. De-esterification and bio hydrogenation of dietary fats occurs in the rumen, mainly due to microbial processes. This process yields short chain-fatty acids, saturated fatty acids, trans fatty acids and conjugated linoleic acid isomers.

Smith and Crouse [88] showed that the deposition of adipose tissue as marbling used glucose as a carbon source as opposed to subcutaneous fat where acetate was the main carbon source. There is evidence to suggest that providing access to glucose at an earlier age promotes the deposition of IMF later in life more so than if the glucose is fed to the animals later in life, although 98% of glucose fed will be degraded in the rumen to propionate [17]. The deposition of IMF is not fully understood, and further studies are needed on the relationship of the plasma lipidome to phenotypic information (such as all carcase attributes i.e. beef marbling score (BMS), HSCW, P8, rump fat). These studies could indicate if there are specific lipids that influence the regulation and deposition of IMF as well as metabolites or small molecules.

1.7.2 Livestock Selection Intensity and Methods

Livestock selection intensity is the amount of pressure placed on selecting the top percentage of animals for a specific trait, e.g. animals with highest 1% marbling of a herd [89]. Increasing the selection intensity on desirable traits such as marbling and HSCW can be difficult as the generation interval (the age at which the parents can produce the next generation) can require up to two years before the first progeny are born. This has resulted in the selection of animals based on data from closely related animals such as siblings or half siblings to determine the genetic merit of an individual. Whilst this approach has historically been beneficial, there are significant limitations that include small numbers of closely related animals, which results in low accuracy for an individual population [90]. The ability to assess breeding values across multiple breeds or herds in beef cattle is limited due to the genetic diversity - there are too many genetic lines to be able to reference across multiple herds. Predictive markers or biomarkers of a trait would be extremely beneficial as it would allow the timely and costly process of progeny testing individual animals to increase genetic progress at a quicker rate [91]. Both genomics and metabolomics could be these tools to allow for prediction of genetic potential of animals at an earlier age and increase genetic progress at a faster rate. This is especially important for traits that are hard to measure such as marbling, which expresses only when the animal is slaughtered. Traditional selection methods involved the selection of superior animals with desired traits such as marbling, fertility, weight, and survivability [92].

Using a metabolomic biomarker as a performance indicator rather than or in addition to genetic linkage may allow for incorporation of environmental influences, and a better understanding of the overall biological system of the animal [93].

Some of the original selection traits included docility (for domestication), coat colour, and animal shape or structure [94]. The selection for some desired traits has become more comprehensive because of the difficulty to measure and being polygenic (influenced by multiple genes). The current method most commonly used by the Australian beef industry to improve the quality of a carcase are Estimated Breeding Values EBV [95]. This process enables the producer to select bulls or females based on the genetic potential of the animal for multiple traits including HSCW, EMA, marbling, rib fat, and P8 fat [95]. The data used to calculate EBV's generally comes from live animal ultrasound scanning or abattoir carcase data plus the pedigree information collected by studs or producers. The ability to select animals on shear eye appeal is very difficult and the ability to predict genetic merit of animals can also be increased by using genomic data such as SNPs included in the genetic analysis. Another method used to identify causal mutations influencing meat quality is using candidate genes and marker assisted selection. In contrast to GWAS, the candidate gene process focuses on the association of a pre-defined set of genes. Ron and Weller [96] proposed a four-step method to identify candidate genes; however, this has been largely unsuccessful. This approach has been applied to studies investigating meat quality; however, due to there being many genes affecting meat quality the proportion explained by one gene is minimal. There is also an argument that genomic variance can influence the expression of a gene and that epigenetics can also silence or activate a gene along the genome [97].

Consumer demand is for consistent high-quality beef. Another unsolved issue is that tenderness can only be estimated after slaughter. There are some factors known to influence tenderness such as the CAPN1 and CAST genes, these genes influence the sheer force of beef [98]. It is widely known that the influence of specific gene markers, gene expression or protein concentrations can be specific to the breed, muscle, or to a contemporary group. This makes it difficult to develop a worldwide strategy for selection [99]. Selection of traits based on carcase data should be important for any enterprise involved in the supply chain [100]. This enables selection criteria to be placed on desirable traits such as marbling and carcase weight. Since both marbling and HSCW are moderately heritable (Table 2), there are bulls that enable higher marbling and carcase weight to be achieved; however, it can be difficult to identify these bulls. Selecting for these traits based on progeny data feedback would allow for genetic progress.

1.8 Residual Feed Intake (RFI)

With increasing world population and finite resources available, it is critical that food production becomes more efficient. The ability to select beef cattle based on the efficiency in which an animal converts expensive feed inputs into kilos of beef would enable the whole system to become more efficient [101]. In addition, the ability to reduce the environmental impact of producing beef would be beneficial [102]. The most widely used trait to measure feed efficiency is the feed conversion ratio (FCR) which is the ratio of dry matter intake (DMI) to average daily gain (ADG) of the animal [102]. However, selection of animals for FCR results in a larger mature cow body weight (BW) with increased feed costs of the herd [103]. FCR is moderately heritable but is impractical to calculate, as it requires the measurement of feed intake for individual animals. An alternative measure of feed efficiency is residual feed intake (RFI) or net feed intake. The RFI is a measure of feed efficiency of animals which takes into consideration the actual intake of an animal minus the predicted intake based on the size and maintenance requirements of the animal [101]. Taking into consideration the phenotypic variation in body size and growth into the calculation enables RFI to improve the feed efficiency without increasing the mature size of the animals or reducing the productivity [104-106]. However, measuring feed efficiency is expensive as each animal needs to be placed in a pen with electronic feeders on scales and electronic identification[107]. Therefore, RFI is also a difficult to measure trait as it is marbling, and potential biomarkers or genetic markers would have great value to assist the industry with the selection process for more efficient animals. Nevertheless, it has been shown that increasing efficiency (lower RFI) increases the leanness of the meat produced because there is an antagonist relationship between the fat in the carcase and the lower RFI animals [108,109]. This could be very important in Wagyu cattle where the key attribute is to produce animals with high fat content to achieve the marbling desired. However, little research exists on the efficiency of feed conversion in Wagyu cattle, and on the underlying metabolomic mechanisms that allow then achieving high marbling scores. Therefore, research is needed measuring feed efficiency, carcase traits and metabolomics in Wagyu cattle.

The ability to select animals based on feed efficiency is multifactorial and is influenced by genetic variation, behaviours, physiology, and environmental factors [110]. Numerous biological factors can lead to sources of variation in phenotypic RFI. The appetite, feeding behaviour and activity of the animals can have a large impact on the measurements recorded [108]. Voluntary feed intake is a complex process of interactions from the neuro-endocrine control mechanisms and the physiological state of the animals [111]. [112] concluded that there was a requirement for

further investigation to understand the endocrine function and gene and protein expression within the hypothalamus to further understand the variation between animals in feed efficiency. Kenny, et al. [113] have undertaken a review into the effect of daily time spent feeding, the results suggested that on average animals with high RFI spent 10.3 minutes longer eating out of an average of 93 min/d than their low RFI counterparts. This indicates the feeding behaviours of the animals have a large impact on the feed intake and RFI which needs to be considered if trying to select animals based on the RFI measurement.

1.8.1 RFI and Genetic Selection

The importance of improving the genetic selection for a specific trait is relative to a clear definition of a breeding objective. In Wagyu cattle, marbling or IMF is the most important breeding objective [10]. The ability to select for animals that eat less without compromising other performance traits is critical. However, beef cattle breeding for RFI has issues due to the genetic diversity of breeds of cattle. The heritability of RFI in Holstein Friesians has been reported from 0.4 to 0.27 [114,115]. This variation is across multiple populations across the world, which indicates there is a requirement for recalculation of the heritability estimates before re-estimating the genetic parameters. Canovas, et al. [116] have shown that there have been numerous candidate regions in the genome associated with commercially relevant traits, which came about with the development of 'omic' technologies such as metabolomics, proteomics, transcriptomics, genomics, and metagenomics.

Weber, et al. [117] examined the underlying molecular networks and physiological traits associated with feed efficiency in beef cattle. The study examined eight steer progenies of two influential Angus bulls with opposing genomic predictions for RFI. The study examined the steers from 8 months of age and the animals were phenotyped for growth and feed intake until slaughter at 14-16 months of age. The gene expression networks were examined, and the results showed that there were differently expressed genes and gene co-expression networks that linked tissue function with transcription factors and genes harbouring GWAS SNP. The findings from this study indicate there are significant genes and gene interaction associated with the regulatory networks and defining pathways associated with RFI.

1.8.2 RFI and Metabolomics

The relationship between metabolomics and RFI was examined in several studies [54-57]. Karisa, et al. [55] was one of the first studies to publish the relationship between plasma metabolites and performance traits in beef cattle. The study investigated the relationship between metabolites

and RFI at three time points throughout the feedlotting process. There were only 2 metabolites (creatine and glycine) significantly correlated with RFI at time point 1, 10 metabolites (hippurate, glutamate, betaine, citrate, lysine, phenylalanine, creatine, acetate, carnitine, and threonine) at time point 2, and 3 metabolites (hydroxyisobutyrate, tyrosine and formate) at time point 3. This study was very important finding because it demonstrated the ability to potentially predict feed efficiency using biomarkers in the plasma of beef cattle. However, the reasons for changes in the importance of metabolites over time is unclear because the study was relatively short period and further research is required to understand and confirm the results. In Wagyu cattle with long feeding period, the time of sampling could be critical to achieve good predictions. However, no research exists to understand the effect of sampling protocols on the relationships between the metabolome and important production traits such as marbling and RFI.

Foroutan, et al. [118] developed a prediction model for feed efficiency using plasma metabolites in young Angus bulls. Two biomarkers formate and Leucine always had a higher relationship in the high RFI bulls than in the low RFI bulls. The latter authors used a logistic regression model to predict the RFI status of the animal based upon the two biomarkers. The NMR panel was the most accurate making them good candidates to be used as biomarkers [118].

1.9 Machine Learning

Livestock production generates a large amount of data including weight, feed intake, treatments, genetics, and metabolomics. The ability to record such data is becoming easier with the development of technology and capacity to measure and record [119]. Machine learning uses multiple approaches to analyse data and produce predictions for specific traits or to help understand biological processes [120]. Machine learning uses a versatile approach to data analysis as there are fewer assumptions and the distribution of the data is not required to be normal. Some of the main methods in machine learning are neural networks, Bayesian models, random forests, deep learning, dimensionality reduction, decision trees, ensemble learning, instance based models and support vector machines [120].

Neethirajan [121] reviewed the role of sensors, big data, and machine learning in animal production. The review highlighted the fact that there are fewer farms available, and more animals required to feed the ever-growing population, with the global demand for various meat products predicted to increase by over 70% in the next three decades. With this in mind, it is critical that the production of animals becomes more efficient to ensure there is less wastage

of vital resources. The optimization of feed efficiency and energy intake is one of the applications to predict requirements of the animals and also select for more efficient converters of energy to protein [122]. Understanding of complex systems is required to use the advanced technologies to examine biological systems and identify complex patterns. The developing technologies require analysis of many types of data from images, text, audio and videos, and complex algorithms then examine this information and identify and predict problems such as disease outbreaks [123].

1.9.1 Machine Learning Methodologies

The basic statistical framework in machine learning in most practical applications involves a pool of candidate probability models that can predict traits or variables based on unobserved data or a process that is better known as 'training' the dataset. This technique is referred to as supervised learning, where the predicted target or phenotype is known whereas if the phenotype is discrete, such as disease status, then is called a classification model. If there is no phenotype available and partially incomplete it is referred to as unsupervised learning [119]. The ability to predict the outcome of a model is undertaken by splitting the dataset into training and validation populations, where the validation dataset is not included in the development of the model. The decision on which model to use is largely dependent on the data that is available, and the model selected needs to be determined to ensure the data is not over fitted and the results are not misrepresenting the actual results. The framework to explain how multiple databases feed back into the analysis process is shown in Figure 1.8.

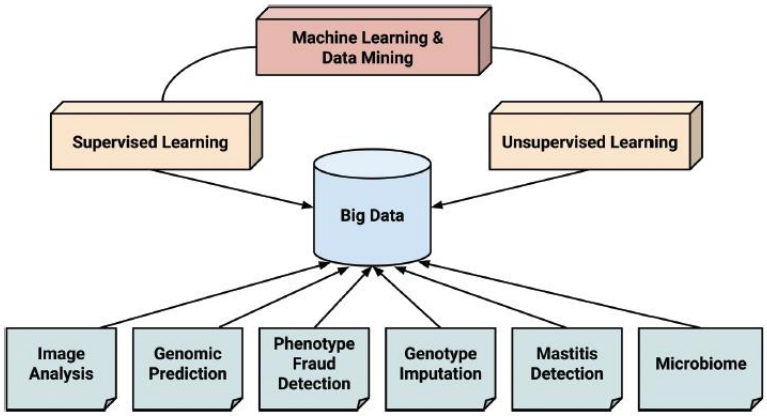


Figure 1.8: An overview of big data analysis when applied in animal science using the machine learning techniques (Sourced from (Morota, et al. [119]).

Principal Component Analysis (PCA) is one of the statistical procedures able to reduce the dimensions inside the data set without removing the variation [124]. This is the most common unsupervised statistical method commonly employed in metabolomics due to the increasing dimensions (features) measured in datasets. PCA is able to reduce the number of dimensions by stripping away unnecessary information or features, such as the differentiation between patients urine to determine which patient has been exposed to drugs, disease or other environmental factors [125]. PCA is generally the starting point of the analysis due to its ability to illustrate both the highest variance and potential outliers within the data set. The disadvantages of PCA include the fact that the greatest directions of variance do not always maximise information within the data set, e.g. there could be an outlier driving the variance in the dataset [126]. PCA is a method designed to examine what is driving the variance in the dataset.

Following the PCA, the most common supervised statistical approach is orthogonal partial least squared-discriminant analysis (OPLS-DA). The OPLS-DA method tries to find a linear relationship between the X and Y vector matrices. The X (predictor) vector matrix is generally the spectrum data (metabolites) compared to the Y (response) vector matrix which is generally the clinical/physiological metadata i.e. marble score or sick/healthy patients [127]. Caution needs to be taken when using the OPLS-DA method in regards to a small data set as it can lead to over fitting and class separation in the absence of any actual variation within the data set [50].

Other statistical methods can be applied to a metabolomics dataset such as classification and regression trees (CART), clustering procedures and random forests. A classification tree aims to target specific variables and identify the specific "Class" to which any individual variable could be assigned. A regression tree examines a specific target variable that is continuous, and the tree is used to be able to predict its value. The basis of the algorithm is a structural sequence of questions that starts at the root node and partitions the dataset using one variable e.g. if the value of the variable is x it is assigned here otherwise it is portioned to the next root node where another question is further proposed to partition the dataset further [128].[126] Random forest are also another method amongst many machine-learning methods that are available to use in predictive modelling. The Random forest method is a supervised learning algorithm that consists of a combination of trees to determine the most efficient predictor [129].

1.9.2 Machine Learning in Livestock

The use of machine learning has many applications in cattle with some more complex than others. Genomic prediction was one of the earliest adopters of the data mining techniques.

Long, et al. [130] used machine learning in genomic selection against early mortality in broiler chickens. The application of machine learning in genomic analysis has been undertaken multiple times since the continued development of the methods [131,132]. Another application of machine learning in the genetics field is the imputation of genotypes to increase the coverage of the genome; the imputation accuracy is measured by the ratio of correct calls compared to the overall call rate. Ventura, et al. [133] investigated the ability to impute data without causing the future analysis to be biased, and the method was improved upon by using machine-learning methods that included other data such as the number of animals, the density of each panel and the breed and composition of the animals that were being genotyped.

Machine learning has been used in dairy cattle, which could be due to the amount of data recorded and measured in dairy herds. Shahinfar, et al. [134] investigated the ability to predict the insemination outcomes of Holstein dairy cattle using machine-learning algorithms. The machine learning algorithms that were used included Naïve bayes, Bayesian networks, decision trees, bootstrap aggregation, and random forests. The study concluded that the random forest method was significantly better at classifying the pregnancy outcome with 72.3% and 73.6% accuracy for primiparous and multiparous cows, respectively. This is one application of machine learning that enables farming practices to become more efficient by identifying which animals will contribute to production goals. Hyde, et al. [135] examined the automated prediction of mastitis infection patterns in dairy herds using data from 290 farms across the UK between 2009 and 2014. The key to the analysis was to identify the route of the pathogen if it was either contagious or environmental transmission into the herd. The model that was used was able to achieve an 86% positive predicted value and 99% accuracy for the negative predicted value. The early diagnosis of mastitis allows rapid intervention to reduce infection within the herd.

An application of machine learning in animal welfare applications includes the behavioural classification using supervised ensemble classifiers by Dutta, et al. [136]. The study aimed to use supervised machine learning techniques to classify cattle behaviour by fitting accelerometer and magnetometer collars to the animals measuring five major behaviour classes. Grazing, ruminating, resting, walking and other behaviours were the five classes used to examine the behaviour of the animals, and the supervised classification models included binary tree, linear discriminant analysis classifier, naïve Bayes classifier, k-nearest neighbour classifier, and adaptive neuro fuzzy inference system classifier. The application of this technology could be to provide early detection and assessment of the animals health problems such as lameness and potentially future applications of management tools like detection of oestrus [136].

Another application of machine learning was to predict marbling score and carcase traits in Korean Hanwoo beef cattle [137]. The data included live weight, ultrasound, biophysical measurements, sires EBV's, ADG and the top ranked SNPs from an earlier performed GWAS. Four machine-learning algorithms were evaluated including Model Trees, Random Forests, Multilayer Perception and Support Vector Machines. These models were evaluated at predicting the carcase attributes twice in the animal's life (early and late). The results indicated that support vector machines with sequential minimal optimization and model trees performed the best within the study. The ability to select animals early in their life enables the whole production system to become more efficient, the key to having a more precise prediction model is ensuring the recording of the data is accurate [137].

Improvements in the efficiency of production is critical in Wagyu given the long period that animals spend in a feedlot. The ability to identify and select individual Wagyu early in life for feed efficiency and carcase quality would significantly improve resource utilization and reduce costs. Machine learning will have an important role in analysing large data sets and contributing to animal selection.

1.10 Summary

This literature review has demonstrated the potential to use a combinatorial approach of metabolomics, routinely collected animal farm data, and machine learning on Wagyu beef cattle to identify and predict cattle with desirable production and carcase traits such as RFI, marbling, HSCW and EMA. In addition, metabolomics could help in elucidating the underlying biological mechanisms involved in the regulation of these traits in a unique breed of cattle such as Wagyu where energy and fat metabolism is very important for the sustainability of the business. For example, the metabolic processes and importance of different metabolites that define marbling and RFI as the animal matures are poorly understood. Therefore, information on the effect of DOF on animal metabolism and the ability of metabolomics to predict importance production traits are lacking in the literature. It is also important that these mechanisms are studied and understood before machine learning is implemented because these could guide sampling and analytical protocols, and the development of prediction models. The metabolome is a product of the interaction between genes, mRNA, proteins, microbiome, and the environment. The literature suggests many potential applications for the combination of each of these technologies to further understand the biological interactions in the bodies of beef cattle.

1.11 References

1 Greenwood, P. L. Review: An overview of beef production from pasture and feedlot globally, as demand for beef and the need for sustainable practices increase. *Animal* **15**, 100295, doi:https://doi.org/10.1016/j.animal.2021.100295 (2021).

2 Greenwood, P. L., Gardner, G. E. & Ferguson, D. M. Current situation and future prospects for the Australian beef industry - A review. *Asian-Australasian journal of animal sciences* **31**, 992-1006, doi:10.5713/ajas.18.0090 (2018).

3 NSW Department of Primary Industries. (ed NSWGovernment) 5 (2015).

4 Goldansaz, S. A. *et al.* Livestock metabolomics and the livestock metabolome: A systematic review. *PLoS ONE* **12**, e0177675, doi:10.1371/journal.pone.0177675 (2017).

5 Singh, H. P., Batish, D. R., Pandher, J. K. & Kohli, R. K. Assessment of allelopathic properties of Parthenium hysterophorus residues. *Agriculture, Ecosystems & Environment* **95**, 537-541, doi:http://dx.doi.org/10.1016/S0167-8809(02)00202-5 (2003).

6 Singh, B., Mal, G., Gautam, S. K. & Mukesh, M. in *Advances in Animal Biotechnology* 397-403 (Springer, 2019).

7 da Costa, M. B. *et al.* Performance, carcass traits and serum metabolomic profile of Nellore males with different genetic potential for post-weaning growth. *Animal* **14**, 873-880, doi:https://doi.org/10.1017/S1751731119002520 (2020).

8 Fontanesi, L. Metabolomics and livestock genomics: Insights into a phenotyping frontier and its applications in animal breeding. *Animal Frontiers* **6**, 73-79, doi:10.2527/af.2016-0011 (2016).

9 Wishart, D. S. in *Genetics meets metabolomics: from experiment to systems biology,* (Springer, 2012).

10 Gotoh, T., Nishimura, T., Kuchida, K. & Mannen, H. The Japanese Wagyu beef industry: current situation and future prospects - A review. *Asian-Australasian journal of animal sciences* **31**, 933-950, doi:10.5713/ajas.18.0333 (2018).

11 Motoyama, M., Sasaki, K. & Watanabe, A. Wagyu and the factors contributing to its beef quality: A Japanese industry overview. *Meat science* **120**, 10-18, doi:10.1016/j.meatsci.2016.04.026 (2016).

12 Aus-Meat. 2 (2010).

13 Aus-Meat. (ed Aus-Meat Limited) (2019).

14 Meat Standards Australia. Vol. 09 (ed Meat and Livestock Australia) 2 (2015).

15 Ridgway, K., Smith, R. M. & Lalljie, S. P. D. in *Comprehensive Sampling and Sample Preparation* (ed Janusz Pawliszyn) 819-833 (Academic Press, 2012).

16 Nakahashi, Y., Maruyama, S., Seki, S., Hidaka, S. & Kuchida, K. Relationships between monounsaturated fatty acids of marbling flecks and image analysis traits in longissimus muscle for Japanese Black steers. *J Anim Sci* **86**, 3551-3556 (2008).

17 Nguyen, D. V., Nguyen, O. C. & Malau-Aduli, A. E. O. Main regulatory factors of marbling level in beef cattle. *Veterinary and Animal Science*, 100219, doi:https://doi.org/10.1016/j.vas.2021.100219 (2021).

18 OECD, Food & Nations, A. O. o. t. U. *OECD-FAO Agricultural Outlook 2021-2030*. (2021).

19 Meat and Livestock Australia. *Red Meats Exports Expand in 2018-2019*, <https://www.mla.com.au/prices-markets/market-news/2019/red-meat-exports-expand-in-2018-19/?utm_campaign=Social&utm_content=1563084840&utm_medium=social&utm_source> (2019).

20 Meat and Livestock Australia. *Value of Australian Beef Exports falls in 2020*, <https://www.mla.com.au/prices-markets/market-news/2021/value-of-australian-beef-exports-falls-in-2020/#> (2021).

21 Victoria, A. *Assessing Fat Depth* <http://agriculture.vic.gov.au/agriculture/livestock/beef/handling-and-management/assessing-fat-depth> (2006).
22 Wood, J. D. *et al.* Manipulating meat quality and composition. *Proceedings of the Nutrition Society* **58**, 363-370, doi:10.1017/S0029665199000488 (1999).
23 Smith, S. B. Marbling and Its Nutritional Impact on Risk Factors for Cardiovascular Disease. *Korean Journal for Food Science of Animal Resources* **36**, 435-444, doi:10.5851/kosfa.2016.36.4.435 (2016).
24 Smith, S. B. & Johnson, B. J. Marbling: Management of cattle to maximize the deposition of intramuscular adipose tissue. *Beef Research*, 1-14 (2014).
25 Australian Wagyu Association. (2021).
26 Australian Wagyu Association. *Wagyu Growth Continues*, <http://www.wagyu.org.au/wagyu-growth-continues/> (2016).
27 Australian Wagyu Association. *Japenese History*, <http://www.wagyu.org.au/breed-history/> (2015).
28 Australian Wagyu Association. *Major Wagyu Bloodlines*, <http://www.wagyu.org.au/major-wagyu-bloodlines/> (2016).
29 Australain Wagyu Association. *Japanese Black Wagyu Prefectural Bloodlines*, <https://www.wagyu.org.au/content/uploads/2020/08/AWA-FACTSHEET_prefectural-bloodlines.pdf> (2020).
30 Jon Condon. in *Beef Central* (2020).
31 Hocquette, J. F., Lehnert, S., Barendse, W., Cassar-Malek, I. & Picard, B. Recent advances in cattle functional genomics and their application to beef quality. *animal* **1**, 159-173, doi:doi:10.1017/S1751731107658042 (2007).
32 Johnston, D. J., Tier, B. & Graser, H. U. Beef cattle breeding in Australia with genomics: opportunities and needs. *Animal Production Science* **52**, 100-106, doi:http://dx.doi.org/10.1071/AN11116 (2012).
33 Dunn, W. B. *et al.* Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat. Protocols* **6**, 1060-1083, doi:http://www.nature.com/nprot/journal/v6/n7/abs/nprot.2011.335.html#supplementary-information (2011).
34 Duarte, I. F., Diaz, S. O. & Gil, A. M. NMR metabolomics of human blood and urine in disease research. *Journal of Pharmaceutical and Biomedical Analysis* **93**, 17-26, doi:http://dx.doi.org/10.1016/j.jpba.2013.09.025 (2014).
35 Dunn, W. B., Broadhurst, D. I., Atherton, H. J., Goodacre, R. & Griffin, J. L. Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chemical Society Reviews* **40**, 387-426, doi:10.1039/B906712B (2011).
36 Alonso, A., Marsal, S. & Julià, A. Analytical Methods in Untargeted Metabolomics: State of the Art in 2015. *Frontiers in Bioengineering and Biotechnology* **3**, 23, doi:10.3389/fbioe.2015.00023 (2015).
37 Laghi, L., Picone, G. & Capozzi, F. Nuclear magnetic resonance for foodomics beyond food analysis. *TrAC Trends in Analytical Chemistry* **59**, 93-102, doi:http://dx.doi.org/10.1016/j.trac.2014.04.009 (2014).
38 Dona, A. C. *et al.* Precision High-Throughput Proton NMR Spectroscopy of Human Urine, Serum, and Plasma for Large-Scale Metabolic Phenotyping. *Analytical Chemistry* **86**, 9887-9894, doi:10.1021/ac5025039 (2014).
39 El-Aneed, A., Cohen, A. & Banoub, J. Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers. *Applied Spectroscopy Reviews* **44**, 210-230, doi:10.1080/05704920902717872 (2009).

40 Emwas, A.-H. M. in *Metabonomics: Methods and Protocols* (ed T. Jacob Bjerrum) 161-193 (Springer New York, 2015).
41 Gu, H., Zhang, P., Zhu, J. & Raftery, D. Globally Optimized Targeted Mass Spectrometry: Reliable Metabolomics Analysis with Broad Coverage. *Analytical Chemistry* **87**, 12355-12362, doi:10.1021/acs.analchem.5b03812 (2015).
42 Dettmer, K., Aronov, P. A. & Hammock, B. D. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews* **26**, 51-78, doi:10.1002/mas.20108 (2007).
43 Keun, H. C. *NMR-based Metabolomics*. (Royal Society of Chemistry, 2018).
44 Gika, H. G., Theodoridis, G. A., Plumb, R. S. & Wilson, I. D. Current practice of liquid chromatography–mass spectrometry in metabolomics and metabonomics. *Journal of Pharmaceutical and Biomedical Analysis* **87**, 12-25, doi:http://dx.doi.org/10.1016/j.jpba.2013.06.032 (2014).
45 Metz, T. O. *Metabolic profiling methods and protocols*. (New York : Springer, 2011).
46 Bowen, B. P. & Northen, T. R. Dealing with the Unknown: Metabolomics and Metabolite Atlases. *Journal of the American Society for Mass Spectrometry* **21**, 1471-1476, doi:http://dx.doi.org/10.1016/j.jasms.2010.04.003 (2010).
47 Zhang, A. h., Sun, H., Qiu, S. & Wang, X. j. NMR-based metabolomics coupled with pattern recognition methods in biomarker discovery and disease diagnosis. *Magnetic Resonance in Chemistry* **51**, 549-556, doi:10.1002/mrc.3985 (2013).
48 Dutta, M. *et al.* A metabonomics approach as a means for identification of potential biomarkers for early diagnosis of endometriosis. *Molecular BioSystems* **8**, 3281-3287, doi:10.1039/C2MB25353D (2012).
49 Neuhaus, D. in *eMagRes* (John Wiley & Sons, Ltd, 2007).
50 Puchades-Carrasco, L., Palomino-Schätzlein, M., Pérez-Rambla, C. & Pineda-Lucena, A. Bioinformatics tools for the analysis of NMR metabolomics studies focused on the identification of clinically relevant biomarkers. *Briefings in Bioinformatics*, doi:10.1093/bib/bbv077 (2015).
51 Dumas, M.-E. Metabolome 2.0: quantitative genetics and network biology of metabolic phenotypes. *Molecular BioSystems* **8**, 2494-2502 (2012).
52 Hao, D. *et al.* Overview of Metabolomic Analysis and the Integration with Multi-Omics for Economic Traits in Cattle. *Metabolites* **11**, 753 (2021).
53 Clemmons, B. A. *et al.* Rumen fluid metabolomics of beef steers differing in feed efficiency. *Metabolomics* **16**, 1-9 (2020).
54 Clemmons, B. A. *et al.* Serum metabolites associated with feed efficiency in black angus steers. *Metabolomics* **13**, 147, doi:10.1007/s11306-017-1282-z (2017).
55 Karisa, B. K. *et al.* Plasma metabolites associated with residual feed intake and other productivity performance traits in beef cattle. *Livestock Science* **165**, doi:10.1016/j.livsci.2014.03.002 (2014).
56 Novais, F. J. *et al.* Identification of a metabolomic signature associated with feed efficiency in beef cattle. *BMC Genomics* **20**, 8, doi:10.1186/s12864-018-5406-2 (2019).
57 Consolo, N. *et al.* Muscle and liver metabolomic signatures associated with residual feed intake in Nellore cattle. *Animal Feed Science and Technology* **271**, 114757 (2021).
58 D'Occhio, M. J., Baruselli, P. S. & Campanile, G. Metabolic health, the metabolome and reproduction in female cattle: a review. *Italian Journal of Animal Science* **18**, 858-867, doi:10.1080/1828051X.2019.1600385 (2019).
59 Sun, H.-Z. *et al.* Metabolomics of Four Biofluids from Dairy Cows: Potential Biomarkers for Milk Production and Quality. *Journal of Proteome Research* **14**, 1287-1298, doi:10.1021/pr501305g (2015).
60 Osorio, M. T., Moloney, A. P., Brennan, L. & Monahan, F. J. Authentication of beef production systems using a metabolomic-based approach. *animal* **6**, 167-172, doi:10.1017/S1751731111001418 (2012).

61 Widmann, P. *et al.* A systems biology approach using metabolomic data reveals genes and pathways interacting to modulate divergent growth in cattle. *BMC Genomics* **14**, 798-798, doi:10.1186/1471-2164-14-798 (2013).
62 Li, J. *et al.* Genomic Heritability and Genome-Wide Association Studies of Plasma Metabolites in Crossbred Beef Cattle. *Frontiers in genetics* **11**, 538600-538600, doi:10.3389/fgene.2020.538600 (2020).
63 Gemmer, M. R. *et al.* Can metabolic prediction be an alternative to genomic prediction in barley? *PLOS ONE* **15**, e0234052, doi:10.1371/journal.pone.0234052 (2020).
64 Marcone, M. F. *et al.* Diverse food-based applications of nuclear magnetic resonance (NMR) technology. *Food Research International* **51**, 729-747, doi:http://dx.doi.org/10.1016/j.foodres.2012.12.046 (2013).
65 Mariette, F. Investigations of food colloids by NMR and MRI. *Current Opinion in Colloid & Interface Science* **14**, 203-211, doi:http://dx.doi.org/10.1016/j.cocis.2008.10.006 (2009).
66 Jung, Y. *et al.* Discrimination of the Geographical Origin of Beef by 1H NMR-Based Metabolomics. *Journal of Agricultural and Food Chemistry* **58**, 10458-10466, doi:10.1021/jf102194t (2010).
67 Tomita, S. *et al.* A NMR-based, non-targeted multistep metabolic profiling revealed l-rhamnitol as a metabolite that characterised apples from different geographic origins. *Food Chemistry* **174**, 163-172, doi:http://dx.doi.org/10.1016/j.foodchem.2014.11.028 (2015).
68 Li, S. *et al.* Recent advances in the application of metabolomics for food safety control and food quality analyses. *Critical Reviews in Food Science and Nutrition* **61**, 1448-1469, doi:10.1080/10408398.2020.1761287 (2021).
69 Pinu, F. R. Early detection of food pathogens and food spoilage microorganisms: Application of metabolomics. *Trends in Food Science & Technology* **54**, 213-215 (2016).
70 Xu, Y., Cheung, W., Winder, C. L. & Goodacre, R. VOC-based metabolic profiling for food spoilage detection with the application to detecting Salmonella typhimurium-contaminated pork. *Analytical and bioanalytical chemistry* **397**, 2439-2449 (2010).
71 Li, C., Schmidt, N. E. & Gitaitis, R. Detection of onion postharvest diseases by analyses of headspace volatiles using a gas sensor array and GC-MS. *LWT-Food Science and Technology* **44**, 1019-1025 (2011).
72 Emwas, A.-H. *et al.* Standardizing the experimental conditions for using urine in NMR-based metabolomic studies with a particular focus on diagnostic studies: a review. *Metabolomics* **11**, 872-894, doi:10.1007/s11306-014-0746-7 (2015).
73 Kenny, L. C. *et al.* Robust Early Pregnancy Prediction of Later Preeclampsia Using Metabolomic Biomarkers. *Hypertension* **56**, 741-749, doi:10.1161/hypertensionaha.110.157297 (2010).
74 Bahado-Singh, R. O. *et al.* Metabolomics and first-trimester prediction of early-onset preeclampsia. *The Journal of Maternal-Fetal & Neonatal Medicine* **25**, 1840-1847, doi:10.3109/14767058.2012.680254 (2012).
75 Barderas, M. G. *et al.* Metabolomic profiling for identification of novel potential biomarkers in cardiovascular diseases. *BioMed Research International* **2011** (2011).
76 Ganna, A. *et al.* Large-scale Metabolomic Profiling Identifies Novel Biomarkers for Incident Coronary Heart Disease. *PLoS Genet* **10**, e1004801, doi:10.1371/journal.pgen.1004801 (2014).
77 Imhasly, S. *et al.* Metabolomic biomarkers correlating with hepatic lipidosis in dairy cows. *BMC veterinary research* **10**, 122-122, doi:10.1186/1746-6148-10-122 (2014).
78 De Buck, J., Shaykhutdinov, R., Barkema, H. W. & Vogel, H. J. Metabolomic Profiling in Cattle Experimentally Infected with Mycobacterium avium subsp. paratuberculosis. *PLoS ONE* **9**, e111872, doi:10.1371/journal.pone.0111872 (2014).
79 Blakebrough-Hall, C., Dona, A., D'occhio, M. J., McMeniman, J. & González, L. A. Diagnosis of Bovine Respiratory Disease in feedlot cattle using blood 1H NMR metabolomics. *Scientific Reports* **10**, 115, doi:10.1038/s41598-019-56809-w (2020).

80 Gómez, E. *et al.* Metabolomic identification of pregnancy-specific biomarkers in blood plasma of BOS TAURUS beef cattle after transfer of in vitro produced embryos. *Journal of Proteomics* **225**, 103883, doi:https://doi.org/10.1016/j.jprot.2020.103883 (2020).
81 Perry, T. W. *Beef cattle feeding and nutrition*. (Elsevier Science, 2012).
82 Maddock, R. (ed North Dakota State University) (2013).
83 Dodson, M. V. *et al.* Lipid metabolism, adipocyte depot physiology and utilization of meat animals as experimental models for metabolic research. *International Journal of Biological Sciences* **6**, 691-699 (2010).
84 Owens, F. N., Gill, D. R., Secrist, D. S. & Coleman, S. W. Review of some aspects of growth and development of feedlot cattle. *Journal of Animal Science* **73**, 3152-3172, doi:10.2527/1995.73103152x (1995).
85 Chung, K. Y. *et al.* Lipid characteristics of subcutaneous adipose tissue and M. longissimus thoracis of Angus and Wagyu steers fed to US and Japanese endpoints. *Meat science* **73**, 432-441, doi:http://dx.doi.org/10.1016/j.meatsci.2006.01.002 (2006).
86 Nafikov, R. A. & Beitz, D. C. Carbohydrate and Lipid Metabolism in Farm Animals. *The Journal of Nutrition* **137**, 702-705 (2007).
87 Rhoades, R. D. *et al.* Effect of dietary energy source on in vitro substrate utilization and insulin sensitivity of muscle and adipose tissues of Angus and Wagyu steers. *Journal of Animal Science* **85**, 1719, doi:10.2527/jas.2006-498 (2007).
88 Smith, S. B. & Crouse, J. D. Relative contributions of acetate, lactate and glucose to lipogenesis in bovine intramuscular and subcutaneous adipose tissue. *The Journal of nutrition* **114**, 792 (1984).
89 Miller, S. Genetic improvement of beef cattle through opportunities in genomics. *Revista Brasileira de Zootecnia* **39**, 247-255 (2010).
90 Hayes, B. J., Lewin, H. A. & Goddard, M. E. The future of livestock breeding: genomic selection for efficiency, reduced emissions intensity, and adaptation. *Trends in Genetics* **29**, 206-214, doi:http://dx.doi.org/10.1016/j.tig.2012.11.009 (2013).
91 Suhre, K. *Genetics meets metabolomics: from experiment to systems biology,*. (Springer, 2012).
92 Barwick, S. A. & Henzell, A. L. Development successes and issues for the future in deriving and applying selection indexes for beef breeding. *Australian Journal of Experimental Agriculture* **45**, 923-933 (2005).
93 Fontanesi, L. in *Systems Biology in Animal Production and Health, Vol. 1* (ed Haja N. Kadarmideen) 43-62 (Springer International Publishing, 2016).
94 Womack, J. E. First steps: bovine genomics in historical perspective. *Animal Genetics* **43**, 2-8, doi:10.1111/j.1365-2052.2012.02382.x (2012).
95 Boerner, V., Johnston, D. J. & Tier, B. Accuracies of genomically estimated breeding values from pure-breed and across-breed predictions in Australian beef cattle. *Genetics Selection Evolution* **46**, 1-11 (2014).
96 Ron, M. & Weller, J. I. From QTL to QTN identification in livestock - winning by points rather than knock-out: a review: From QTL to QTN identification in livestock. *Animal Genetics* **38**, 429-439, doi:10.1111/j.1365-2052.2007.01640.x (2007).
97 Berry, D. P., Conroy, S., Pabiou, T. & Cromie, A. R. Animal breeding strategies can improve meat quality attributes within entire populations. *Meat science*, doi:https://doi.org/10.1016/j.meatsci.2017.04.019.
98 Bolormaa, S. *et al.* A genome-wide association study of meat and carcass traits in Australian cattle. *Journal of animal science* **89**, 2297-2309, doi:10.2527/jas.2010-3138 (2011).
99 Hocquette, J. F. *et al.* Win–win strategies for high beef quality, consumer satisfaction, and farm efficiency, low environmental impacts and improved animal welfare. *Animal Production Science* **54**, 1537-1548, doi:http://dx.doi.org/10.1071/AN14210 (2014).

100 Arnold, J., Bertrand, J., Benyshek, L. & Ludwig, C. Estimates of genetic parameters for live animal ultrasound, actual carcass data, and growth traits in beef cattle. *Journal of animal science* **69**, 985-992 (1991).
101 Arthur, J. P. & Herd, R. Residual feed intake in beef cattle. *Revista Brasileira de Zootecnia* **37**, 269-279 (2008).
102 Moore, S. S., Mujibi, F. D. & Sherman, E. L. Molecular basis for residual feed intake in beef cattle. *Journal of Animal Science* **87**, E41-E47, doi:10.2527/jas.2008-1418 (2009).
103 Carstens, G. E. Net feed intake: Potential selection tool to improve feed efficiency in beef cattle. (2019).
104 Basarab, J. *et al.* Residual feed intake and body composition in young growing cattle. *Canadian Journal of Animal Science* **83**, 189-204 (2003).
105 Lancaster, P., Carstens, G., Ribeiro, F., Tedeschi, L. & Crews Jr, D. Characterization of feed efficiency traits and relationships with feeding behavior and ultrasound carcass traits in growing bulls. *Journal of animal science* **87**, 1528-1539 (2009).
106 Wang, Z. *et al.* Test duration for growth, feed intake, and feed efficiency in beef cattle using the GrowSafe System. *Journal of animal science* **84**, 2289-2298, doi:10.2527/jas.2005-715 (2006).
107 Miller, M. D. *Associations between RFI, and metabolite profiles and feeding behavior traits in feedlot cattle* Masters of Science - Major Animals Science thesis, Texas A&M University, (2016).
108 Herd, R. & Pitchford, W. Residual feed intake selection makes cattle leaner and more efficient. *Recent Advances in Animal Nutrition* **18**, 45-58 (2011).
109 Kelly, D. N. *et al.* Feed efficiency and carcass metrics in growing cattle. *Journal of Animal Science* **97**, 4405-4417 (2019).
110 Lancaster, P. A. *Biological sources of variation in residual feed intake in beef cattle* 3321725 thesis, Texas A&M University, (2008).
111 Allen, M. S. Drives and limits to feed intake in ruminants. *Animal Production Science* **54**, 1513-1524 (2014).
112 Fitzsimons, C., McGee, M., Keogh, K., Waters, S. M. & Kenny, D. A. Molecular physiology of feed efficiency in beef cattle. *Biology of domestic animals*, 122-165 (2017).
113 Kenny, D. A., Fitzsimons, C., Waters, S. M. & McGee, M. Invited review: Improving feed efficiency of beef cattle – the current state of the art and future challenges. *Animal: an International Journal of Animal Bioscience* **12**, 1815-1826, doi:http://dx.doi.org/10.1017/S1751731118000976 (2018).
114 Williams, Y. J. *et al.* Variation in residual feed intake in Holstein-Friesian dairy heifers in southern Australia. *J Dairy Sci* **94**, 4715-4725, doi:10.3168/jds.2010-4015 (2011).
115 Hurley, A. M. *et al.* Inter-relationships among alternative definitions of feed efficiency in grazing lactating dairy cows. *J Dairy Sci* **99**, 468-479, doi:10.3168/jds.2015-9928 (2016).
116 Canovas, A., Casellas, J., Thomas, M. & Medrano, J. in *Proc. Assoc. Advmt. Anim. Breed. Genet.* 377-383.
117 Weber, K. L. *et al.* Identification of Gene Networks for Residual Feed Intake in Angus Cattle Using Genomic Prediction and RNA-seq. *PLOS ONE* **11**, e0152274, doi:10.1371/journal.pone.0152274 (2016).
118 Foroutan, A., Fitzsimmons, C., Mandal, R., Berjanskii, M. V. & Wishart, D. S. Serum Metabolite Biomarkers for Predicting Residual Feed Intake (RFI) of Young Angus Bulls. *Metabolites* **10**, 491 (2020).
119 Morota, G., Ventura, R. V., Silva, F. F., Koyama, M. & Fernando, S. C. BIG DATA ANALYTICS AND PRECISION ANIMAL AGRICULTURE SYMPOSIUM: Machine learning and data mining advance predictive big data analysis in precision animal agriculture1. *Journal of Animal Science* **96**, 1540-1550, doi:10.1093/jas/sky014 (2018).

120 Liakos, K. G., Busato, P., Moshou, D., Pearson, S. & Bochtis, D. Machine learning in agriculture: A review. *Sensors* **18**, 2674, doi:https://doi.org/10.3390/s18082674 (2018).
121 Neethirajan, S. The role of sensors, big data and machine learning in modern animal farming. *Sensing and Bio-Sensing Research* **29**, 100367, doi:https://doi.org/10.1016/j.sbsr.2020.100367 (2020).
122 da Rosa Righi, R., Goldschmidt, G., Kunst, R., Deon, C. & André da Costa, C. Towards combining data prediction and internet of things to manage milk production on dairy cows. *Computers and Electronics in Agriculture* **169**, 105156, doi:https://doi.org/10.1016/j.compag.2019.105156 (2020).
123 Ellis, J. L. *et al.* Review: Synergy between mechanistic modelling and data-driven models for modern animal production systems in the era of big data. *Animal* **14**, s223-s237, doi:https://doi.org/10.1017/S1751731120000312 (2020).
124 Ringner, M. What is principal component analysis? *Nat Biotech* **26**, 303-304 (2008).
125 Potts, B. C. M. *et al.* NMR of biofluids and pattern recognition: assessing the impact of NMR parameters on the principal component analysis of urine from rat and mouse. *Journal of Pharmaceutical and Biomedical Analysis* **26**, 463-476, doi:http://dx.doi.org/10.1016/S0731-7085(01)00430-7 (2001).
126 Ringner, M. What is principal component analysis? *Nature biotechnology* **26**, 303-304, doi:10.1038/nbt0308-303 (2008).
127 Triba, M. N. *et al.* PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters. *Molecular BioSystems* **11**, 13-19, doi:10.1039/C4MB00414K (2015).
128 Loh, W.-Y. Classification and regression trees. *Wiley Interdisciplinary Reviews: Data Mining and Knowledge Discovery* **1**, 14-23, doi:10.1002/widm.8 (2011).
129 Breiman, L. Random Forests. *Machine Learning* **45**, 5-32, doi:10.1023/A:1010933404324 (2001).
130 Long, N., Gianola, D., Rosa, G. J., Weigel, K. A. & Avendano, S. Machine learning classification procedure for selecting SNPs in genomic selection: application to early mortality in broilers. *Journal of animal breeding and genetics* **124**, 377-389 (2007).
131 González-Recio, O., Rosa, G. J. & Gianola, D. Machine learning methods and predictive ability metrics for genome-wide prediction of complex traits. *Livestock Science* **166**, 217-231 (2014).
132 Pérez-Enciso, M. (Wiley-VCH, 2017).
133 Ventura, R. V. *et al.* Assessing accuracy of imputation using different SNP panel densities in a multi-breed sheep population. *Genetics Selection Evolution* **48**, 1-20 (2016).
134 Shahinfar, S. *et al.* Prediction of insemination outcomes in Holstein dairy cattle using alternative machine learning algorithms. *Journal of Dairy Science* **97**, 731-742, doi:10.3168/jds.2013-6693 (2014).
135 Hyde, R. M. *et al.* Automated prediction of mastitis infection patterns in dairy herds using machine learning. *Scientific reports* **10**, 1-8 (2020).
136 Dutta, R. *et al.* Dynamic cattle behavioural classification using supervised ensemble classifiers. *Computers and electronics in agriculture* **111**, 18-28 (2015).
137 Shahinfar, S., Al-Mamun, H. A., Park, B., Kim, S. & Gondro, C. Prediction of marbling score and carcass traits in Korean Hanwoo beef cattle using machine learning methods and synthetic minority oversampling technique. *Meat science* **161**, 107997, doi:https://doi.org/10.1016/j.meatsci.2019.107997 (2020).

Chapter 2: Relationship of the blood metabolome to subsequent carcase traits at slaughter in feedlot Wagyu crossbred steers

Connolly, S., Dona, A., Wilkinson-White, L., Hamblin, D., D'Occhio, M. J & González, L. A. Relationship of the blood metabolome to subsequent carcase traits at slaughter in feedlot Wagyu crossbred steers. *Scientific Reports* **9**, 15139, doi:10.1038/s41598-019-51655-2 (2019).

Overview

This chapter was focused on examining the relationship between the blood metabolome and carcase traits at slaughter in Wagyu crossbred steers. This could enable the identification of steers that will produce carcases with high marbling earlier in the feedlotting process. Understanding the relationships between the fat and muscle metabolism could also unravel the biological mechanisms driving carcase value.

2.1 Abstract

The aim of the present study was to determine the relationships in feedlot cattle between the blood metabolome and (1) carcase traits with a focus on intramuscular fat (marbling), and (2) the length of time cattle consumed a high-starch diet. Blood samples were obtained from 181 Wagyu-crossbred steers between 300-400 days before slaughter when carcase data was collected. ^{1}H-NMR spectroscopy identified 35 metabolites with 7 positively associated with marbling (3-hydroxybutyrate, propionate, acetate, creatine, histidine, valine, and isoleucine; $P \leq 0.05$). Subcutaneous rump fat thickness was positively associated with glucose, leucine, and lipids ($P \leq 0.05$) and negatively associated with anserine and arabinose ($P \leq 0.05$). Carcase weight and growth rate were negatively associated with 3-hydroxybutyrate ($P < 0.05$), and growth rate was negatively associated with creatine ($P < 0.05$) and positively associated with aspartate ($P < 0.05$). Glucose and arginine showed a significant interaction between marbling and number of days animals consumed a high-starch diet ($P < 0.05$). Sire was the single variable with the largest effect on the relative concentration of metabolites and carcase and production traits. Blood metabolomics helps understanding fat and muscle metabolism, and is associated with genotype, and carcase and production traits in cattle offering potential biomarkers to be able to select animals based on their blood metabolome.

Keywords

Metabolome, growth, fat deposition, carcase, Wagyu cattle

2.2 Introduction

Carcase quality and value in beef cattle are determined primarily by intramuscular fat (IMF; marbling), carcase weight, eye muscle area (EMA; *Longissimus Thoracis et Lumborum*, LTL), and subcutaneous fat thickness at the 12th rib or rump site [1]. These are proxy indicators of carcase composition, meat yield, and quality, and are therefore widely used by livestock industries globally [1,2]. In some markets, marbling is the dominant commercial trait because of the relationship between marbling and sensory and eating quality of beef [3]. Wagyu cattle are renowned for high marbling and this trait is the major factor that determines carcase price together with carcase weight [4]. Phenotypic and genetic selection for marbling is difficult as it can only be accurately measured after slaughter and it has a relatively moderate heritability of 0.38 [5]. The ability to identify animals with superior carcase traits early in the production cycle would improve productivity and profitability and enable faster genetic progress.

Synthesis of adipose and muscle tissue in cattle occurs from metabolic precursors such as glucose, propionate, acetate, amino acids and lipids, amongst many others [6]. Different fat

deposits preferentially utilize certain metabolites as precursors such as IMF preference for propionate and glucose, and subcutaneous fat (SC) preference for acetate [7]. Therefore, the concentrations of metabolites in the blood of cattle would be expected to be correlated with the mass of IMF and SC. The type of diet consumed has also been associated with the blood metabolome at the time of slaughter in cattle [8] because diet affects fat and muscle deposition [7]. Beef cattle are often raised on pastures and then inducted into feedlots where high grain diets are fed to increase lipogenesis and growth rate [7]. Therefore, the blood metabolome could also be affected by the length of time animals consume a high-grain diet (days on feed, DOF). Reports are lacking on the relationship between the blood metabolome and fat and muscle tissue mass in cattle, or the effect of the number of days animals consume a high grain diets on this relationship.

The present study sought to determine the relationship between the blood metabolome and carcase traits in Wagyu-crossbred steers. ^{1}H-NMR spectroscopy of plasma was used to measure the relative concentration of metabolites as this technique can measure a wide variety of metabolites, is fast and relatively simple [9]. A significant relationship between carcase traits and the concentration of blood metabolites could potentially lead to the identification of biomarkers to predict those traits or assist with genetic improvement. The hypotheses of the present study were (1) that concentrations of blood metabolites were associated with marbling and other carcase traits in Wagyu-cross steers, and (2) that such association was not affected by the length of time animals were fed a high-energy, grain-based feedlot diet. The blood metabolome of steers was ascertained at 65, 119 and 163 DOF and steers were slaughtered after approximately 400 to 440 DOF.

2.3 Material and methods

2.3.1 Animals and experimental design

Three mixed groups of F1 (n = 127), F2 (n = 22), and F3 (n = 32) Wagyu-crossbred steers (initial LW 330 ± 1 kg SEM) were inducted on three separate occasions into a commercial feedlot in southern Queensland, Australia. The genotypes of the females crossed with Wagyu bulls were Angus (n = 16), Brahman (n = 28), Brahman crossbred (n = 26), Jersey (n = 3) and Shorthorn (n = 108). The steers generated for the study were the progeny of 23 sires (Japanese Black Wagyu full blood bulls). Group 1 had 49 steers inducted at day 0 (start of the study); Group 2 had 63 steers inducted at day 44; Group 3 had 69 steers inducted at day 97. Animals were housed in one pen but had entered the feedlot as three groups on different dates with Groups 2 and 3 entering 44 and 97 days, respectively, after Group 1. Animals were fed to allow

for *ad libitum* consumption of diets that were changed during the period in the feedlot as shown in Table 2.1.

Table 2.1: Diet formulation and chemical composition of the four rations fed at different stages in the feedlot to Wagyu crossbred steers.

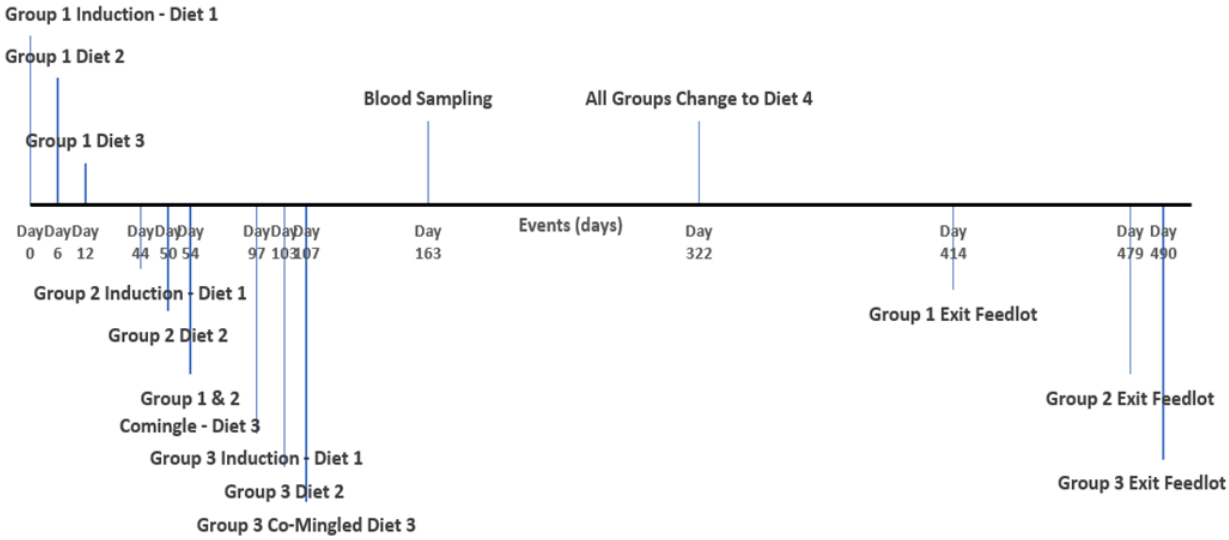
	Unit	Diet 1	Diet 2	Diet 3	Diet 4
Ingredient [1]					
Days fed Diet		0 to 6	7 to 11	12 to 323	324 to 450
Steam flaked barley	%	19	25	35	42
Steam flaked wheat	%	19	25	13	23
Grower Supplement	%	5	5	0	2
Finisher Supplement	%	0	0	5	4
Molasses	%	14	10	5	4
Vegetable oil	%	0	1	1	1
Brewers sweet grain	%	0	0	19	10
Sunflower Meal	%	9	6	2	0
Corn Silage	%	12	13	15	10
Barley Straw	%	12	11	6	5
Cereal Hay	%	12	4	0	0
Chemical composition					
Crude Protein	% DM	13.58	13.56	13.93	13.51
Neutral Detergent Fibre	% DM	31.05	26.53	25.18	20.96
Net Energy of Gain	MCAL/kg	0.99	1.15	1.25	1.35
Net Energy of Maintenance	MCAL/kg	1.61	1.79	1.91	2.02
Metabolisable Energy	MJ/KG	10.46	11.33	11.85	12.42
Ionophore	PPM	21.13	22.26	22.08	22.45

[1] *As fed basis*

Feeding changes, blood sampling and time of slaughter are shown in Figure 2.1. Each group of steers was fed diets 1 and 2 for the first 11 days in the feedlot in separate pens then were commingled with other steers in the study. Groups 2 and 3 spent less time on diet 3 compared to Group 1 as they were commingled on different days. Blood samples for metabolome analysis

were taken on the same day for all animals which meant that the number of days each group was on diet 3 was: Group 1 (152 days); Group 2 (108 days); Group 3 (54 days).

Figure 2.1: Timeline of events over the 490 days the animals were in the feedlot illustrating the individual group diet changes, blood sampling and feedlot exit in relation to experimental day.



On the day of blood sampling, animals were removed from their pen at 0600 h before feed distribution and samples were taken between 0700 and 1030 h. Blood was collected from the coccygeal vein using an 18G needle and evacuated lithium heparin tubes (Vacutainer BD, Becton Dickinson, Frankland Lakes, NJ). Samples were immediately placed on ice until centrifugation at 10,000 × g for 15 min. Plasma was stored at -80^{0}C until analysis. Days in the feedlot at slaughter were 414, 435 and 393 days for Groups 1, 2, and 3, respectively.

The Aus-Meat carcase grading was performed by an accredited assessor [10] at a commercial abattoir [10]. Information recorded included hot standard carcase weight (HSCW), a camera measure of marbling, eye muscle area (EMA), subcutaneous fat depth of the 12th rib, and subcutaneous rump fat thickness at the P8 site. The AUS-meat marbling grading scale ranges from 0 to 9+ with 0 being the lowest and 9+ the greatest marbling. The LTL muscle of each carcase was also examined for percentage marbling using high-quality digital hyperspectral images which is referred to as camera marbling (CM) (HK-333, Hayasaka Rikoh Co. Ltd., Sapporo, Japan) [11]. Marbling score is a subjective and discrete measurement whereas CM is objective and continuous.

2.3.2 Sample Preparation for metabolome profiling

Sample preparation for metabolic profiling used methods from a published protocol [12]. Samples were thawed at room temperature and an aliquot (350 uL) was mixed with 350 µL of aqueous (80% H_2O:20% D_2O) phosphate buffer solution including 0.075 M NaH_2PO_4, pH 7.4 (KOH adjusted), 0.1% sodium azide, and 1 mM 3-141 trimethylsilyl-1- [2,2,3,3, -2H4] propionate (TSP) as an internal standard. Samples were vortexed for 30 sec and then centrifuged at 6,000 x g for 10 min. An aliquot of the supernatant (600 uL) for each plasma sample was transferred to 5 mm NMR tubes (Bruker, SampleJet 5mm, Billerica MA, USA) for ^{1}H-NMR analysis.

Samples were analysed with a Bruker Advance III 600 MHz spectrometer equipped with a 5-mm TCI cryoprobe. Samples were run under automation mode using a Sample Jet with all samples refrigerated at 278˚ K until just prior to acquisition. Data were collected at 310˚ K for a total of 20 min. ^{1}H-NMR spectra were acquired using the noesygrrp1d and cpmgpr1d pulse sequences (32 scans collected for each experiment). Irradiation of the solvent (water) resonance is applied during pre-saturation delay (4.0 s) for all spectra and for the noesy also during the mixing time (0.01 s). The pulse sequence parameters, most notably the 90° pulse (~ 12 µs) are optimised for each sample. The data were collected with approximately 96 k (noesy) or 32 k (cpmg) real data points and processed with an exponential line broadening of 0.3 Hz prior to Fourier transformation.

Data were imported into Matlab 7.0 Software (Mathworks, Natick, MA). NMR spectra were aligned and normalised by automatically phasing, baseline correcting and referencing the dataset to the α-C_1H-Glucose doublet (5.233 ppm) [13]. The residual water (2.42-3.14 ppm) was truncated from the dataset to reduce analytical variability. Statistical recoupling of variables was performed on the aligned and normalised spectrum which selected the start and end points of clusters [14]. Therefore, this output contains several clusters or buckets which are chosen as they represent features in the spectral matrix. The cluster value for each sample is simply the area under the curve for each cluster (component or peak). These values are used as relative concentrations and were multiplied by $1x10^6$ to reduce the number of decimal places. In parallel, the raw spectra were imported into Chenomx® for the assignment of metabolites to these clusters, with metabolites identified using the profiler and library manager models within. This was achieved by comparing ^{1}H-NMR spectra to the spectral library of Chenomx® NMR Suite Professional (Chenomx Inc., Edmonton, AB, Canada) as well as referencing from published literature and the Livestock Metabolite Database [9,15,16]. Once clusters were assigned

to a metabolite, the sum of the area under the curve for all clusters belonging to a metabolite was calculated.

2.3.3 Statistical analyses

A general linear model was used to analyse the fixed effect of days in the feedlot (DOF), breed, generation (F1, F2 or F3), and sire (bull) on carcase and performance traits including Aus-Meat marble, CM, rib fat, P8 fat and HSCW, amongst others. Least Square Means were calculated for each DOF and differences between means adjusted for multiple comparisons using the Tukey method. The same general linear model was used to perform analysis of covariance adding each carcase trait as a covariate to determine their association with the relative concentration of metabolites (dependent variable). This model allowed estimating partial correlation coefficients between carcase traits and the relative metabolite concentration. The model also included the covariate × DOF interaction to test the hypothesis that the relationship (slope) remains constant across DOF. All statistical analyses were done using SAS 9.4 (SAS Institute Inc., Cary, New Jersey, USA). Significant statistical differences were declared at $P \leq 0.05$ and tendencies discussed at $0.05 < P \leq 0.10$.

Principal component analysis' (PCA) were conducted using the relative concentration of the 35 identified metabolites as variables in the model. Then, PC1 and PC2 were plotted against each other to visualise the clustering of animals according to DOF and CM. The first 5 PC were selected for the final PCA as these had an eigenvalue >1.

2.4 Results

Table 2.2 shows the mean values for carcase and production traits for each DOF group of animals. The DOF groups at the time of blood sampling affected all variables except marbling and Wagyu percentage ($P > 0.05$; Table 2.2). Group 1 sampled at 163 DOF showed thinner rump fat, thicker rib fat, and were older compared to animals sampled at 65 (Group 3) and 119 DOF (Group 2) ($P < 0.05$). Group 2 (119 DOF) had heavier carcases and live weight compared to Group 3 (65 DOF) ($P < 0.05$). Sire was the most important fixed effect affecting most carcase and production traits ($P<0.05$) except rib fat and EMA ($P > 0.10$). The accompanying breed (crossbreed) only affected age and Wagyu percentage ($P < 0.05$). Generation (F1, F2, F3) only affected growth rate ($P < 0.009$) and Wagyu percentage ($P < 0.001$) but did not affect other carcase traits ($P > 0.05$).

Table 2.2: Carcase traits, age and live weight of Wagyu cross steers that were blood sampled for metabolomics analysis at different days on feed (DOF).

	Days on Feed			P-Value			
	65	119	163	DOF	Breed	Generation	Sire
No. Animals	69	63	49	-	-	-	-
Aus-Meat Marble Score	6.2 ± 0.56	5.8 ± 0.52	5.9 ± 0.52	0.279	0.404	0.384	0.002
Camera marbling (%)	25.8 ± 2.22	25.4 ± 2.07	24.0 ± 2.07	0.182	0.996	0.599	0.008
Rump Fat (mm)	27.6 ± 1.06 ^{A}	27.2 ± 0.97 ^{A}	23.7 ± 1.01 ^{B}	0.006	0.628	0.520	<0.001
Rib fat (mm)	11.2 ± 0.91 ^{A}	10.8 ± 0.84 ^{A}	15.8 ± 0.86 ^{B}	<0.001	0.799	0.519	0.516
Eye muscle area (cm^2)	40.7 ± 0.89 ^{A}	36.7 ± 0.83 ^{B}	41.4 ± 0.85 ^{A}	<0.001	0.567	0.998	0.308
Growth rate (kg/d)	0.99 ± 0.031 ^{AB}	1.00 ± 0.030 ^{B}	1.05 ± 0.032 ^{A}	0.009	0.097	0.009	<0.001
Carcase weight (kg)	428 ± 5.4 ^{B}	449 ± 6.0 ^{A}	434 ± 5.2 ^{AB}	0.007	0.784	0.755	0.044
Age at Induction (days)	688 ± 29.9 ^{A}	666 ± 28.0 ^{A}	721 ± 28.0 ^{B}	0.001	0.021	0.066	<0.001
Age at Slaughter (days)	1111 ± 18.1 ^{A}	1112 ± 17.2 ^{A}	1147 ± 18.9 ^{B}	0.026	0.030	0.065	<0.001
Induction Live Weight (kg)	326 ± 3.1 ^{A}	336 ± 2.8 ^{B}	321 ± 2.9 ^{A}	<0.001	0.373	0.310	0.028
Exit Live Weight (kg)	745 ± 9.0 ^{B}	783 ± 8.3 ^{A}	766 ± 8.6 ^{AB}	0.003	0.816	0.402	0.054
Wagyu content (%)	72.6 ± 0.76	73.2 ± 0.71	73.0 ± 0.71	0.254	0.004	<0.001	0.002

$^{A, B, C}$ *Means within rows without a common superscript differ (P < 0.05).*

The standard recoupling of variables identified 315 features or peaks from the ^{1}H-NMR spectrum. From these clusters, 35 metabolites were identified using the available Chenomx® database of compounds, and their identity validated from previous literature and the livestock metabolome database. A representative ^{1}H-NMR spectrum indicating 11 identified metabolites is shown in Figure 2.2 to illustrate the multiple features. Also noted are unknown peaks that could not be identified and data on these are not presented in this report.

Figure 2.2: Representative ^{1}H-NMR spectrum of plasma from a Wagyu crossbred steer showing clusters assigned to different metabolites.

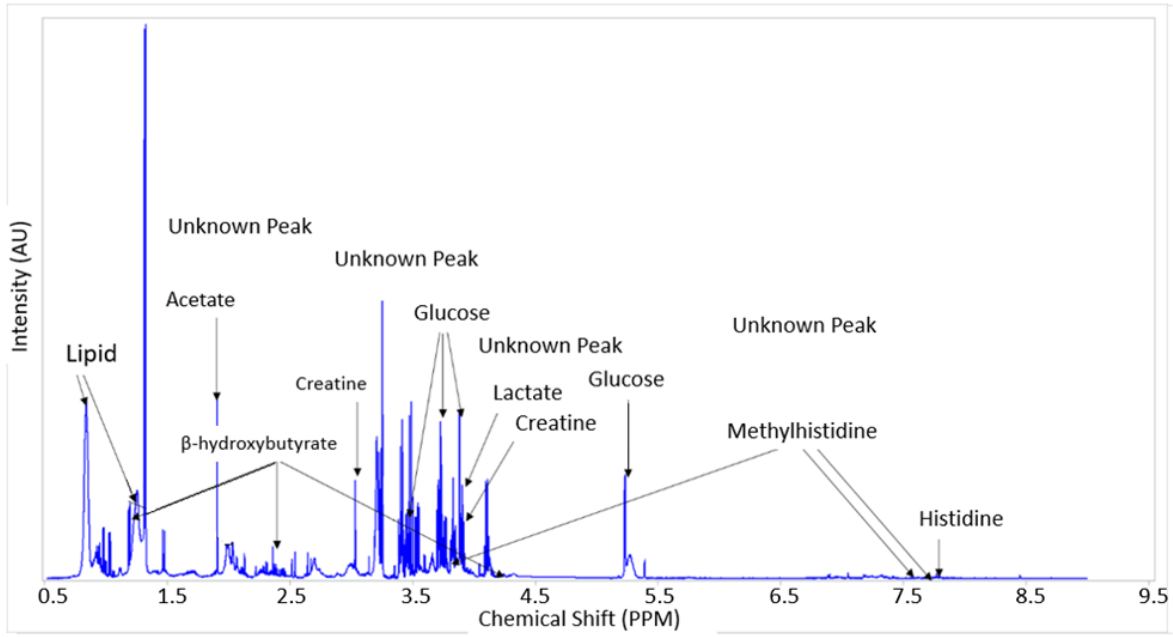


Table 2.3 shows descriptive statistics for each metabolite. Glucose had the largest number of clusters due to the large size of the molecule with the greatest area under the curve; however, glucose showed low variability (CV) amongst animals. Smaller metabolites such as formate and acetate had lower number of features, lower area under the curve (relative concentrations) and larger variability across animals.

Table 2.3: Descriptive statistics of the relative concentration of metabolites identified in blood plasma of Wagyu cross steers using H NMR (N=181).

Variable	No. Clusters	Minimum	Mean	Maximum	STD Error	CV
3-hydroxybutyrate	2	108.9	174.0	284.6	2.30	17.9
Acetate	1	65.6	197.9	371.2	4.40	30.1
Acetone	1	17.0	25.3	49.2	0.34	18.2
Anserine	1	280.6	339.5	336.3	1.97	7.8
Arabinose	1	51.8	89.4	120.2	0.97	14.6
Arginine	1	307.8	387.2	484.4	2.46	8.6
Aspartate	1	17.5	24.4	30.6	0.19	10.7
Carnosine	1	63.7	87.7	124.5	0.69	10.6

Choline	1	63.7	87.6	124.5	0.69	10.6
Citrate	2	47.0	86.0	114.4	0.87	13.7
Citrulline	1	13.1	19.5	19.5	0.17	11.5
Creatine	2	136.2	174.5	221.6	1.14	8.8
Creatinine	2	158.0	238.1	320.7	1.86	10.6
Dimethyl sulfone	1	24.2	35.0	46.9	0.32	12.3
Formate	1	1.91	3.8	32.9	0.17	60.2
Glucose	16	2622	3248	4005	19.23	8
Glutamate	1	16.3	24.2	35.0	0.21	11.9
Glutamine	8	157.7	218.6	280.9	1.40	9.0
Glycine	1	98.7	138.6	200.9	1.39	13.6
Histidine	2	246.0	351.7	490.4	3.52	13.5
Isobutyrate	3	201.9	246.2	301.2	1.20	6.6
Isoleucine	2	116.1	145.2	176.0	0.83	7.7
Lactate	2	323.0	649.8	1484.4	13.88	28.9
Leucine	2	127.9	168.0	209.5	1.06	8.5
Lipid	12	1378.4	1968.5	2409.3	12.85	8.8
Mannose	1	7.8	11.0	17.3	0.12	14.9
Methionine	3	118.6	168.2	215.5	1.11	9.0
Methylamine	1	11.5	36.6	54.3	0.52	19.2
Methyl histidine	3	165.4	200.2	241.6	1.05	7.1
Phenylalanine	3	24.3	34.4	43.82	0.25	9.8
Proline	5	39.7	58.6	101.1	0.64	14.8
Propionate	1	8.3	18.1	24.61	0.15	11.1
Serine	3	126.9	176.0	297.58	1.49	11.4
Tyrosine	3	155.4	191.6	229.83	1.11	7.8
Valine	4	284.4	381.9	480.16	2.36	8.4

The general linear models indicated that the relative concentration of metabolites was not affected by breed, generation, or the interactions between fixed effects ($P > 0.05$), and these factors were therefore excluded from the models (data not shown). Results from the analysis of covariance showing partial correlation coefficients between carcase traits and the relative

concentration of metabolites are presented in Figure 2.3. A strong positive correlation was found between CM and marbling score, and growth rate and carcase weight (*P < 0.001*). A modest positive correlation was found between growth rate or carcase weight and subcutaneous rump fat (P < 0.001), and a weak negative correlation between marbling and growth rate or carcase weight (*P < 0.05*). Marbling was not correlated with subcutaneous rump or rib fat (P > 0.10) and a positive correlation was found between marbling score and EMA (P < 0.01; Table 3). Eye muscle area tended to be correlated with growth rate (*P < 0.10*) but not with other carcase traits (P > 0.10).

Camera marbling was positively correlated with 3-hydroxybutyrate, propionate, acetate, histidine, creatine, and isoleucine *(P ≤ 0.05;* Figure 2.3). Similar positive correlations were found between Aus-Meat marble score and the relative concentration of those metabolites although valine also reached significance *(P < 0.05*). No negative correlations were found between CM or marble score and the relative concentration of metabolites (*P > 0.05*). Subcutaneous rib fat showed a negative correlation with dimethyl sulfone (P < 0.05) and a negative tendency with acetate and isobutyrate (P < 0.10). Subcutaneous rump fat depth was positively correlated with lipids, glucose, and leucine (*P < 0.05*), and tended to be positively correlated with acetate and lactate (P < 0.10). Rump fat was negatively correlated with anserine and arabinose (*P < 0.05*; Figure 2.3). Carcase weight, growth rate and eye muscle area did not show positive correlations with metabolites (*P > 0.05*) except for that between aspartate and growth rate (P < 0.05). Carcase weight and growth rate were negatively correlated 3-hydroxybutyrate, and growth rate also with creatine (*P < 0.05*). Eye muscle area did not show significant correlations with any metabolite except for a negative trend with mannose, leucine, and citrate (P ≤ 0.10; Figure 2.3).

Figure 2.3: Heat map illustrating partial correlation coefficients of the relationship between metabolites and carcase traits.

Dependent	Camera Marbling	Marbling Score	Rib Fat	Rump Fat	Growth rate	Carcase weight	Eye Muscle Area
Camera marbling		0.79***	-0.01	0.04	-0.16*	-0.15*	0.07
Marbling Score			0.03	0.00	-0.12†	-0.12	0.19**
Rib Fat				0.07	0.10	0.03	-0.08
Rump Fat					0.36***	0.41***	0.05
Growth rate						0.88***	0.13†
Carcase weight							0.09
Eye Muscle Area							
3-Hydroxybutyrate	0.29***	0.25***	-0.07	0.03	-0.17*	-0.15*	-0.03
Propionate	0.27****	0.22**	-0.10	-0.04	0.04	0.04	0.02
Acetate	0.22**	0.21**	-0.13†	0.12†	-0.10	-0.07	-0.05
Histidine	0.19**	0.17*	-0.08	0.03	-0.02	-0.01	-0.05
Creatine	0.15*	0.17*	-0.02	-0.06	-0.14*	-0.09	0.04
Isoleucine	0.15*	0.16*	0.02	-0.06	0.07	0.02	-0.09
Acetone	0.14†	0.13†	-0.08	0.09	-0.06	-0.06	-0.06
Isobutyrate	0.13†	0.14†	-0.13†	-0.11	0.00	-0.05	-0.04
Valine	0.12†	0.20**	0.06	0.10	-0.04	-0.01	-0.09
Arginine	0.08	0.05	-0.00	-0.08	-0.11	-0.10	-0.01
Anserine	0.07	0.00	-0.01	-0.16*	-0.10	-0.10	-0.04
Methyl-histidine	0.06	0.03	-0.03	-0.14†	-0.07	-0.11	-0.06
Glutamine	0.05	0.12†	-0.08	-0.00	0.05	-0.02	-0.04
Glucose	0.04	0.00	-0.02	0.15*	-0.10	-0.10	-0.03
Glycine	0.04	-0.01	-0.06	0.02	0.13†	0.08	-0.05
Mannose	0.02	0.03	0.05	0.06	-0.09	-0.04	-0.13†
Leucine	0.02	0.13†	0.02	0.14*	0.04	0.01	-0.13†
Glutamate	0.02	0.05	0.06	0.01	0.05	0.01	-0.06
Serine	0.02	0.04	0.00	-0.00	0.01	-0.08	-0.05
Lactate	0.01	0.03	-0.08	0.13†	0.03	0.00	0.09
Tyrosine	0.01	0.02	-0.02	0.07	0.02	0.04	-0.04
Methionine	0.01	0.06	-0.02	-0.02	-0.08	-0.02	-0.10
Dimethyl sulfone	0.00	0.06	-0.14*	-0.02	-0.09	-0.05	0.08
Choline	-0.01	-0.05	-0.03	0.02	0.02	0.00	-0.03
Aspartate	-0.03	-0.04	-0.04	0.01	0.14*	-0.06	-0.08
Phenylalanine	-0.04	0.02	0.06	0.03	0.04	0.02	-0.07
Creatinine	-0.05	-0.10	-0.06	-0.10	0.03	0.00	-0.03
Formate	-0.05	0.03	0.03	-0.11	0.01	0.03	0.02
Proline	-0.05	-0.05	0.06	-0.14†	0.12	0.07	-0.01
Carnosine	-0.06	-0.02	-0.11	0.02	0.07	0.00	-0.10
Methylamine	-0.08	-0.02	-0.10	0.01	0.09	0.0	-0.10
Arabinose	-0.09	-0.10	0.08	-0.14*	0.01	0.04	0.00
Citrate	-0.09	-0.01	-0.11	0.02	0.08	0.00	-0.12†
Lipids	-0.10	-0.09	0.07	0.18**	0.05	0.10	-0.02
Citrulline	-0.14	-0.11	-0.08	0.08	0.06	0.03	-0.01

****, **, *, † is for $P \leq 0.001$, $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.10$, respectively.*

The analysis of covariance for CM showed that sire affected the relative concentration of 16 metabolites ($P \leq 0.05$) and tended to affect another 6 metabolites ($P \leq 0.05$; Table 2.4). Confirming results from correlation analysis, the relative concentration of 3-hydroxybutyrate, propionate, acetate, creatine, and histidine increased with CM ($P < 0.05$) and no metabolites decreased with CM. The main effect of DOF affected anserine, arginine, glucose, and methyl histidine ($P \leq 0.05$); however, these metabolites also showed a DOF × CM interaction as it was the trend observed for lipids as well ($P \leq 0.10$; Table 2.4). Figure 2.4 illustrates the linear relationship between the relative concentration of glucose and marbling for each DOF group. Glucose showed a linear decrease with marbling at 65 DOF *($P < 0.05$*), no effect at 119 DOF (*$P > 0.10$*) and increased with marbling at 163 DOF (*$P < 0.05$*). In contrast, the relative concentration of arginine (data not shown) and lipids (Figure 2.4) increased with marbling at 65 DOF (*$P < 0.05$*), no change at 119 DOF (*$P > 0.05$*) and decreased at 163 DOF (*$P < 0.05$*).

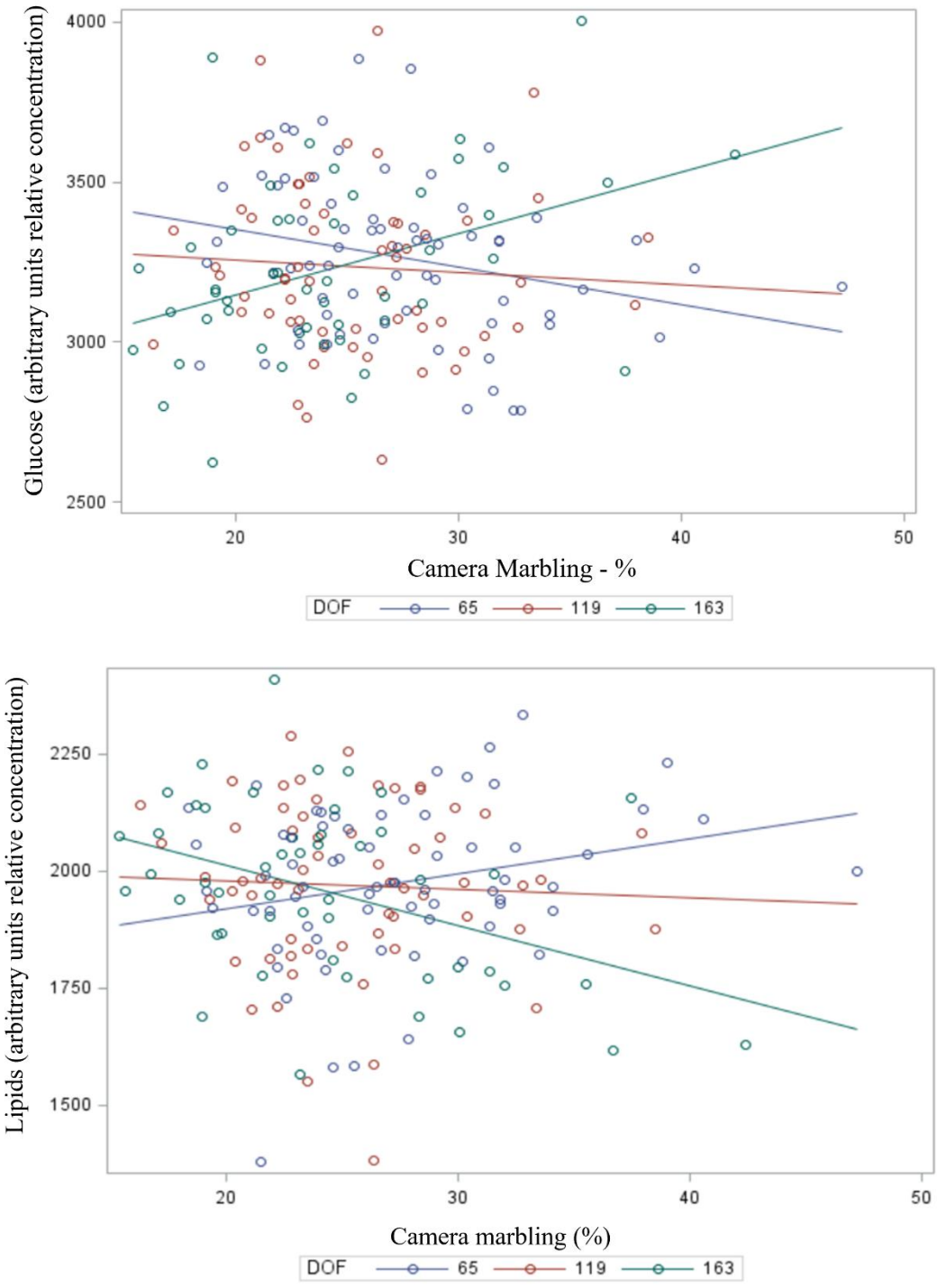
Table 2.4: Effect of days on feed (DOF) and camera marbling on the relative concentration of blood metabolites in Japanese Black Wagyu crossbred steers. [A, B, C] Means without a common superscript differ (*$P < 0.05$*).

	Days on Feed (DOF)			Marbling	P-value			
Metabolite	65	119	163	Regression ± SE	DOF	Marbling	Marb × DOF	Sire
3-Hydroxybutyrate	170.4 ± 5.46	181.0 ± 4.96	180.4 ± 5.15	1.82 ± 0.504	0.488	<0.001	0.249	0.482
Acetate	202.3 ± 10.61	211.9 ± 9.62	181.9 ± 10.01	3.04 ± 0.985	0.156	0.003	0.159	0.529
Acetone	24.1 ± 0.83	25.6 ± 0.75	26.6 ± 0.78	0.1 ± 0.077	0.668	0.055	0.354	0.657
Anserine	347.1 ± 4.51 A	335.1 ± 4.09 B	335.8 ± 4.26 B	0.43 ± 0.426	0.005	0.332	0.014	0.062
Arabinose	91.7 ± 2.18	90.6 ± 1.98	87.7 ± 2.06	-0.17 ± 0.2	0.575	0.207	0.353	0.008
Arginine	393.2 ± 5.76 A	379.5 ± 5.22 B	390.9 ± 5.43 AB	0.51 ± 0.538	0.032	0.240	0.026	0.071
Aspartate	24.9 ± 0.46	24.6 ± 0.42	24.0 ± 0.43	0.03 ± 0.042	0.401	0.685	0.350	0.303
Carnosine	48.2 ± 1.3	50.6 ± 1.18	47.6 ± 1.22	0.12 ± 0.119	0.779	0.424	0.642	0.093
Choline	88.1 ± 1.59	87.4 ± 1.44	85.6 ± 1.5	0.05 ± 0.144	0.887	0.937	0.747	0.023
Citrate	86.4 ± 2.08	88.6 ± 1.89	84.1 ± 1.96	0.29 ± 0.19	0.551	0.219	0.359	0.126
Citrulline	19.0 ± 0.4	19.2 ± 0.36	19.5 ± 0.37	-0.06 ± 0.036	0.117	0.153	0.150	0.024
Creatine	387.7 ± 6.5	388.9 ± 5.9	376.1 ± 6.14	1.49 ± 0.594	0.499	0.033	0.721	0.012
Creatinine	31.1 ± 0.66	32.7 ± 0.6	33.7 ± 0.62	-0.08 ± 0.062	0.829	0.483	0.903	0.012
Dimethyl sulfone	34.5 ± 0.76	34.7 ± 0.69	33.5 ± 0.71	0.01 ± 0.069	0.915	0.952	0.962	0.028
Formate	3.67 ± 0.12	3.62 ± 0.11	3.49 ± 0.11	0.01 ± 0.011	0.775	0.501	0.600	0.116
Glucose	3,263 ± 43.8 A	3,182 ± 39.69 B	3,240 ± 41.29 A	1.22 ± 4.059	0.033	0.599	0.031	0.007
Glutamate	25.0 ± 0.48	25.3 ± 0.43	23.6 ± 0.45	0.04 ± 0.045	0.430	0.760	0.749	0.000
Glutamine	219.9 ± 3.37	221.4 ± 3.06	212.4 ± 3.18	0.37 ± 0.31	0.952	0.441	0.977	0.006
Glycine	145.9 ± 3.19	137.1 ± 2.89	131.6 ± 3.01	0.09 ± 0.304	0.484	0.628	0.490	0.285
Histidine	346.4 ± 8.22	356.4 ± 7.46	361.2 ± 7.76	1.8 ± 0.751	0.609	0.009	0.462	0.035
Isobutyrate	243.2 ± 2.83	247.3 ± 2.57	246.2 ± 2.67	0.44 ± 0.257	0.963	0.068	0.893	0.011
Isoleucine	148.6 ± 1.99	143.4 ± 1.8	139.0 ± 1.88	0.38 ± 0.189	0.657	0.186	0.973	0.992
Lactate	620.4 ± 31.88	657.3 ± 28.92	748.0 ± 30.08	-1.24 ± 2.984	0.881	0.854	0.868	0.064
Leucine	166.7 ± 2.51	167.6 ± 2.27	163.9 ± 2.37	0.1 ± 0.227	0.946	0.768	0.929	0.076
Lipid	1,989 ± 28.7	1,977 ± 26.04	1,931 ± 27.08	-2.15 ± 2.66	0.185	0.167	0.073	0.014
Mannose	10.9 ± 0.29	11.3 ± 0.26	11.0 ± 0.27	-0.01 ± 0.026	0.897	0.749	0.970	0.046
Methionine	171.0 ± 2.57	171.1 ± 2.33	162.2 ± 2.42	0.16 ± 0.24	0.758	0.897	0.998	0.012
Methylamine	36.4 ± 1.25	38.3 ± 1.14	35.7 ± 1.18	0.15 ± 0.115	0.707	0.287	0.533	0.210
Methyl histidine	204.0 ± 2.47 A	198.8 ± 2.24 B	197.5 ± 2.33 B	0.23 ± 0.23	0.040	0.404	0.096	0.096

Phenylalanine	33.9 ± 0.63	34.2 ± 0.57	33.9 ± 0.59	-0.03 ± 0.056	0.946	0.612	0.915	0.618
Proline	58.7 ± 1.55	57.7 ± 1.4	57.4 ± 1.46	-0.08 ± 0.14	0.968	0.472	0.910	0.204
Propionate	17.2 ± 0.32	17.9 ± 0.29	18.6 ± 0.31	0.1 ± 0.031	0.904	< 0.001	0.899	0.004
Serine	174.5 ± 3.26	172.7 ± 2.95	172.6 ± 3.07	-0.09 ± 0.294	0.786	0.791	0.837	0.260
Tyrosine	192.5 ± 2.49	192.9 ± 2.26	190.3 ± 2.35	0.04 ± 0.226	0.604	0.864	0.599	0.000
Valine	375.1 ± 5.65	380.4 ± 5.13	374.7 ± 5.33	0.84 ± 0.511	0.936	0.104	0.882	0.109

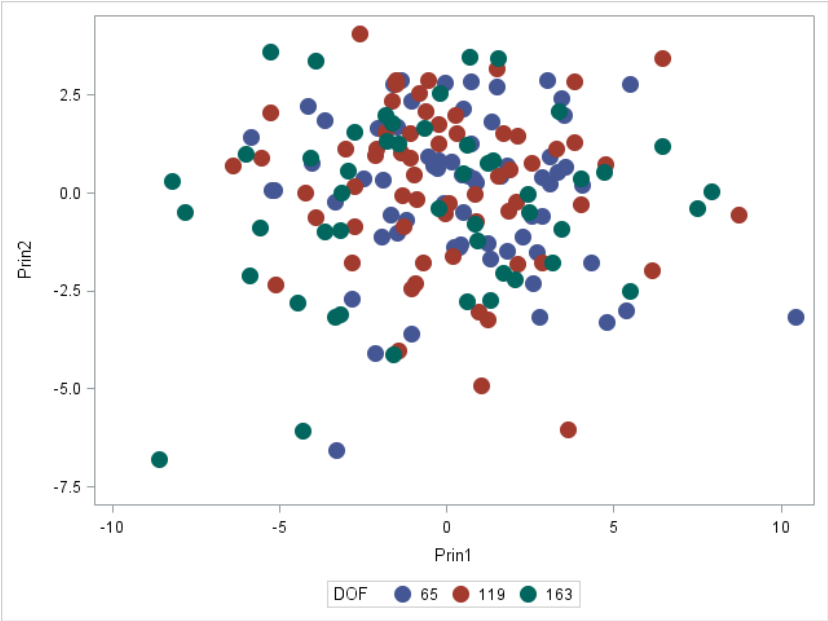
[A, B, C] *Means without a common superscript differ (P < 0.05).*

Figure 2.4: Analysis of covariance for lipids and glucose showing the DOF and CM interaction.



The PC1 explained only 11.61% of the variability within the dataset of 35 identified metabolites and PC2 explained only 4.46% of the variability. The plot with both PC1 and PC2 demonstrated that there was no clustering of animals according to DOF (Figure 2.5) with the data points from different groups randomly distributed. Similarly, this plot did not highlight a strong relationship between PC and CM relationship between (Figure 2.6).

Figure 2.5: Principal component analysis of 35 blood metabolites of Wagyu-cross steers showing PC 1 vs PC 2 with the days on feed (DOF) group coloured for each data point.



2.5 Discussion

A hypothesis tested in the present study was that the concentrations of blood metabolites were associated with marbling and other carcase traits in Wagyu-cross steers. This hypothesis was supported by significant associations between the relative concentration of metabolites and carcase traits. The blood metabolome was ascertained at approximately 300 days before steers were slaughtered to collect data on carcase traits. This is highly important because it suggests that the metabolome could be used for the early identification of steers with the propensity to marble, which could have major implications for efficient utilization of feed in steers that produce a carcase of high value.

2.5.1 Genetics

In addition, the potential for metabolomics to inform genetic selection is supported by the significant effect of sire on both carcase traits and the metabolic profile of animals. Sire was the single most important factor affecting carcase and performance traits and the relative concentration of metabolites. Sixteen of 35 metabolites were affected, and a further 6 metabolites tended to be affected, by sire. On average, sire explained 21.5% and marbling 4.8% of the variability in the relative concentration of these significant metabolites. The effect of sire on carcase traits is expected because of the known heritability of these in Wagyu cattle [1]. However, the effect of genotype on blood metabolomics of cattle has to the knowledge of the authors not previously been reported. A recent study reported that blood metabolites were associated with feed efficiency in feedlot cattle, a trait that also affects carcase traits [2]. Genetic progress could be improved if metabolomic information is considered together with pedigree and genomic information as previously suggested [3,4].

In addition to the early identification of animals with propensity to marble and genetic selection, the identification of metabolites correlated with performance and body composition could improve the understanding of fat and muscle biology, metabolic pathways, and the function of metabolites in cattle. The interpretation of metabolomics data in cattle is challenging due to limited information on the synthesis and utilization of metabolites for tissue metabolism, deposition, and mobilization. Complex interactions can affect the concentration of metabolites in blood such as absorption of metabolites from the gastrointestinal tract, synthesis of metabolites in organs and tissues, and uptake of metabolites by tissues for deposition and degradation (e.g., complete oxidation). A recent review found 79 articles that identified 8 or more metabolites in cattle [5], suggesting that the use of metabolomics in bovine studies is relatively unexplored. The present study found positive and negative associations between multiple metabolites and the extent of tissue accrued in different depots (intramuscular and subcutaneous fat, growth rate, eye muscle area and carcase weight at slaughter). Therefore, blood metabolomics in cattle could help to unravel metabolic pathways and mechanisms of fat and muscle in body systems. Blood proteomics has shown potential for biomarkers of tenderness in cattle but the complexity of biological systems makes it unlikely that any single biomarker will have an outstanding effect [6]. In the present study none of the metabolites were strongly correlated to carcase traits. However, the fact several metabolites showed significant

correlations with carcase traits indicates that further research is warranted on the identification and potential applications of using multiple biomarkers or metabolites.

2.5.2 Fat Metabolism

Marble scores ranged from 3 to 9+ in the present study (average of 5.97) and such variability across animals decreases with increased Wagyu content [7]. Japanese Black Wagyu cattle may be a good model to study fat metabolism in cattle due to such extent of marbling in this breed. Both carcase weight and marbling should be considered together in any balanced breeding program because the value of a carcase is determined by both traits and there is often a trade-off with a negative correlation between these two traits [8]. In the present study animals that grew faster tended to have larger EMA, heavier carcases, thicker subcutaneous rump fat and lesser marbling. The negative association between marbling and carcase weight may suggest that animals that direct more nutrients to IMF deposition may direct less nutrients to skeletal muscle and bones resulting in a lower carcase weight. Some metabolites reflected this negative association such as 3-hydroxybutyrate and creatine which were positively associated with marbling and negatively with growth rate or carcase weight. Both 3-hydroxybutyrate and creatine are key energy sources for cattle; however, 3-hydroxybutyrate is a key metabolite involved in fat tissue metabolism [9]. Creatine is a key metabolite facilitating the recycling of ATP predominantly in brain tissue and muscle [10]. In agreement with the present study, double-muscled Belgian Blue cattle had heavier carcases with higher proportion of lean tissue in the 7th rib cut which coincided with lower plasma concentration of creatine and higher of creatinine compared to conventional Belgian Blue cattle. A previous study [11] reported higher concentration of 3-hydroxybutyrate for genetic lines with lighter carcases and lower body fat proportion in Charolais x Holstein crosses although marbling score was not different between lines. The metabolite 3-hydroxybutyrate originates from either absorption of acetate from the rumen (~70%) or hepatic oxidation of long chain fatty acids, particularly from fat mobilization during negative energy balance [12]. Animals in the present study were growing and in positive energy balance, so it is speculated that the positive association between 3-hydroxybutyrate and marbling is either due to greater absorption of acetate from the rumen or faster fat turnover rate in animals with higher marbling. Therefore, circulating 3-hydroxybutyrate in cattle seems to reflect different metabolic pathways depending on whether animals are in positive or negative energy balance. Furthermore, the present study suggested that 3-hydroxybutyrate is one of the most important metabolites for IMF deposition in Wagyu feedlot cattle under positive energy

balance and fast growth rates. Mobilization of protein from muscle and fat under negative energy balance is also reflected through an increase of 3-methylhistidine [9] and decrease of creatinine [13]. However, these metabolites were not correlated to any carcase trait in the present study which could be due to the positive energy balance of steers.

Most of the energy used by ruminants comes from ruminal microbial degradation of feed which produces volatile fatty acids (VFA) with acetic, propionic and butyric acids being the most important [14]. Acetate is a key lipogenic substrate in ruminants and once absorbed in the blood most of the acetate is converted to 3-hydroxybutyrate, oxidized via the tricarboxylic acid cycle (TCA) or used for fatty acid synthesis [15]. Propionate reaches the liver where it is either oxidized or enters the TCA cycle as succinyl-CoA to form glucose. However, there is no apparent agreement in the literature as to which metabolites are the most important precursors of the different fat depots and muscle defining body composition in cattle. It has been reported that acetate and glucose are the major precursors for fatty acid biosynthesis, with glucose being preferred by intramuscular adipocytes and acetate by subcutaneous fat depots [12,16-18]. It has been shown that plasma propionate increases the secretion of insulin which activates lipogenic enzymes and accelerates fatty acid synthesis increasing intramuscular fat [19]. Smith and Crouse [20] demonstrated in vitro that 70-80% of the acetyl units contributed to lipogenesis in subcutaneous fat were from acetate, 50-75% in intramuscular fat were from glucose, and 15-30% in either intramuscular or subcutaneous fat were from lactate. A previous review [12] also concluded that the main precursors for IMF deposition in ruminants are lactate and glucose, and acetate to a lower extent. The present study supports the hypothesis that both circulating propionate and acetate have a similar positive influence on marbling however 3-hydroxybutyrate seems to play the most important role. In contrast, we did not find significant relationships between lactate or glucose and marbling to support previous observations.

Acetate was the only metabolite that tended to be positively correlated with both intramuscular and subcutaneous rump fat depots, and negatively with subcutaneous rib fat thickness. In addition, subcutaneous rump fat tended to be positively associated with glucose, lipids, leucine, and lactate. However, none of these metabolites were correlated with marbling, subcutaneous rib fat, carcase weight or growth rate. The positive correlation between lipids and rump fat contrasts with trends reported in post-partum dairy cows losing weight under negative energy balance, which showed that circulating lipids (triglycerides, phospholipids and cholesterol)

increased concomitantly with a decrease in body weight, condition score, backfat thickness and LTL muscle diameter [13].

2.5.3 Amino Acids and Muscle Metabolism

Valine, isoleucine and leucine are branched chain amino acids known to enhance lipolysis at insufficient or excessive concentrations but can also increase lipogenesis [21]. In the present study, these amino acids were positively associated with marbling and in the case of leucine with rump fat as well. Isobutyrate is a branched chain volatile fatty acid produced by rumen fermentation of amino acids [22] however their effect on fat synthesis and deposition in cattle seems unknown. Based on the positive associations between these metabolites and IMF reported in the present study, we speculate that branched chain amino acids, histidine and isobutyrate could promote lipogenesis or fat deposition, or both in IMF. However, these are just speculations and further research is required to understand the role of these metabolites on fat and muscle metabolism.

In the present study, some metabolites seemed to be involved in both muscle and IMF metabolism (3-hydroxybutyrate and creatine), others in both intramuscular and subcutaneous fat depots but not in body growth (e.g., acetate), others in IMF only (e.g., propionate), and others only significant for one of the subcutaneous fat depots only (e.g., lipids on rump fat). The fact that some metabolites were correlated with only one of the fat depots or tissues could allow more targeted genetic progress for one (e.g., marbling) against other tissues or depots which have lower commercial value (e.g., subcutaneous fat). The marked differences found between rib and rump fat metabolism of the present study requires further research to understand the reasons for this finding.

2.5.4 Effect of DOF on the Blood Metabolome

The second objective of the present study was to investigate the influence of the length of time that cattle consumed a high grain and starch diet (DOF) on the blood metabolome and determine if the correlation between metabolites and marbling was affected by DOF. This is critical information that needs to be known before an attempt is made to use metabolomics for the prediction of important carcase traits or genetic selection in cattle as the amount of DOF at sampling may alter the metabolomic profile of the animals. Both carcase and production traits were affected by DOF, which was unexpected because animals in the present study were randomly selected from a commercial producer of feeder cattle and the animals were all taken

from one breeding cohort of animals. Importantly, marbling was not different between DOF groups, indicating that the results reported on the relationship between the metabolome and marbling is not confounded by DOF groups having different marbling. No metabolites were affected by the DOF main factor only and, therefore, the relative concentration of metabolites does not seem to be affected by the length of time animals consume a high grain diet in the ranges evaluated in the present study. These results were supported by the PCA which did not show any clustering of data points according to DOF.

A similar study [23] to the present one, identified 45 metabolites to examine the relationship between the blood metabolome and residual feed intake of feedlot cattle sampled at 14, 42 and 70 DOF. The metabolites selected as predictors of residual feed intake differed amongst DOF; however, it was unclear if these results were related to recent diet changes, the length of time animals consumed a high-grain diet, or different environmental conditions across sampling dates, amongst others. The present study used animals well adapted to the high-grain diet (animals were consuming the high grain diet for at least 54 days at time of sampling) and all animals were sampled on the same date to avoid the effect of environmental conditions

Arginine, glucose, and lipids were the only metabolites influenced by the interaction between DOF and CM. The relative concentration of glucose increased, and lipids decreased, as marbling increased in animals sampled later (163 DOF); however, the opposite trend was reported for animals sampled earlier in the feeding period (65 DOF). The findings in the present study suggest that the relationship between marbling and arginine, glucose and lipids is affected by DOF, and thus DOF needs to be considered to predict marbling from these metabolites. This is an important finding as the ability to sample and identify desirable animals at an earlier stage can reduce the economic cost of feeding these animals for a longer time. Therefore, the most promising metabolites to predict marbling are those not affected by DOF, or the length of time animals consume a high grain diet in the feedlot.

2.6 Conclusion

Blood metabolomics in cattle shows potential biomarkers that could help to better understand fat and muscle metabolism and predict economically important carcase traits at 10 to 14 months before slaughter. These could be used to identify and select individual animals with desirable carcase traits. The length of time in the feedlot when animals are sampled appears not to be a critical factor affecting the blood metabolome. Genotype has a large influence on both blood

metabolomics and carcase and production traits suggesting that ^{1}H NMR metabolomics could assist with genetic improvement of cattle for relevant production and carcase traits and meat quality.

Animal Ethics

The study had animal ethics approval from The University of Sydney Animal Ethics Committee: Protocol no. 1125. The study was undertaken in accordance with the Australian code for the care and use of animals for scientific purposes 8th Edition 2013.

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Competing Interests

The research was undertaken with a commercial partner Hamblin Pty Ltd who provided the animals and partly funded the project along with the Australian Government. Two authors (SC and DH) are employed by Hamblin Pty Ltd.

Availability of data and materials

The data and computing programs used in this manuscript may be available from the corresponding author on request and if approved by funding bodies to do so. Restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available.

Authors' contributions

SC contributed to sample collection, data analyses, and writing; MD contributed to study design and writing; DH contributed to sample collection and provided animals; LW undertook ^{1}H-NMR analyses and contributed to writing; AD contributed to data analysis and data

interpretation, and writing; LAG contributed to study design, statistical analyses, and writing. All authors read and approved the manuscript for publication.

2.7 References

1 Xie, Y. *et al.* Effect of Breed and SIre on Carcass Characteristics and Fatty Acid Profiles of Crossbred Wagyu and Angus Steers. *Meat science* **43**, 167-177 (1996).

2 Novais, F. J. *et al.* Identification of a metabolomic signature associated with feed efficiency in beef cattle. *Bmc Genomics* **20**, doi:ARTN 810.1186/s12864-018-5406-2 (2019).

3 Fontanesi, L. Metabolomics and livestock genomics: Insights into a phenotyping frontier and its applications in animal breeding. *Animal Frontiers* **6**, 73-79, doi:10.2527/af.2016-0011 (2016).

4 Fontanesi, L. in *Systems Biology in Animal Production and Health, Vol. 1* (ed Haja N. Kadarmideen) 43-62 (Springer International Publishing, 2016).

5 Goldansaz, S. A. *et al.* Livestock metabolomics and the livestock metabolome: A systematic review. *Plos One* **12**, doi:ARTN e017767510.1371/journal.pone.0177675 (2017).

6 Moloto, K. W. *et al.* Is there a Possibility of Meat Tenderness ProteinBiomarkers on the Horizon? *International Journal of Agriculture Innovations and Research* **6**, 467-472 (2017).

7 Mir, P. S. *et al.* Growth, carcass and meat quality characteristics of beef cattle with 0, 50 and 75 percent Wagyu genetic influence. *Canadian Journal of Animal Science* **79**, 129-137, doi:10.4141/A98-114 (1999).

8 Ponzoni, R. W. & Newman, S. Developing breeding objectives for australian beef cattle production. *Animal Science* **49**, 35-47, doi:10.1017/S0003356100004232 (2010).

9 van der Drift, S. G. A., Houweling, M., Schonewille, J. T., Tielens, A. G. M. & Jorritsma, R. Protein and fat mobilization and associations with serum β-hydroxybutyrate concentrations in dairy cows. *Journal of Dairy Science* **95**, 4911-4920, doi:https://doi.org/10.3168/jds.2011-4771 (2012).

10 Albin, R. C. & Clanton, D. C. Factors contributing to the variation in urinary creatinine and creatinine-nitrogen ratios in beef cattle. *Journal of animal science* **25**, 107-112, doi:10.2527/jas1966.251107x (1966).

11 Hammon, H. M. *et al.* Differences in milk production, glucose metabolism, and carcass composition of 2 Charolais × Holstein F2 families derived from reciprocal paternal and maternal grandsire crosses1. *Journal of Dairy Science* **93**, 3007-3018, doi:https://doi.org/10.3168/jds.2009-2931 (2010).

12 Hocquette, J. F. *et al.* Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. *animal* **4**, 303-319 %R doi:310.1017/S1751731109991091 (2010).

13 Bruckmaier, R. M., Gregoretti, L., Jans, F., Faissler, D. & Blum, J. W. Longissimus Dorsi Muscle Diameter, Backfat Thickness, Body Condition Scores and Skinfold Values Related to Metabolic and Endocrine Traits in Lactating Dairy Cows Fed Crystalline Fat or Free Fatty Acids. *Journal of Veterinary Medicine Series A* **45**, 397-410, doi:10.1111/j.1439-0442.1998.tb00842.x (1998).

14 Perry, T. W. in *Beef Cattle Feeding and Nutrition (Second Edition)* (eds Tilden Wayne Perry & Michael J. Cecava) xv (Academic Press, 2012).

15 Church, D. C. *The ruminant animal: digestive physiology and nutrition*. Rev. edn, (Waveland, 1993).

16 Duarte, M. S. *et al.* Enhancement of adipogenesis and fibrogenesis in skeletal muscle of Wagyu compared with Angus cattle. *Journal of animal science* **91**, 2938-2946, doi:10.2527/jas.2012-5892 (2013).

17 Smith, S. B. & Johnson, B. J. Marbling: Management of cattle to maximize the deposition of intramuscular adipose tissue. *Beef Research*, 1-14 (2014).
18 Ladeira, M. M. *et al.* Review: Nutrigenomics of marbling and fatty acid profile in ruminant meat. *animal*, 1-13, doi:10.1017/S1751731118001933 (2018).
19 Mori, A. *et al.* Comparison of Plasma Metabolite Concentrations and Enzyme Activities in Beef Cattle Raised by Different Feeding Systems in Korea, Japan and New Zealand. *Journal of Veterinary Medicine Series A* **54**, 342-345, doi:10.1111/j.1439-0442.2007.00964.x (2007).
20 Smith, S. B. & Crouse, J. D. Relative contributions of acetate, lactate and glucose to lipogenesis in bovine intramuscular and subcutaneous adipose tissue. *The Journal of nutrition* **114**, 792 (1984).
21 Zhang, S., Zeng, X., Ren, M., Mao, X. & Qiao, S. Novel metabolic and physiological functions of branched chain amino acids: a review. *J Anim Sci Biotechnol* **8**, 10-10, doi:10.1186/s40104-016-0139-z (2017).
22 Andries, J. I., Buysse, F. X., Debrabander, D. L. & Cottyn, B. G. Isoacids in Ruminant Nutrition - Their Role in Ruminal and Intermediary Metabolism and Possible Influences on Performances - a Review. *Anim Feed Sci Tech* **18**, 169-180, doi:Doi 10.1016/0377-8401(87)90069-1 (1987).
23 Karisa, B. K. *et al.* Plasma metabolites associated with residual feed intake and other productivity performance traits in beef cattle. *Livestock Science* **165**, doi:10.1016/j.livsci.2014.03.002 (2014).

Chapter 3: Changes in the blood metabolome of Wagyu crossbred steers with time in a feedlot and relationships with marbling

Connolly, S., Dona, A., Hamblin, D., D'Occhio, M. J. & González, L. A. Changes in the blood metabolome of Wagyu crossbred steers with time in the feedlot and relationships with marbling. *Scientific Reports* **10**, 18987, doi:10.1038/s41598-020-76101-6 (2020). (Published)

Overview

The focus of this chapter was to study the relationship between the blood metabolome at two different sampling points and to examine the relationship with carcase traits, particularly marbling. The objective was to determine changes in the metabolome from middle to late in the feedlotting process due to animals being at different stages of physiological maturity. This information is required for potential commercial applications of biomarkers of marbling.

3.1 Abstract

Wagyu crossbred steers (n = 167) were used to (1) compare the metabolome of individual animals at two distant time-points (days 196 and 432) in a feedlot (this corresponded to 272 and 36 days before slaughter); and (2) determine relationships between the metabolome and marbling, and the effect of days in the feedlot (time-points) on these relationships. ^{1}H-NMR spectroscopy followed by standard recoupling of variables analysis produced 290 features or 'peaks' from which 38 metabolites were identified. There was a positive correlation between the relative concentration (RC) at days 196 and 432 for 35 of 38 metabolites ($P > 0.05$). The RC of 21 metabolites mostly involved in muscle energy and glucose metabolism increased ($P < 0.05$) from day 196 to 432, and the RC of 13 metabolites mostly involved in lipid metabolism decreased ($P < 0.05$). There were 14 metabolites correlated with marbling including metabolites involved in energy and fat metabolism (glucose, propionate, 3-hydroxybutyrate, lipids). The relationship between marbling and the RC of metabolites was affected by time-point, being positive for 3-hydroxybutyrate and acetate ($P < 0.05$) at day 432 but not at day 196. The findings indicate that the blood metabolome in Wagyu crossbred steers changes with time in a feedlot. Notwithstanding, the metabolome has potential to predict marbling in Wagyu. The ability to predict marbling from the blood metabolome appears to be influenced by days in a feedlot and presumably the stage of development towards a mature body conformation.

3.2 Introduction

Cattle with Wagyu (*Bos taurus*) genetics have a high propensity to accumulate intramuscular fat (marbling) and are targeted at markets for premium beef [1,2]. Wagyu and Wagyu crossbred cattle typically undergo periods of 350 to 650 days in a feedlot to achieve high marbling. Animals that fail to achieve the necessary marbling are heavily discounted, and the cost of production can be greater than the market value. The final grading of Wagyu carcases occurs after slaughter, and hence there is considerable interest in identifying ways to predict the carcase outcome for individual animals. Marbling has a relatively high heritability (0.38-0.50) [2,3] in Wagyu.

The blood metabolome has emerged as an important source of biomarkers that have the potential to predict production, health and disease in livestock[4-6]. In a recent study, the blood metabolome of Wagyu crossbred steers was found to be associated with important production traits such as growth rate, carcase weight, subcutaneous rump fat, and marbling (intramuscular fat)[7]. Certain metabolites were positively associated with different production traits and other

metabolites had a negative association. Irrespective, the findings showed that the blood metabolome has potential to offer biomarkers that can be used to select individual Wagyu steers for performance in a feedlot. Sire of the steers was the single most important factor affecting the blood metabolome, which suggests that metabolome biomarkers could potentially also be used to select Wagyu sires[7].

In the study in Wagyu steers, the blood metabolome was determined 300-400 days before animals were slaughtered[7]. Cattle undergo metabolic and physiological adjustments during growth and development towards a mature body conformation and size. Bone growth is fastest in younger animals and precedes muscle growth [8,9]. Fat deposition increases with age and as animals reach a mature body size. Amongst the different fat deposits, abdominal fat is the earliest to accumulate followed by intermuscular fat, subcutaneous fat, and then marbling[1]. Based on the results within the present study, features of the blood metabolome in cattle change significantly with time in a feedlot. It is entirely feasible that specific associations of the metabolome with production and carcase traits may also differ over time. For example, it could be predicted that metabolites linked to marbling may have a stronger association with marbling in older, maturing animals. With the above background in mind, the aims in the present study were (1) to compare the metabolome for individual Wagyu crossbred steers at days 196 and 432 in a feedlot (this corresponded to 272 and 36 days before slaughter); and (2) to determine relationships between the metabolome and marbling, and the effect of days in the feedlot (time-points) on these relationships. The sampling time-points were 236 days apart which should have meant that animals would be in different metabolic and physiological status related to degree of maturity. The findings may help to build the body of knowledge on relationships between the metabolome and important production traits in cattle. The findings could also have the potential to identify specific metabolites that might be used as predictive biomarkers of high market value in cattle.

3.3 Materials & Methods

3.3.1 Animals and experimental design

Wagyu crossbred steers (n = 167) from a single cohort and same management were used in the study. There was an even distribution of animals with different Wagyu genetics: there were 54, 56 and 53 animals within the first cross (50% Wagyu), second cross (75% Wagyu) and third cross and above (≥87.5% Wagyu), respectively. Fullblood Wagyu were predominantly crossed with Shorthorn (50%), Angus (10%), Brahman (38%) and other breeds (12%). Animals were

maintained in a commercial feedlot in southern Queensland, Australia, and allowed ad-libitum consumption of feed. Changes in diet were implemented at days 6, 12 and 263 in the feedlot (Figure 3.1). Diet composition and number of days each diet was fed are shown in Table 3.1 with chemical analysis done for diets 3 and 4 when blood sampling occurred. Animals were sampled at day 196 (early feedlot period) and day 432 (late feedlot period) (Figure 3.1). These sampling time-points corresponded to 272 and 36 days before animals were slaughtered (Figure 3.1).

Figure 3.1: Timeline of events relative to experimental day for Wagyu crossbred feedlot cattle to measure blood metabolomics**.**

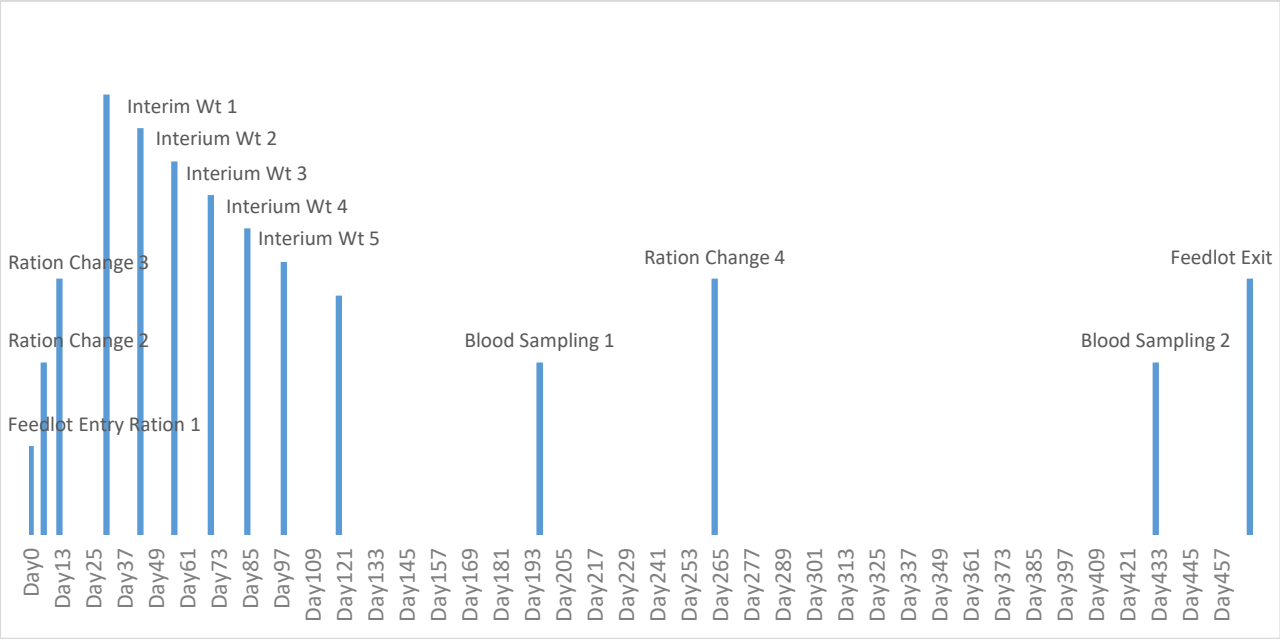


Table 3.1 Diet ingredients and composition.

Ingredient	Unit	Diet 1	Diet 2	Diet 3	Diet 4
Steam Flaked Barley	% as fed	21.5	28.5	37.5	47.0
Steam Flaked Wheat	% as fed	21.0	22.5	14.5	19.0
Finisher Supplement	% as fed	5.0	5.2	0.0	1.7
Growth Supplement	% as fed	0.0	0.0	5.0	3.5
Molasses	% as fed	12.0	10.1	4.8	5.0
Vegetable Oil	% as fed	0.0	1.2	1.4	1.5
Brewers Sweet Grain	% as fed	0.0	0.0	15.0	8.0
Sunflower Meal	% as fed	5.5	5.0	1.5	0.0
Corn Silage	% as fed	12.0	12.0	12.8	9.8
Barley Straw	% as fed	12.0	9.5	7.5	4.5
Lucerne Hay	% as fed	11.0	6.0	0.0	0.0
Chemical Composition					
Dry Matter	%	-	-	94.8	95.2
Moisture	%	-	-	5.2	4.9
Acid Detergent Fibre	%	-	-	9.0	7.0
Dry Matter Digestibility	%	-	-	81.0	85.0
Inorganic Ash	%	-	-	6.0	6.0
Organic Matter	%	-	-	94.0	94.0
Crude Fat	%	-	-	4.0	4.1
Crude Protein	%	14.1*	13.8*	12.5	12.4
Neutral Detergent Fibre	% DM	29.3*	25.6*	20.0	17.0
Metabolizable Energy	MJ/kg DM	10.9*	11.6*	13.0	13.5
Ionophore (monesin)	ppm	21.3*	22.2*	23.4*	23.9*
Net Energy of Gain	MJ/kg DM	4.4*	4.9*	5.5*	6.0*
Net Energy of Maintenance	MJ/kg DM	7.0*	7.6*	8.3*	8.9*

**Values are estimates from feed composition tables of the ingredients making up the diet.*

Blood samples for metabolome analysis were obtained at days 196 and 432. On sampling days, animals were removed from their pens at 0600 h before feeding, and blood was collected between 0700 h and 1030 h. An 18G needle and evacuated lithium heparin tube (Vacutainer BD, Becton Dickinson, Frankland Lakes, NJ) were used to take coccygeal vein samples. Samples were immediately placed on ice for up to 20 min and centrifuged at 10,000 × g for 15 min. Plasma was stored at -80°C until metabolome analysis.

3.3.2 Carcase data

Carcase grading data was recorded by an accredited assessor using the Aus-Meat method [10] with measurements of hot standard carcase weight (HSCW), marbling score, rib eye muscle area (EMA), and subcutaneous rib and rump fat thickness. The subjective Aus-Meat marbling score was assessed by a trained, accredited assessor on a scale of 0 to 9+ with 0 being the least and 9+ the greatest marbling. Marbling percentage was measured objectively using a hyperspectral camera (camera marbling, CM) (HK-333 camera; Hayasaka Rikoh Co. Ltd., Sapporo, Japan)[11].

3.3.3 Metabolite profiling

Plasma samples were prepared for metabolome analysis in accordance with a published protocol [12]. Samples were thawed at room temperature and an aliquot (350 µL) was mixed with 350 µL of aqueous (80% H_2O:20% D_2O) phosphate buffer solution including 0.075 M NaH_2PO4, pH = 7.4 (KOH adjusted), 0.1% sodium azide, and 1 mM 3-141 trimethylsilyl-1-[2,2,3,3, -2H4] propionate (TSP) as an internal standard. Samples were mixed on a vortex for 30 sec and centrifuged at 6,000 × *g* for 10 min. Aliquots (600 µL) of supernatant for each sample were pipetted into 5 mm NMR tubes for ^{1}H-NMR analysis (Bruker, SampleJet 5 mm, Billerica MA, USA). A quality control comprising equal volumes of approximately 10 samples was included after every 15 samples.

A Bruker Advance III 600 MHz spectrometer equipped with a 5 mm TCI cryoprobe was used to analyse the samples. Samples were refrigerated at 4.85 ˚C prior to acquisition and run using a Sample Jet in automatic mode. Data was collected at 36.85 ˚C for 20 min. Noesygr and cpmgpr1d pulse sequences (32 scans collected for each experiment) were used to acquire the 1D ^{1}H-NMR spectra. Irradiation of the solvent (water) resonance was applied during pre-saturation delay (4.0 s) for all spectra and for the noesy also during the mixing time (0.01 s). The pulse sequence parameters were optimized for each sample, particularly the 90° pulse (~12 µs). The data was collected for each sample with approximately 32k (cpmg) or 96 k (noesy) real data points and processed with an exponential line broadening of 0.3 Hz prior to Fourier transformation.

Spectral data was imported into Matlab 7.0 software (Mathworks, Narick, MA). Each individual spectra was aligned and automatically phased, baseline corrected and referenced to the α-C_1H-Glucose doublet at 5.233 ppm [13]. Spectra was then normalized using probabilistic quotient normalization. Statistical recoupling of variables (SRV) was used on the processed spectrum to calculate the start and endpoint of components or clusters. Bucketing is also

another name used for the statistical method in SRV [14]. The SRV output contains the clusters or peaks with the area under the curve calculated which is equivalent to the relative concentration (RC) of each individual peak. Sample spectra were then imported into Chenomx® NMR Suite Professional (Chenomx Inc., Edmonton, AB, Canada), which was used as the reference library to identify peaks or features that belong to a metabolite according to its ppm using the spectral library along with published literature [15]. The RC of all peaks that belonged to the same metabolite were added to obtain the total RC of each metabolite for analysis.

3.3.4 Statistical analyses

The feature or cluster dataset was multiplied by a factor of 10^6 to reduce the number of decimal places. All statistical analyses were performed using SAS (v 9.4; SAS Institute Inc., Cary, NJ, USA). Pearson correlation coefficients were calculated between the RC of the identified metabolites at days 196 and 432 to determine the relationship between them across animals. Principal component analysis (PCA) was conducted using 38 identified metabolites to reduce the dimensionality of the dataset and then examine the effect of sampling time-point on the clustering and separation along the principal components (PC). The components with an eigenvalue >1 were used in the final PCA and data is presented for the first three PC explaining the largest variation in the dataset. A generalized linear model (GLM) was used on the PCA scores output to examine the effect of time-point and Pearson correlation coefficients between marbling and the PC scores were also calculated.

The differences between time-points in the RC of metabolites were analysed using a mixed-effects linear regression model containing the fixed effect of camera marbling (CM) as a covariate, time-point as the repeated measure, and the CM × time-point interaction. This model tested for different slopes between time-points, i.e., the regression between CM and the dependent variable for each time-point. Animal ID was a random effect. Any factor which was not significant was removed from the model and the model was re-run. All data was checked for normality and log-transformed where required. Outliers were detected using studentized residual and those strong residuals with a value > 3.5 or < – 3.5 were removed from the dataset. There were 50 outliers in total that were removed out of 21,759 data points analysed.

3.4 Results

3.4.1 Carcase attributes

Descriptive statistics of carcase measurements, weights and feed intake are shown in Table 3.2. Feedlot exit weight had greater variation than the feedlot induction weight. The average marbling score was 6.66 and average camera marbling (CM) was 27.81%; however, these values showed wide ranges. Marbling score had greater variability compared to CM. Carcase weight and eye muscle area (EMA; *Longissimus Thoracis et Lumborum*, LTL) had the lowest coefficients of variation (CV) of all measurements. The feed intake in relation to percentage BW had an average of 2.86 and variation of 10.25.

Table 3.2: Descriptive statistics of Wagyu crossbred steers (n = 167).

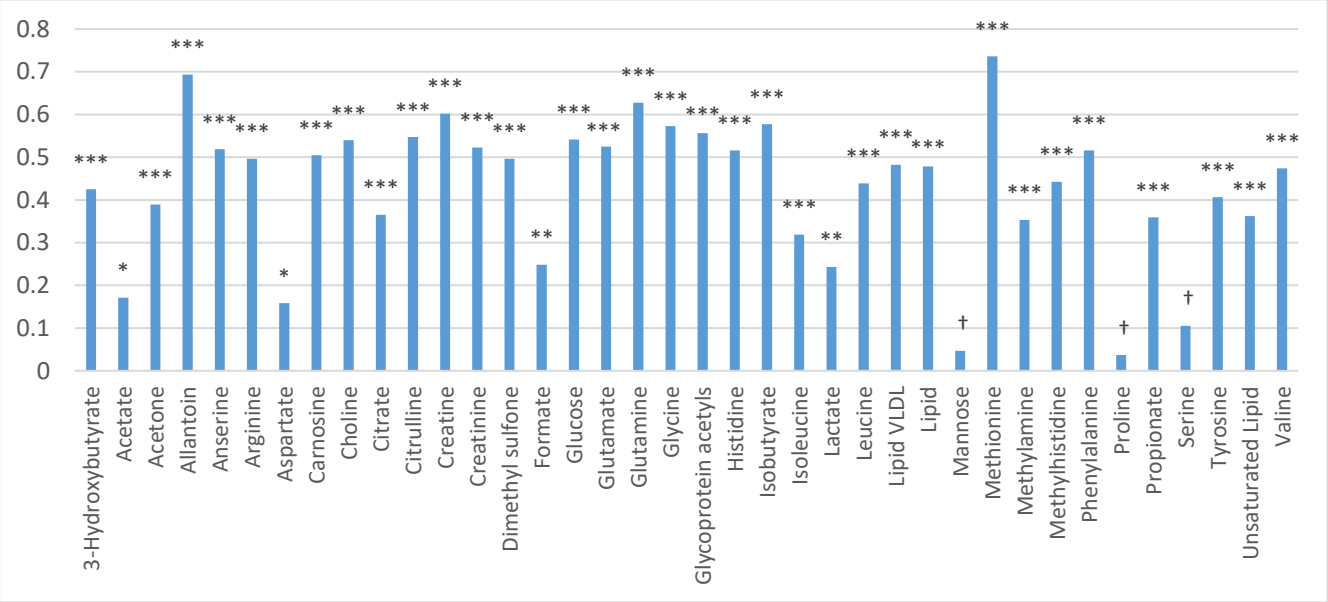
Variable	Minimum	Mean	Maximum	Standard Error	Coefficient of Variation
Wagyu Percent (%)	50	71.7	98.7	0.99	24.87
Age at Induction (Days)	460	614	1041	6.89	20.14
Feedlot Induction Weight (kg)	246	332.0	430	1.63	8.81
Feedlot Exit Weight (kg)	554	731.4	929	3.96	9.55
ADG Feedlot (kg)	0.42	0.87	1.30	0.008	17.34
Aus-Meat Marble Score	2.00	6.66	9.00	0.112	10.89
Camera Marbling (%)	16.3	27.81	44.5	0.325	13.82
HSCW (kg)	323	427	542	2.253	6.40
EMA (cm^2)	60	79.4	98	0.469	1.87
Rump Fat (mm)	10	16.4	37	0.301	29.84
Rib Fat (mm)	3	7.10	22	0.172	24.67
Feed intake (% Body Weight)	2.05	2.86	3.61	0.016	10.25

3.4.2 Metabolome spectral data

The dataset produced from analysis of the metabolome spectral data using statistical recoupling of variables (SRV) contained 290 peaks or clusters which were mapped to the spectral library of Chenomx®. This identified 38 metabolites based on the ppm of the individual clusters from the spectral library and published literature[15]. Pearson correlation coefficients between the RC of each feature and each metabolite at days 196 and 432 were calculated. The correlation between the RC at days 196 and 432 ranged from -0.13 to +0.78 across all 290 features (data not shown). None of the negative correlation coefficients were significantly different than zero ($P > 0.05$) and 215 of 290 features with r > +0.15 were significant ($P < 0.05$). Of 38 identified metabolites, only serine, proline and mannose were not

significant ($P > 0.05$) with the remaining 35 metabolites showing a positive correlation between the RC at days 196 and 432 (Figure 3.2).

Figure 3.2: Pearson correlation coefficient between the relative concentration of plasma metabolites at days 196 and 432 in a feedlot in Wagyu crossbred steers



****, **, *, † $P \leq 0.001$, $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.10$, respectively.*

3.4.3 Principal component analysis

The principal component analysis (PCA) score plot indicated a clear separation between samples taken at day 196 compared with day 432, with the first three components explaining 61.22% of the variation in the dataset (Figure 3.3). Further analyses of the principal component (PC) scores showed that most animals showed positive values for PC2 and PC3 on day 196 but negative PC2 and PC3 on day 432 (Table 3.3). PC1 and PC4 were negative at day 196 and positive at day 432, although the difference between time-points was smaller compared to PC2 and PC3 (Table 3.3).

Figure 3.3 Score plot of the top 3 principal components (PC1 to PC3) obtained from 38 blood metabolites of Wagyu crossbred steers sampled at day 196 (blue) and day 432 (red) in a feedlot.

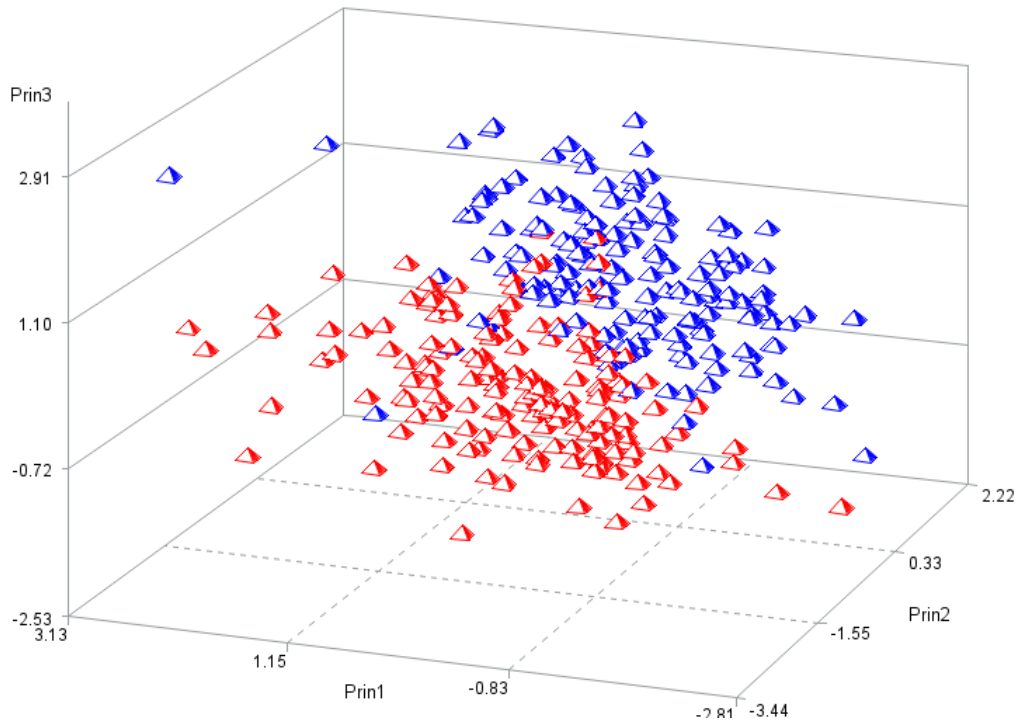


Table 3.3 The effect of time-point in a feedlot on five principal components 1 to 3 (Prin1-Prin3) and Pearson correlation coefficients between principal component scores and marbling of Wagyu crossbred steers.

	Time-point				
	Day 196	Day 432	P-Value	R^2	Pearson r with marbling
PC1	-0.279 ± 0.0758	0.277 ± 0.0756	<0.001	0.077	0.110*
PC2	0.561 ± 0.0654	-0.558 ± 0.0652	<0.001	0.314	-0.183***
PC3	0.517 ± 0.0676	-0.514 ± 0.0674	<0.001	0.267	0.161**
PC4	-0.224 ± 0.0769	0.223 ± 0.0767	<0.001	0.050	-0.159**
PC5	0.021 ± 0.0789	-0.021 ± 0.0787	0.708	0.0004	0.155**

***, **, * *$P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$, respectively, for the Pearson correlation coefficients.*

PC = Principal Component

The R^2 values indicated that PC2 and PC3 were the two components that explained the largest proportion of the variability between time-points. All PC were significantly correlated with marbling ($P < 0.05$); however, PC2 had the largest Pearson coefficient. Both PC2 and PC4 were negatively correlated with marbling ($P < 0.05$) whereas PC1, PC3 and PC5 were positively correlated with marbling ($P < 0.05$; Table 3.3).

Figure 3.4 shows the loading or pattern plot for PC1 and PC2 which explained approximately half of the variability in the dataset. Lipid groups (lipids, very low-density lipoprotein (VLDL), and glycoprotein acetyls) and choline showed negative loading on PC1 and positive loading on PC2 which characterised samples at day 196 as shown in Table 3. Metabolites with positive loading on PC1 and negative loading on PC2 were glucose, methyl histidine, arginine, anserine and creatinine (Figure 3.4) which characterised samples at day 432. Only lactate and the lipid groups showed negative loading on PC1 (Figure 3.4). Allantoin, acetate and amino acids (aspartate, leucine, isoleucine, carnosine, and proline) showed high positive loadings on both PC1 and PC2 (Figure 3.4). Figure 3.5 shows the loading plot for PC2 and PC3 where approximately 30% of the variation was explained and the lipids (VLDL, glycoprotein acetyls and lipids) and choline clustered together with high positive loading on PC2 and negative on PC3. Arginine, anserine, unsaturated lipids, and glucose had high positive loading on PC3, whereas creatinine, citrate and methylamine showed high negative loading (Figure 3.5).

Figure 3.4 Loading plot for principal components 1 and 2 with 38 metabolites identified in blood of Wagyu crossbred steers.

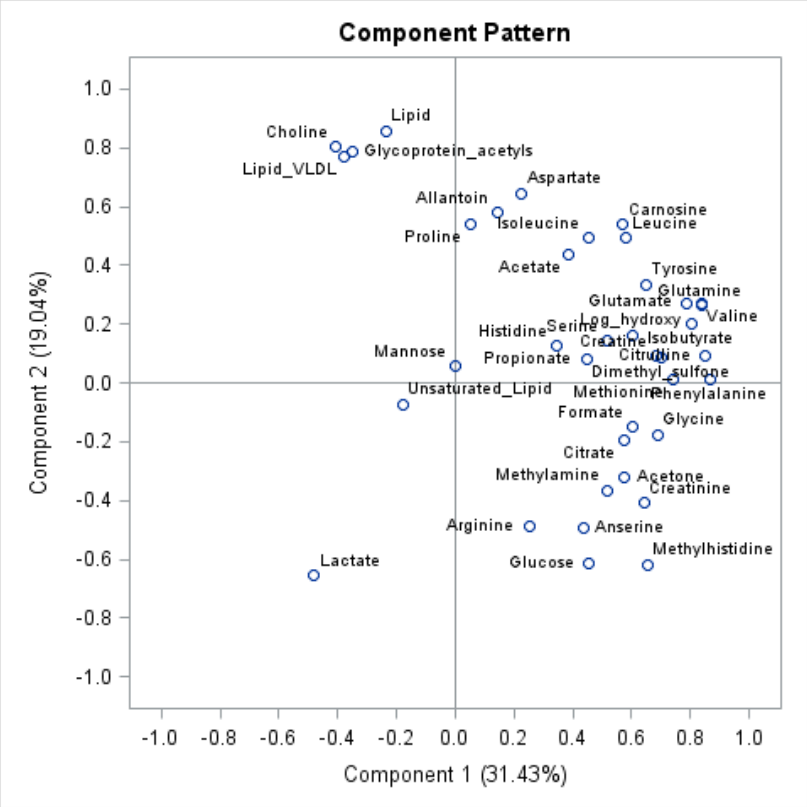
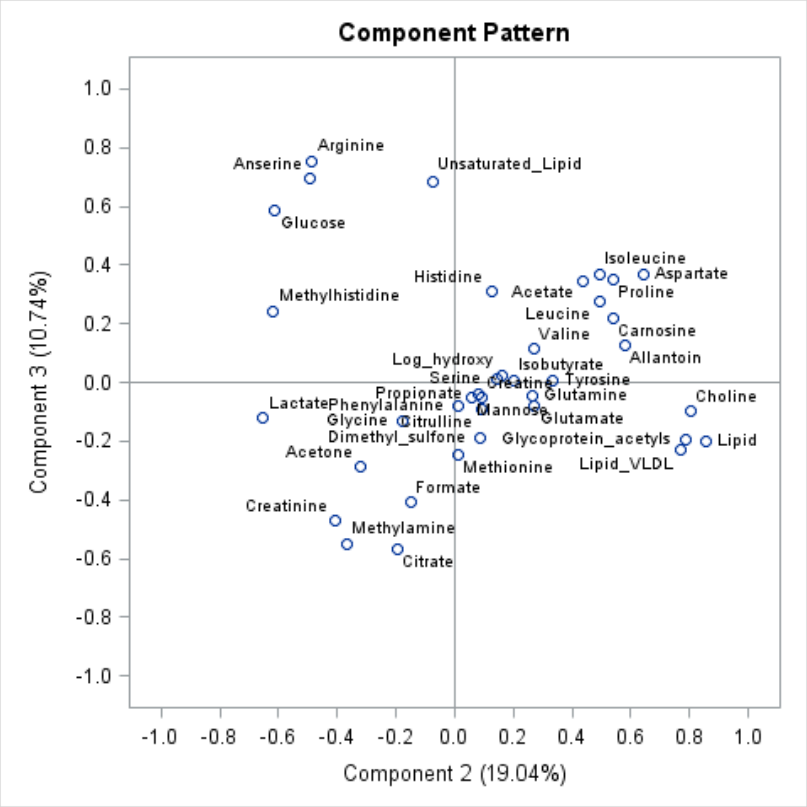


Figure 3.5 Loading plot for principal components 2 and 3 of 38 blood metabolites of Wagyu crossbred steers sampled at days 196 and 432 in a feedlot.



3.4.4 Metabolite relative concentrations and relationships with marbling

The average relative concentration (RC) of each metabolite for each sampling point and the regression coefficient between the RC and marbling are shown in Table 3.4. The CM × time interaction was significant (P > 0.05) for 8 metabolites (3-hydroxybutyrate, acetate, allantoin, histidine, isobutyrate, methyl histidine, tyrosine, and valine). Only 5 of 38 identified metabolites were not affected by time-point (*P > 0.05*); these were: arginine, mannose, methyl histidine, propionate, and serine (Table 3.4). Of those metabolites with no significant CM × time interaction, fifteen metabolites showed an increase (*P < 0.05*) in RC from day 196 to day 432; these were: acetone, anserine, citrate, citrulline, creatine, creatinine, dimethyl-sulphone, formate, glucose, glutamate, glutamine, glycine, lactate, methionine, and methylamine. In contrast, ten metabolites showed a decrease (*P < 0.05*) in RC from day 196 to day 432; these were aspartate, carnosine, choline, glycoprotein acetyls, isoleucine, leucine, VLDL, lipids, proline, and unsaturated lipids. The average RC did not differ (*P < 0.05*; Table 3.4) between days 196 and 432 for 3-hydroxybutyrate, tyrosine and valine; however, both the intercept and slope differed between time-points.

There were 11 metabolites with significant ($P < 0.05$; Table 3.4) regression coefficient between marbling and RC as indicated by the main effect of marbling with non-significant interaction. These included 5 metabolites (choline, formate, glycoprotein-acetyls, VLDL and lipids) that had a negative correlation with CM ($P < 0.05$). Six metabolites had a positive correlation with CM ($P < 0.05$; Table 3.4) including anserine, arginine, citrate, glucose, methylamine, and propionate). In addition, creatine showed a tendency for a positive relationship with marbling ($P = 0.06$).

Table 3.4 The effect of days in a feedlot (time) on the relative concentration of blood metabolites, and the relationship between metabolites and marbling (CM) in Wagyu crossbred steers.

Metabolite	Time		P-Value	Marbling Regression Coefficient	CM P-value	CM × time P-value
	Day 196	Day 432				
3-Hydroxybutyrate $_{log}$	4.92 ± 0.0132	4.94 ± 0.0132	*<0.001*	0.0082 ± 0.2133	*0.049*	*<0.001*
Acetate $_{log}$	4.74 ± 0.025	4.48 ± 0.025	*<0.001*	0.94 ± 0.429	*0.418*	*0.016*
Acetone	18.02 ± 0.236	22.38 ± 0.235	*<0.001*	-0.023 ± 0.034	*0.497*	*0.723*
Allantoin	23.37 ± 0.307	19.08 ± 0.307	*<0.001*	-0.054 ± 0.052	*0.044*	*0.042*
Anserine	262.9 ± 1.71	266.9 ± 1.71	*0.024*	0.93 ± 0.252	*<0.001*	*0.069*
Arginine	655.1 ± 3.92	657.4 ± 3.91	*0.575*	1.80 ± 0.57	*0.002*	*0.209*
Aspartate	14.07 ± 0.18	11.52 ± 0.180	*<0.001*	0.021 ± 0.024	*0.378*	*0.522*
Carnosine	27.11 ± 0.233	25.36 ± 0.232	*<0.001*	0.0043 ± 0.035	*0.898*	*0.540*
Choline	455.9 ± 3.71	401.3 ± 3.70	*<0.001*	-2.09 ± 0.549	*<0.001*	*0.749*
Citrate	139.9 ± 1.42	167.7 ± 1.42	*<0.001*	0.47 ± 0.197	*0.017*	*0.098*
Citrulline	20.42 ± 0.175	21.34 ± 0.175	*<0.001*	0.026 ± 0.026	*0.333*	*0.099*
Creatine	265.9 ± 2.66	272.3 ± 2.66	*0.008*	0.77 ± 0.407	*0.061*	*0.288*
Creatinine	36.31 ± 0.416	45.46 ± 0.416	*<0.001*	0.012 ± 0.062	*0.850*	*0.581*
Dimethyl sulfone	24.49 ± 0.232	25.99 ± 0.231	*<0.001*	-0.025 ± 0.034	*0.465*	*0.858*
Formate	3.89 ± 0.059	4.92 ± 0.059	*<0.001*	-0.016 ± 0.008	*0.045*	*0.116*
Glucose	1613.5 ± 10.63	1677.9 ± 10.60	*<0.001*	5.71 ± 1.573	*<0.001*	*0.073*
Glutamate	21.0 ± 0.196	21.7 ± 0.20	*<0.001*	-0.016 ± 0.029	*0.591*	*0.273*
Glutamine	251.6 ± 1.93	256.9 ± 1.92	*0.002*	-0.11 ± 0.296	*0.712*	*0.098*
Glycine	116.9 ± 1.28	130.8 ± 1.28	*<0.001*	0.0656 ± 0.186	*0.727*	*0.402*
Glycoprotein acetyls	256.4 ± 1.80	236.7 ± 1.79	*<0.001*	-1.04 ± 0.268	*<0.001*	*0.110*
Histidine	286.5 ± 2.55	273.0 ± 2.53	*<0.001*	0.92 ± 0.428	*0.382*	*0.007*
Isobutyrate	113.9 ± 0.99	115.4 ± 0.98	*0.012*	0.43 ± 0.166	*0.185*	*0.003*
Isoleucine	184.8 ± 1.16	173.8 ± 1.16	*<0.001*	0.007 ± 0.162	*0.965*	*0.286*
Lactate	723.7 ± 17.79	830.8 ± 17.69	*<0.001*	2.23 ± 2.559	*0.388*	*0.364*
Leucine	156.6 ± 1.25	148.1 ± 1.245	*<0.001*	0.09 ± 0.18	*0.622*	*0.289*
Lipid	1201.4 ± 6.36	1133.1 ± 6.34	*<0.001*	-3.82 ± 0.933	*<0.001*	*0.134*
VLDL	807.7 ± 5.40	752.7 ± 5.36	*<0.001*	-3.53 ± 0.773	*<0.001*	*0.062*
Mannose	4.67 ± 0.091	4.89 ± 0.091	*0.089*	-0.021 ± 0.011	*0.062*	*0.289*
Methionine	224.9 ± 2.058	245.6 ± 2.06	*<0.001*	0.09 ± 0.327	*0.775*	*0.075*
Methylamine	29.27 ± 0.493	41.05 ± 0.495	*<0.001*	0.197 ± 0.069	*0.005*	*0.099*
Methyl histidine	194.5 ± 1.23	209.6 ± 1.22	*0.695*	0.61 ± 0.208	*0.033*	*0.047*
Phenylalanine	35.77 ± 0.297	38.04 ± 0.296	*<0.001*	0.009 ± 0.044	*0.897*	*0.054*
Proline	37.81 ± 0.544	31.24 ± 0.543	*<0.001*	-0.024 ± 0.067	*0.719*	*0.558*
Propionate	17.08 ± 0.156	17.25 ± 0.156	*0.352*	0.08 ± 0.022	*<0.001*	*0.245*
Serine	17.06 ± 0.231	17.07 ± 0.231	*0.965*	0.009 ± 0.029	*0.754*	*0.542*
Tyrosine	66.58 ± 0.656	67.52 ± 0.654	*0.026*	0.057 ± 0.111	*0.273*	*0.010*
Unsaturated Lipid	440.1 ± 1.79	428.6 ± 1.78	*<0.001*	0.21 ± 0.252	*0.401*	*0.365*
Valine	275.4 ± 2.13	275.8 ± 2.12	*0.032*	0.73 ± 0.36	*0.322*	*0.026*

VLDL: very low-density lipoprotein

The results for the metabolites with significant CM × time interactions are shown in Table .5 through the regression coefficient for each time-point. Allantoin and tyrosine showed a

negative association (*P < 0.05*) with marbling at day 196 but no association at day 432 (*P > 0.05*). The metabolites 3-Hydroxybutyrate, acetate, histidine, isobutyrate, methyl histidine and valine showed a positive association (*P < 0.05*) with marbling at day 432 but there was no apparent association at day 196 (Table 3.5).

Table 3.5 Regression coefficients of the relative concentration of blood metabolites against marbling for metabolites with a significant interaction between marbling and days in a feedlot for Wagyu crossbred steers.

	Days in a feedlot			
	Day 196		**Day 432**	
Metabolite	**Reg. Coeff ± SE**	**P-Value**	**Reg. Coeff ± SE**	**P-Value**
3-Hydroxybutyrate $_{log}$	-0.002 ± 0.002	0.545	0.008 ± 0.002	*<0.001*
Acetate $_{log}$	-0.004 ± 0.004	*0.384*	0.009 ± 0.004	*0.030*
Allantoin	-0.142 ± 0.053	*0.008*	-0.054 ± 0.052	*0.298*
Histidine	-0.022 ± 0.458	*0.962*	0.920 ± 0.448	*0.041*
Isobutyrate	-0.031 ± 0.170	*0.855*	0.429 ± 0.167	*0.011*
Phenylalanine	-0.010 ± 0.052	*0.842*	0.055 ± 0.051	*0.282*
Methyl histidine	0.156 ± 0.213	*0.465*	0.608 ± 0.208	*0.004*
Tyrosine	-0.255 ± 0.1138	*0.026*	0.055 ± 0.1109	*0.622*
Valine	-0.114 ± 0.3695	*0.758*	0.746 ± 0.3614	*0.040*

Reg. Coeff. = Regression Coefficient, SE = Standard Error

3.5 Discussion

The first aim of the present study was to compare the metabolome for individual Wagyu crossbred steers at days 196 and 432 in a feedlot. These time-points were 236 days apart, and 272 and 36 days before animals were slaughtered and carcase traits measured. Previous studies sampling cattle at different times for metabolomic analysis had shorter time intervals and were undertaken in younger animals earlier in the feedlotting process [7, 16-18]. Two important differential features of the present study were (1) the long interval between the two sampling points and (2) the sampling of older animals that were presumed to be undergoing a greater rate of intramuscular fat (IMF) accretion rate because of greater physiological maturity. The

relative concentrations for 33 out of 38 metabolites were different between days 196 and 432. These findings indicated that the blood metabolome in steers changes with time in a feedlot. These changes could be due to a number of factors including age, body maturation (e.g. rate of IMF accretion), prevailing environment, and diet. Yang, et al. [19] reported differences in the relative abundance of 56 plasma metabolites between steers fed a diet with low corn grain (29% of DM) and those fed a diet with high corn grain (49% of DM). The changes in diet from day 196 to 432 in the present study were minor, with the diet fed at day 432 having 3.85% lower NDF and 6.0% lower forage. Furthermore, laboratory analysis of diets 3 (196 days sampling) and 4 (432 days sampling) indicated no significant differences in the chemical composition. The concentration of crude fat of both diets was of interest because it suggests that the availability of lipids was similar at both sampling times. Therefore, the changes in the RC of metabolites and lipid groups, and the relationships presented between metabolites and marbling reflect changes in animal metabolism rather than changes in the diet consumed. The average ambient temperature in the sub-tropical climate of the present study also showed only a minor difference between time-points (day 196 was 34˚C; day 432 was 30˚C). Therefore, the changes in the plasma metabolome between days 196 and 432 were presumed to be due to age, body maturation, and metabolic and physiological status.

The second aim of the present study was to determine the relationships between the metabolome and marbling, and the effect of DOF (time points) on these relationships. Fourteen metabolites which were associated with marbling had correlation coefficients of 0.35 to 0.60 between days 196 and 432 (3-hydroxybutyrate, propionate, 54, choline, anserine, arginine, citrate, methylamine, methyl histidine, and lipid groups - lipids, VLDL and glycoprotein-acetyls). This finding indicated that the ranking of individual animals based on the RC of these metabolites was consistent from days 196 to 432. In addition to this, the fact that many of these metabolites were associated with marbling encourage potential applications of metabolomics to aid in selecting animals for propensity to marble. Other metabolites (creatine, allantoin, glutamine, and methionine) had relatively high correlation coefficients ($r > 0.60$) between days 196 and 432; however, none of these metabolites were correlated with marbling in the present study except for a positive trend for creatine. Arginine, citrate, glucose, and propionate were associated with marbling as the main factor. Propionate enters the TCA cycle and is converted to glucose in the liver (gluconeogenic pathway), and glucose is then used for fatty acid synthesis to be finally used for IMF deposition [2]. Furthermore, arginine and citrate can also be converted to acetyl-coA in the TCA cycle and later used for fatty acid synthesis [20]. The positive

relationship between propionate and marbling of the present study was also observed in an earlier study with Wagyu crossbred steers with a similar genetic background[7]. Similar to the present study, the earlier study[7] also reported positive associations between marbling and the RC of creatine, 3-hydroxybutyrate, acetate, histidine, isobutyrate, and valine. The similarities between the two studies could be explained by the similar genetic background and comparable management and feeding systems. In addition, the consistency of these relationships between metabolites and marbling may represent common critical metabolic pathways involved in IMF synthesis and deposition. However, more metabolites were associated with marbling in the present study compared to the previous sampling younger animals [7]. In addition, more metabolites were associated with marbling at 432 compared to 196 days (3-hydroxybutyrate, acetate, histidine, isobutyrate, methyl histidine, and valine). These results suggest that the association between the metabolome and marbling increased with age or degree of maturity. However, further studies are required to confirm this hypothesis.

Glucose showed a positive association with marbling independently of sampling time however there was a tendency for a marbling × DOF interaction (P = 0.07) because the slope of the regression coefficient tended to be greater at day 196 compared to day 432 (data not shown). In the earlier study with Wagyu crossbred steers, glucose showed a linear decrease with marbling at day 65, no association at day 119, and positive association at day 163 in the feedlot[7]. The findings from the present and earlier studies could be interpreted to suggest that the relationship between glucose and marbling becomes stronger and more positive as steers mature and glucose demand for marbling increases. It has been reported that dairy cows can respond to increased glucose demand during post-partum by doubling liver gluconeogenesis [21]. Therefore, it is plausible that animals with higher propensity to marble have faster gluconeogenic rate producing glucose needed for *de novo* synthesis of both fatty acids and triglycerides as previously reviewed [1,2].

Similarly, there was a negative association between VLDL and marbling in the present study with a tendency for a marbling × time interaction (P = 0.06) because the regression coefficient (slope) was lesser at day 196 compared to day 432 (data not shown). In the earlier study with Wagyu, the association between lipids and marbling was positive at day 65, not significant at day 119, and negative at day 163[7]. In both studies, therefore, the negative association between lipids and marbling became stronger in older animals (i.e., more negative). It is possible that the greater rate of IMF accretion as steers mature is achieved, in part, by a more rapid uptake of circulating lipids for marbling or IMF deposition, which results in lesser

blood concentrations. In contrast, higher marbling seems to be 'fuelled' by higher concentration of glucose and its precursor propionate in blood.

Five metabolites (3-hydroxybutyrate, histidine, isobutyrate, methyl histidine and valine) showed a positive linear relationship with marbling at day 432 but not day 196. In contrast, the metabolites allantoin and tyrosine showed a negative association with marbling at day 196 but not day 432. These results further illustrate the complexity of relationships between blood metabolites and marbling and as noted above, further studies are needed to gain a deeper understanding on the metabolome and marbling in cattle throughout different stages of maturity.

The principal component analysis (PCA) and linear models revealed metabolic patterns related to stage of physiological maturity and the relationships with the metabolism of IMF. The PCA score plot indicated that PC2 and PC3 accounted for a lower proportion of the variance of the dataset compared to PC1. However, PC2 and PC3 seemed more suitable to differentiate between DOF based on the proportion of the variance explained in the GLM models. Furthermore, PC2 and PC3 showed the highest negative and positive correlation with marbling, respectively. Therefore, PC2 and PC3 seem to explain stage of maturity and IMF deposition better compared to the rest of the PC's. Positive values for both PC2 and PC3 were found in animals sampled at 196 DOF, and negative values for both PC2 and PC3 at 432 DOF. The loading plots highlighted those metabolites with positive loading on PC2 including choline, lipid groups (lipids, VLDL and glycoprotein acetyls), acetate, allantoin, and a group of amino acids (aspartate, proline, isoleucine, leucine, and carnosine). Furthermore, these metabolites showed a significant decrease of the RC from 196 to 432 DOF and choline, lipids and VLDL were also negatively associated with marbling in the present experiment. Therefore, maturity seems to be associated with a reduction in the circulating concentration of metabolites involved in lipid metabolism. Lipids are the primary components of fat tissue in animal bodies whereas choline is a precursor for the synthesis of hepatic VLDL formed by choline phospholipids[22,23]. In post-partum dairy cows, choline plays an important role in the export of triacylglycerol from the liver promoting phosphatidylcholine synthesis in the Kennedy pathway to improve coping with negative energy balance and increase milk production [22,24]. Therefore, this group of metabolites seem to be important during maturity and fat deposition showing lower concentration with higher maturity when IMF deposition is expected to be faster. In addition, animals with higher ability to marble show lower concentration of

metabolites involved in lipid metabolism. It is plausible that this is a result of faster uptake of circulating lipids by body tissues required for fat deposition.

In contrast to lipid groups, glucose, anserine, and arginine had high negative loading on PC2 and positive on PC3. The RC of these metabolites were positively associated with marbling and increased with DOF. Therefore, it seems plausible that these metabolites act as metabolic fuels for lipid synthesis which are then used for fat deposition as the animal matures with age. Thus, animals with higher availability of these metabolic fuels may favour lipid synthesis and fat deposition, and these animals seem to uptake circulating lipids at a faster rate clearing them from the bloodstream. Furthermore, there were 15 metabolites that increased from 196 to 432 DOF, some of these metabolites included molecules that are important in glycolysis to produce pyruvate and ATP including creatine, glucose, glutamate, glycine, lactate, methionine and phenylalanine[25]. Pyruvate then enters the TCA cycle, as it is glutamate which is involved with the α-Ketoglutarate section of the TCA. Methionine can be converted to succinyl-CoA and then used for glycogenesis whereas phenylalanine can enter the fumarate part of the TCA[25]. These findings may suggest that the ability of animals to produce energy via both glycolysis and TCA increases with age or maturity, and this may be linked to increased synthesis and uptake of lipids.

3.6 Conclusions

Significant changes in the relative concentration of metabolites and of the metabolic profile occurred in crossbred Wagyu steers sampled at two distinct time points (early and late) in the feedlotting process. These changes demonstrate the importance of stage of maturity on metabolic processes and are likely related to fat metabolism and deposition, at least partially. Maturity is accompanied by an increase in the relative concentration of metabolites that participate in metabolic pathways for energy production and precursors used for fat acid synthesis such as citrate, creatine, creatinine, formate, glucose, glutamate, glycine, lactate, and methionine. In contrast, the concentration of circulating metabolites related to lipid metabolism and fat deposition decrease with stage of maturity such as choline, lipids, and acetyl groups. Several amino acids involved in protein metabolism also decreased with time including proline, leucine, isoleucine, histidine, carnosine, allantoin and aspartate. Further to this, Wagyu steers with higher marbling at the time of slaughter tend to show greater concentrations of propionate, 3-hydroxybutyrate, acetate, creatine, glucose, anserine, and arginine but lower blood

concentration of lipid groups, choline, and acetyl groups. Sampling time in relation to stage of maturity needs to be considered to understand results from metabolic studies and for practical applications including the prediction of valuable carcase traits such as marbling.

Animal Ethics

The protocol of the present study was approved by the institutional animal ethics committee of The University of Sydney (approval #1125). The study was undertaken in accordance with the Australian code for the care and use of animals for scientific purposes 8th Edition 2013.

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Competing Interests

The research was undertaken with a commercial partner Hamblin Pty Ltd which provided the animals and partly funded the study. Two authors (SC and DH) are employed by Hamblin Pty Ltd. The other authors (AD, MD, and LAG) declare no conflict of interest.

Availability of data and materials

The data and computing programs used in this manuscript may be available from the corresponding author on request and if approved by funding bodies to do so. Restrictions apply

to the availability of these data, which were used under license for the current study, and so are not publicly available.

Authors' contributions

SC contributed to sample collection, data analyses, and writing; MD contributed to study design and writing; DH contributed to sample collection and provided animals; AD contributed to data analysis and data interpretation, and writing; LAG contributed to study design, statistical analyses, and writing. All authors read and approved the manuscript for publication.

3.7 References

1 Gotoh, T., Nishimura, T., Kuchida, K. & Mannen, H. The Japanese Wagyu beef industry: current situation and future prospects - A review. *Asian-Australasian journal of animal sciences* **31**, 933-950, doi:10.5713/ajas.18.0333 (2018).

2 Park, S. J. *et al.* Genetic, management, and nutritional factors affecting intramuscular fat deposition in beef cattle - A review. *Asian-Australasian journal of animal sciences* **31**, 1043-1061, doi:10.5713/ajas.18.0310 (2018).

3 Oyama, K. Genetic variability of Wagyu cattle estimated by statistical approaches. *Animal Science Journal* **82**, 367-373, doi:10.1111/j.1740-0929.2011.00895.x (2011).

4 Goldansaz, S. A. *et al.* Livestock metabolomics and the livestock metabolome: A systematic review. *PLoS ONE* **12**, e0177675, doi:10.1371/journal.pone.0177675 (2017).

5 D'Occhio, M. J., Baruselli, P. S. & Campanile, G. Metabolic health, the metabolome and reproduction in female cattle: a review. *Italian Journal of Animal Science* **18**, 858-867, doi:10.1080/1828051X.2019.1600385 (2019).

6 Fontanesi, L. in *Systems Biology in Animal Production and Health, Vol. 1* (ed Haja N. Kadarmideen) 43-62 (Springer International Publishing, 2016).

7 Connolly, S. *et al.* Relationship of the blood metabolome to subsequent carcass traits at slaughter in feedlot Wagyu crossbred steers. *Scientific Reports* **9**, 15139, doi:10.1038/s41598-019-51655-2 (2019).

8 Owens, F. N., Gill, D. R., Secrist, D. S. & Coleman, S. W. Review of some aspects of growth and development of feedlot cattle. *Journal of Animal Science* **73**, 3152-3172, doi:10.2527/1995.73103152x (1995).

9 Shahin, K. A. & Berg, R. T. Growth Patterns of Muscle, Fat and Bone, and Carcass Composition of Double Muscled and Normal Cattle. *Canadian Journal of Animal Science* **65**, 279-293, doi:10.4141/cjas85-035 (1985).

10 Aus-Meat. (ed Aus-Meat Limited) (2019).

11 Nakahashi, Y., Maruyama, S., Seki, S., Hidaka, S. & Kuchida, K. Relationships between monounsaturated fatty acids of marbling flecks and image analysis traits in longissimus muscle for Japanese Black steers. *J Anim Sci* **86**, 3551-3556 (2008).

12 Dona, A. C. *et al.* Precision High-Throughput Proton NMR Spectroscopy of Human Urine, Serum, and Plasma for Large-Scale Metabolic Phenotyping. *Analytical Chemistry* **86**, 9887-9894, doi:10.1021/ac5025039 (2014).

13 Dona, A. C. *et al.* A guide to the identification of metabolites in NMR-based metabonomics/metabolomics experiments. *Computational and Structural Biotechnology Journal* **14**, 135-153, doi:10.1016/j.csbj.2016.02.005 (2016).

14 Blaise, B. J. *et al.* Statistical Recoupling Prior to Significance Testing in Nuclear Magnetic Resonance Based Metabonomics. *Analytical Chemistry* **81**, 6242-6251, doi:10.1021/ac9007754 (2009).
15 Nicholson, J. K., Foxall, P. J. D., Spraul, M., Farrant, R. D. & Lindon, J. C. 750 MHz 1H and 1H-13C NMR Spectroscopy of Human Blood Plasma. *Analytical Chemistry* **67**, 793-811, doi:10.1021/ac00101a004 (1995).
16 De Buck, J., Shaykhutdinov, R., Barkema, H. W. & Vogel, H. J. Metabolomic Profiling in Cattle Experimentally Infected with Mycobacterium avium subsp. paratuberculosis. *PLoS ONE* **9**, e111872, doi:10.1371/journal.pone.0111872 (2014).
17 Imhasly, S. *et al.* Metabolomic biomarkers correlating with hepatic lipidosis in dairy cows. *BMC veterinary research* **10**, 122-122, doi:10.1186/1746-6148-10-122 (2014).
18 Karisa, B. K. *et al.* Plasma metabolites associated with residual feed intake and other productivity performance traits in beef cattle. *Livestock Science* **165**, doi:10.1016/j.livsci.2014.03.002 (2014).
19 Yang, Y. *et al.* Rumen and plasma metabolomics profiling by UHPLC-QTOF/MS revealed metabolic alterations associated with a high-corn diet in beef steers. *PLOS ONE* **13**, e0208031, doi:10.1371/journal.pone.0208031 (2018).
20 Ladeira, M. M. *et al.* Nutrigenomics and Beef Quality: A Review about Lipogenesis. *International Journal of Molecular Sciences* **17**, 918, doi:10.3390/ijms17060918 (2016).
21 Aschenbach, J. R., Kristensen, N. B., Donkin, S. S., Hammon, H. M. & Penner, G. B. Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. *IUBMB Life* **62**, 869-877, doi:10.1002/iub.400 (2010).
22 Davidson, S. *et al.* Supplementing Limited Methionine Diets with Rumen-Protected Methionine, Betaine, and Choline in Early Lactation Holstein Cows. *Journal of Dairy Science* **91**, 1552-1559, doi:https://doi.org/10.3168/jds.2007-0721 (2008).
23 Chandler, T. L. & White, H. M. Glucose metabolism is differentially altered by choline and methionine in bovine neonatal hepatocytes. *Plos One* **14**, e0217160, doi:10.1371/journal.pone.0217160 (2019).
24 Zhou, Z., Vailati-Riboni, M., Luchini, D. N. & Loor, J. J. Methionine and Choline Supply during the Periparturient Period Alter Plasma Amino Acid and One-Carbon Metabolism Profiles to Various Extents: Potential Role in Hepatic Metabolism and Antioxidant Status. *Nutrients* **9**, 10, doi:10.3390/nu9010010 (2016).
25 Church, D. C. *The ruminant animal: digestive physiology and nutrition*. Rev. edn, (Waveland, 1993).

Chapter 4: Predicting intramuscular fat (marbling) in longissimus muscle of crossbred Wagyu cattle with animal and feedlot performance data, and the plasma metabolome using machine learning

Connolly, S.K, Dona, A.C, Hamblin, D.W, D'Occhio, M.J, González, L.A 2021. Predicting intramuscular fat (marbling) in longissimus muscle of crossbred Wagyu cattle with animal and feedlot performance data, and the plasma metabolome using machine learning

Overview

The ability to identify which animals are going to produce a highly marbled carcase using the plasma metabolome and routinely recorded animal farm and feedlot data was investigated using machine learning methods. The ability to select animals prior to slaughter would increase the efficiency of the Wagyu production system.

4.1 Aim

To develop a predictive model for high and low marbling at slaughter in crossbred Wagyu cattle with plasma metabolome and animal farm data using machine learning

4.2 Abstract

The aim was to incorporate the plasma metabolome with farm-collected data using machine learning to predict marbling before slaughter of Wagyu crossbred steers in a feedlot production system. Naïve Bayes, classification and decision tree, and random forest predictive modelling, was applied to predict marbling across five datasets that included routinely recorded animal farm data (sire, wagyu percentage, weaning weight, feedlot BW data, and metabolomics data) alone or in combination. Prediction models that used farm and feedlot data yielded accuracy of 73 and 63%, respectively. The metabolomics datasets with either identified metabolites or all metabolic features or peaks resulted in 65 and 63% accuracy, respectively. This was independent of whether the metabolome was measured at 196 or 432 days in a feedlot. Using both metabolomics datasets measured at 196 and 432 days increased the accuracy to 67% without farm or feedlot data. The model, which included animal farm data, feedlot weight data, and two metabolomic sampling points, produced an accuracy of 69.6% on the validation dataset. The findings indicated that the ability to predict high or low marbling animals using animal farm data (sire, Wagyu percentage, weaning weight) is greater than that of the metabolomics; however, not all commercial systems record relevant information. The use of metabolomic data coupled with farm and feedlot data in prediction models has the potential to improve the efficiency of Wagyu production. Individual animals with predicted high carcase value can be selected and retained in the production system to full maturity.

Key words: intramuscular fat, metabolites, body composition, cattle

4.3 Introduction

Wagyu and Wagyu crossbred cattle occupy a market niche in the beef cattle industry because of high intramuscular fat (marbling) which confers high eating quality [1]. Wagyu carcases that are scored high for marbling attract a premium price (score 7 or higher on a scale of 1 to 9). However, there is a very high cost of production due to the long period in a feedlot required to assimilate sufficient intramuscular fat [2]. The ability to predict marbling in Wagyu cattle would allow the selection of individual animals either for breeding or meat production. This would increase the overall efficiency of feedlot production in Wagyu cattle. In recent studies, the plasma metabolome of Wagyu crossbred steers undergoing feedlot production was shown to be correlated to an industry-accepted trait of marbling [3-5].

Given the relationship between the metabolome and marbling in Wagyu steers[3,4], it is possible that the plasma metabolome could potentially be used to predict the marbling outcome of individual animals. This could be combined with other information collected throughout the life of the animal such as sire and growth rate to improve the predictions. There is considerable interest in the application of machine learning modelling to predict carcase traits in livestock. Maltecca, et al. [6] utilised machine learning in crossbred pigs, including microbiome data collected at 15 and 22 weeks, to predict loin traits and backfat thickness. The latter authors reported a Pearson correlation coefficient between observed and predicted values of 0.30 and 0.55 for loin traits and backfat thickness, respectively. Shahinfar, et al. [7] examined machine-learning methods to predict carcase traits of Korean Hanwoo beef cattle with correlations between predicted and observed values of 0.95 and 0.60for carcase weight and marbling, respectively. The model used to predict carcase traits in Hanwoo beef included phenotypic traits such as weights measured at 6, 12, 18 and 24 months of age and 50K SNP data. A study in sheep predicted carcase traits using the random forest algorithm from lifetime phenotypic traits with correlation coefficients between the actual and predicted values of 0.88 and 0.56 for

carcase weight and intramuscular fat, respectively[8]. Each of the above studies indicated that the application of machine learning to predict marbling of Wagyu crossbred steers is promising and needs to be evaluated. The application of machine learning in animal production could potentially result in the evaluation of performance and detection of health and welfare issues in real time [9]. This approach could enable decisions to be made quickly and efficiently resulting in production improvements. There are significant knowledge gaps which machine learning is reducing due to the ability to combine multiple data streams such as genomics, transcriptomics, metabolomics and phenotypic information [10]. Machine learning methods have been previously applied to but not limited to dairy cattle to predict the insemination outcomes [11], the survival of second lactation cows [12], and heat stress in dairy cows [13]. Grzesiak and Zaborski [14] illustrate there are multiple ways of applying machine learning methods such as classification and regression trees, interactive classification trees, naïve Bayes classifier, artificial neural networks, and support vector machines to animal breeding.

The ability to determine carcase quality traits of an animal such as marbling prior to slaughter is currently difficult due to the phenotypic measurement of the trait not being measured accurately until slaughter. Previous studies published by Connolly, et al. [3,4] indicated that there is a relationship between plasma metabolites and carcase traits, and that time in the feedlot can affect the metabolome. However, the ability of metabolomic information to predict marbling has not been published.

In addition to novel datasets from metabolomics, data routinely recorded by beef cattle producers could also be used to improve the prediction of marbling from traits. This includes age, pedigree (sire and dam), breed percentages in crossbreeding programs, and on-farm and interim feedlot weights. However, metabolomics could be useful to predict carcase traits in Wagyu cattle when animal farm data for the animals is not available such as when feedlots

purchase store steers where the sire and dam, and weights from birth are unknown. Therefore, plasma metabolomics could enable selection decisions to be made quicker and more efficiently.

The objective of the present study was to utilise a machine learning technique to develop predictive models of marbling based on phenotypic data collected on farm and metabolomic data from Wagyu crossbred steers. The present study applied naïve bayes, decision trees, and random forest classifiers, to predict marbling prior to slaughter from routinely recorded animal farm data from birth, feedlot BWs, and metabolomics data.

4.4 Materials and Methods

The study was approved by The University of Sydney Animal Ethics Committee (Approval # 1124).

4.4.1 Animals and management

The study utilized 156 Wagyu (*Bos taurus*) crossbred steers that were classified as high marbling if marbling score was above 6 at slaughter (scale 1-9) or low marbling otherwise. Descriptive statistics are shown in Table 4.1 for each marbling group. All animals were born from artificial insemination and sire identification was recorded after the calves were tagged at birth. The breed of dams was Shorthorn (n = 77), Angus (n = 17), Brahman (n = 52), and dairy (n = 10), and the proportion of Wagyu (F1, F2, F3 and F4) in the study progeny was calculated. Calves were raised with their dams on pasture until 191 ± 29 days old, weighed and weaned, and then grazed together as a cohort until a feedlot entry weight of approximately 350 kg. The steers were fed in a commercial feedlot for 468 days with ad-libitum feed and water. During the first 84 DOF at the feedlot, the steers were weighed every 14 days. The first weight was at day 0 for induction, 30, 44, 58, 72, 86, and 100 days. The time period average daily weight gain (ADG; kg/d) calculated was calculated using the weights measured. Animals were slaughtered after 468 DOF and carcase data was recorded by an accredited Aus-Meat[15] assessor

and marbling of the muscle *Longissimus thoracis et lumborum* was measured objectively using a hyperspectral camera (HK-333 camera; Hayasaka Rikoh Co. Ltd., Sapporo, Japan)[5].

4.4.2 Plasma metabolome

Plasma samples for metabolome analysis were obtained when the steers were 812 ± 124 days old and at day 196 on feed (early feedlot period). A second plasma sample was collected when the animals were 1048 ± 123 days old and at day 432 on feed (late feedlot period). The metabolome profiles were generated using a Bruker Advanced II 600 MHz spectrometer as previously described[3]. The metabolite data was transferred into Matlab 7.0 software (Mathworks, Narick, MA) where each individual spectra was aligned, automatically phased, baseline corrected, and referenced to the α-C_1H-Glucose doublet at 5.233 ppm also as previously described[3]. Metabolite peaks were identified using the spectral library in Chenomx® NMR Suite Professional (Chenomx Inc., Edmonton, AB, Canada) as well as published literature[16]. There were 290 peaks or clusters mapped within the dataset and 38 specific metabolites were identified (Table 4.2).

4.4.3 Machine learning modelling

Statistical analyses were performed on the dataset of phenotypic data in R Core Team (2020)[17] using the caret package for the naïve bayes, decision tree, and random forest methods [18]. The naïve Bayes method is based on the Bayesian techniques that assumes that the presence of a specific feature is unrelated to the presence of any other features [12]. The decision tree 1 SE is a classifier decision tree used to predict a qualitative response using the standard error method to prune the tree. The random forest algorithm is a supervised learning method that consists of a combination of trees to determine the most efficient predictors [19].

The dataset was randomly split into training (70%) for algorithm development and validation (30%) to evaluate model performance. Animals with marbling < 25.7 % or marbling

score 6 were considered ‘low’ (n = 59) and animals above this value were considered to have high marbling (n = 97). Models were developed to predict a two-class marbling outcome from datasets containing the predictor variables from different sources or datasets as described in Table 4.1. Models were optimised or ‘tuned’ based on the highest value for receiver operating curve (ROC), and resampling done with repeated cross-validation with 5 folds and 3 repeats. The predictors included three datasets from variables collected on-farm before entry to the feedlot (dataset 1), data collected in the feedlot (dataset 2), identified metabolites (dataset 3), all metabolite features (dataset 4), and all variables available from the combination of previously mentioned datasets (dataset 5; Table 4.1).

The accuracy of the prediction models were evaluated by the following performance metrics on the validation dataset only where the high marbling group is the positive class: accuracy, sensitivity, specificity, area under the curve (AUC), and precision [20]. The accuracy indicates the proportion of data points or animals correctly classified in the corresponding marbling group, the sensitivity is the proportion of true positives the model is predicting correctly, and the specificity is the proportion of true negatives the model is correctly classifying. The AUC indicates the overall performance of the model and shows how capable the model is at distinguishing between classes whereas the precision indicates the ability of the model to return the correct result rather than the incorrect result [21].

Table 4.1. Datasets and predictor variables in each used by the naïve bayes, decision tree 1SE, and random forest classifiers to predict marbling from crossbred Wagyu steers.

Dataset	Variables included in dataset
Animal Farm Dataset (1)	Wagyu percentage, weaning weight, and sire.
Feedlot Dataset (2)	Age at feedlot induction, body weight at 0 (induction), 30, 44, 58, 72, 86, and 100 DOF; weight gain at 30, 44, 58, 72, 86, and 100 DOF, and overall ADG from induction to slaughter.
Identified Metabolites Dataset (3)	Formate, Allantoin, Mannose, Creatinine, Serine, Anserine, Glycine, Choline, Dimethyl sulfone, Citrulline, Citrate, Carnosine, Aspartate, Methylamine, Acetone, Glutamate, Acetate, Propionate, 3-Hydroxybutyrate, Arginine, Citrate, Creatine, Glucose, Glutamine, Glycoprotein acetyls, Histidine, Isobutyrate, Isoleucine, Lactate, Leucine, Lipid, Lipid VLDL, Unsaturated Lipid, Methionine, Methylhistidine, Phenylalanine, Proline, Tyrosine, Valine
All Metabolomics Features Dataset (4)	Relative abundance of all 290 features or peaks found in the NMR spectra using Standard Recoupling of Variables
All Variables Available (5)	All variables in dataset 1, 2, 3 and 4

The analysis included two datasets from the animal data one collected from the farm and one from the feedlot. There were also three series of databases assembled from the metabolomic analyses of the plasma samples collected at 196 and 432 DOF as described in Table 4.1. In the first instance, a metabolite dataset (dataset 3) and an NMR features data (dataset 4) were independently built from the 196 DOF metabolomic analysis. An ‘all variables’ dataset (dataset

5) was then compiled by combining the animal data, the feedlot data, the 196 DOF metabolite data, and corresponding NMR features data (Table 4.1). Thus, three datasets unique to 196 DOF were available as inputs to machine learning.

Likewise, a second series of three datasets unique to the 432 DOF plasma metabolomic analyses were compiled. Therefore 'all variables' dataset (dataset 5) contained the 432 DOF metabolite data and NMR features data along with the animal data and feedlot data. Finally, a third series of three datasets, were generated. These datasets merged the data from the from the two time points, such that the metabolite dataset contained the combined metabolite data from the 196 and 432 DOF plasma samples; similarly, the NMR features dataset combined the NMR features data from both time points. The 'all variables' dataset 5 then contained the animal data, feedlot data, and the combined 196 and 432 DOF metabolite and NMR features data.

4.5 Results

Table 4.2 shows the descriptive statistics of the Aus-meat marbling and camera marbling. Interestingly, Aus-meat marbling score showed greater CV between animals compared to camera marbling, whereas feedlot induction weight and HSCW showed the lowest CV.

Table 4.2: Descriptive statistics of Wagyu crossbred steers split into two classes of high (n = 97) and low (n = 59) marbling including the min, mean, max, standard error and coefficient of variation .

Variable	Minimum	Mean	Maximum	Standard Error	Coefficient of Variation
High marbling group					
Wagyu percent (%)	50.0	76.8	100.0	1.63	20.85
Age At Induction (Days)	460	654	1041	12.32	18.57
Feedlot Induction Weight (kg)	246	338	430	3.01	8.76
ADG Feedlot (kg)	1.03	1.39	1.98	0.019	13.34
Aus-meat Marble Score	4	7.62	9.00	0.162	20.96
Camera Marbling (%)	25.7	31.39	44.50	0.402	12.62
HSCW (kg)	323.5	425.4	542.0	3.814	8.83
Low marbling group					
Wagyu percent (%)	50	64.19	96.88	2.09	25.02
Age At Induction (Days)	465	547	1,005	12.9	18.05
Feedlot Induction Weight (kg)	262	323	370	3.19	7.59
ADG Feedlot (kg)	0.98	1.44	1.86	0.029	15.22
Aus-meat Marble Score	2	5.05	9.00	0.207	31.50
Camera Marbling (%)	16.3	21.86	25.60	0.345	12.11
HSCW (kg)	323	431.9	531.5	5.74	10.20

Table 4.3 shows the results of model performance metrics for the naïve bayes, decision tree 1SE and random forest models (accuracy, sensitivity, specificity, AUC, and precision) for the datasets 1 and 2 of animal farm data and feedlot data. The greatest accuracy was using the random forest and decision tree 1 SE for dataset 1 as shown below. The animal farm data outperformed the feedlot data in accuracy, sensitivity, AUC, and precision when the prediction of marbling into two classes high and low. Random forest modelling performed best with

Animal farm dataset 1 compared to the Naïve Bayes and decision trees. In contrast, Naïve Bayes performed best with Feedlot dataset 2 although the specificity was only 0.41 (Table 4.3).

Table 4.3: Performance of naïve bayes, decision tree 1 SE and random forest models to classify crossbred Wagyu steers into low or high marbling using animal farm data (dataset 1), feedlot data (dataset 2).

	Accuracy	Sensitivity	Specificity	AUC*	Precision
Animal farm dataset 1					
Naïve Bayes	0.622	1.000	0.000	0.776	0.630
Decision Tree 1SE	0.739	0.793	0.647	0.749	0.793
Random Forest	0.739	0.759	0.706	0.785	0.815
Feedlot Dataset 2					
Naïve Bayes	0.630	0.759	0.412	0.637	0.688
Decision Tree 1SE	0.609	0.793	0.294	0.465	0.657
Random Forest	0.587	0.793	0.235	0.577	0.639

** Area under the curve*

Table 4.4 shows the classification results for the three datasets 3, 4 and 5 which included identified metabolites, all metabolite features, and all variables available at the first sample point for metabolomics at 196 DOF. The most accurate model was using the decision tree 1SE on dataset 5 which is a combination of all the available variables, however there were small differences in accuracy between the three datasets. The naïve Bayes model had the second highest accuracy for dataset 4. The greatest sensitivity and precision were produced using the naïve Bayes model in dataset 5 which was the combination of all variables. Sensitivity was high (>0.72) with all datasets and machine learning models, but specificity was low (<0.47; Table 4.4).

Table 4.4: Performance of naïve bayes, decision tree 1 SE and random forest models to classify crossbred Wagyu steers into low or high marbling using 36 identified plasma metabolites (dataset 3), all features or peaks from plasma metabolomics (dataset 4), and all variables available (dataset 5) using metabolomic data sampled at **196 days on feed**.

	Accuracy	Sensitivity	Specificity	AUC*	Precision
Metabolites Dataset 3					
Naïve Bayes	0.565	0.862	0.059	0.604	0.610
Decision Tree 1SE	0.500	0.621	0.294	0.511	0.600
Random Forest	0.609	0.793	0.294	0.618	0.657
Metabolomic features Dataset 4					
Naïve Bayes	0.630	0.724	0.471	0.598	0.700
Decision Tree 1SE	0.565	0.621	0.471	0.573	0.667
Random Forest	0.609	0.862	0.176	0.583	0.641
All data Dataset 5					
Naïve Bayes	0.587	0.931	0.000	0.631	0.614
Decision Tree 1SE	0.652	0.828	0.353	0.559	0.686
Random Forest	0.609	0.931	0.059	0.681	0.628

** Area under the curve*

The results shown in table 4.5 includes model performance using datasets 3, 4 and 5 when blood was sampled at 432 DOF for metabolomics. The most accurate prediction model was using the decision tree 1SE and random forest on dataset 5, which included all available variables. The highest sensitivity was produced using random forest on dataset 4 and the highest precision was the naïve Bayes on dataset 3, which only included the 36 identified metabolites. Specificity of the model was below 50% except for naïve Bayes with Dataset 3 and decision trees with Dataset 5.

Table 4.5: Performance machine learning models to classify crossbred Wagyu steers into low or high marbling using 36 identified plasma metabolites (dataset 3), all features or peaks from plasma metabolomics (dataset 4), and all variables available (dataset 5) using metabolomic data sampled **at 432 days on feed**.

	Accuracy	Sensitivity	Specificity	AUC*	Precision
Metabolites Dataset 3					
Naïve Bayes	0.630	0.552	0.765	0.730	0.800
Decision Tree 1SE	0.587	0.724	0.353	0.688	0.656
Random Forest	0.652	0.897	0.235	0.735	0.667
Metabolic features Dataset 4					
Naïve Bayes	0.587	0.586	0.588	0.644	0.708
Decision Tree 1SE	0.587	0.586	0.588	0.632	0.708
Random Forest	0.652	0.931	0.176	0.736	0.659
All data Dataset 5					
Naïve Bayes	0.652	0.759	0.471	0.694	0.710
Decision Tree 1SE	0.674	0.690	0.647	0.708	0.769
Random Forest	0.674	0.897	0.294	0.732	0.684

** Area under the curve*

Combining both metabolomics datasets measured at 196 and 432 DOF to be used as predictors yielded the highest accuracy using the naïve Bayes and decision tree 1SE for dataset 5 which included all available variables at both sample points of the feedlotting process. However, this latter model had low specificity (59%) and thus naïve Bayes with dataset 3 showed prediction with all performance statistics above 65% (Table 4.6).

Table 4.6: Performance of machine learning models to classify crossbred Wagyu steers into low or high marbling using 36 identified plasma metabolites (dataset 3), all features or peaks from plasma metabolomics (dataset 4), and all variables available (dataset 5) using both metabolomic data sampled **at 196 and 432 days on feed**.

	Accuracy	Sensitivity	Specificity	AUC*	Precision
Metabolites Dataset 3					
Naïve Bayes	0.674	0.655	0.706	0.684	0.792
Decision Tree 1SE	0.609	0.586	0.647	0.647	0.739
Random Forest	0.652	0.897	0.235	0.771	0.667
Metabolic features Dataset 4					
Naïve Bayes	0.674	0.793	0.471	0.694	0.719
Decision Tree 1SE	0.543	0.655	0.353	0.496	0.633
Random Forest	0.652	1.000	0.059	0.685	0.644
All data Dataset 5					
Naïve Bayes	0.696	0.828	0.471	0.333	0.727
Decision Tree 1SE	0.696	0.759	0.588	0.625	0.759
Random Forest	0.674	0.931	0.235	0.704	0.675

** Area under the curve*

4.6 Discussion

The first analysis of the present study examined the accuracy of animal farm data and feedlot data that was collected on farm prior to feedlot entry and then induction and BWs during the feedlotting period. This analysis with dataset 1 and 2 returned the best performance compared to all other datasets 2, 3 4, and 5 with all statistics greater than 0.70 using random forest or greater than 0.65 for decision trees. Dataset 1 included sire, wagyu percentage, and weaning weight, which demonstrate the large influence of genetics on marbling and the importance to predict this trait. It is important to note that the dataset of the present study contained 62% of steers with high marbling and, therefore, the higher the accuracy for the prediction model the more accurate the model is therefore the higher the accuracy the better. Although there is a trade-off between sensitivity and specificity, accuracy above 0.62 was achieved with many datasets in the present study suggesting the models could improve, the industry practice of

feeding all Wagyu steers which results in sensitivity of 1 and specificity of 0. The fact that many classification models and datasets had high sensitivity and modest specificity suggest this approach could help to identify those animals, which will not achieve the desired marbling.

Nevertheless, it is important to note that other measurement methods of intramuscular fat in live animals such as ultrasound could potentially yield similar accuracy in a more practical and cost-effective way. However, no studies attempted to predict carcase marbling from ultrasound images over 265 days before slaughter as done in the present study.

The ability to select cattle based on their marbling potential early in the feedlot process is currently difficult and there are other methods being trialled to enable selection of animals prior to slaughter. de las Heras-Saldana, et al. [22] evaluated the use of whole genome sequence information to improve the accuracies of genomic prediction in Hanwoo cattle for the traits backfat thickness, carcase weight, eye muscle area, and marbling score. The latter authors used a database of 13,717 animals with carcase phenotypes and imputed sequence genotypes as there were no pedigrees available due to the data being collected commercially across multiple years and slaughterhouses. The data was then segregated into two datasets with one used for independent GWAS discovery and the other for validation of prediction. The results indicated accuracies of 0.50, 0.47, 0.58 and 0.47 for eye muscle, backfat thickness, carcase weight, and marbling score, respectively [22]. These values are lower than those obtained in the present study using only metabolomics data although the calculation of accuracy in the latter study differs significantly from the present study. Therefore, the models developed and evaluated in the present study to predict marbling could help to improve the current production systems similar to those achieved with genomic information.

Both metabolomic datasets 3 and 4 collected at 196 and 432 DOF showed lower accuracy compared to the dataset containing the animal farm data only. However, the difference between

the precision for each of the different datasets was small. The ability to select cattle at 32 days prior to slaughter (432 DOF) would not improve the production system greatly as the animals have already consumed a large portion of the feed required. Previous results demonstrated that the metabolome of Wagyu cattle was affected by DOF and correlated to the degree of maturity and amount of fat deposition [3,4]. Thus, we hypothesized that using metabolomics information from samples obtained later in the feedlot could improve the prediction of marbling. This hypothesis was correct although the differences in accuracy were very small compared to 196 DOF. Using both metabolomics datasets from 196 and 432 DOF yielded improved accuracy compared to either dataset independently, or to feedlot data. This suggested that one sampling either early or late in the feedlot does not affect the accuracy of the predictions although the combination of both blood sampling points produced a small improvement.

Shahinfar, et al. [7] have shown that it is effective to use machine learning to predict carcase traits such as marble score and carcase weight in Hanwoo cattle. The study examined four machine learning models including multilayer perceptron, model tree, random forest and support vector machines using 52,924 SNPs as well as traits such as live weight, ultrasound, biophysical measurements, sire EBV's, and average daily gain. The support vector machines returned accuracy of 0.94 for carcase weight and 0.64 for marbling. Similarly, Lee, et al. [23] undertook a study in Hanwoo cattle to develop a model using body size measurements shortly before slaughter such as cold carcase weight, backfat thickness, EMA, side length, forequarter length, hindquarter length, cervical vertebrae length, thoracic vertebrae length and many more. The objective was to estimate the carcase weight of 132 head using three different modelling approaches of multiple regression analysis, partial least squares, and neural networks. The neural networks model produced the greatest accuracy with an R^2 of 0.92 from testing the models.

The ability to select wagyu cattle when entering the feedlot with no previously measured animal farm data available using metabolomics could enable commercial feedlots to become more efficient. Currently, some large-scale producers of feeder cattle are not recording specific traits such as weaning weights, sire, and dam identification. The classification of animals into high and low marbling groups in the present study using feedlot induction weight and multiple weights throughout the feedlot process is not as accurate as dataset 1 using animal farm data such as sire, Wagyu percentage, and weaning weight. Kalaiselvi, et al. [24] conducted a review of metabolomics in livestock and have shown findings to suggest metabolomics combined with genomics could enable a more comprehensive system understanding. The present study is a step towards this application and demonstrates that metabolomics information could assist in improving the accuracy of genomic predictions. Li, et al. [25] highlighted the ability to integrate metabolomics and genomics into animal breeding. Utilising multiple levels of data, the interaction between plasma metabolites and genes or genetic variants can be understood further. Li, et al. [25] explored if higher heritability was available for plasma metabolites because markers with higher heritability for specific traits could enable directional selection in favour of the concentration of that specific metabolite. The inclusion of feedlot recorded data and metabolomics in Wagyu cattle could enable better selection of steers that are going to produce a superior carcase.

4.7 Conclusion

Naïve bayes, decision tree, and random forest machine learning modelling is useful to predict high and low marbling Wagyu crossbred steers. The accuracy of the predictions increases with the integration of animal farm data, feedlot weight data, and metabolomics data measured early and late in the feedlot. Metabolomics data later in the feedlot seems to improve the prediction

models to a greater extent than earlier samples. Pedigree and Wagyu content seem the most important information to predict marbling in Wagyu crossbred cattle.

4.8 References

1 Park, S. J. *et al.* Genetic, management, and nutritional factors affecting intramuscular fat deposition in beef cattle - A review. *Asian-Australasian journal of animal sciences* **31**, 1043-1061, doi:10.5713/ajas.18.0310 (2018).

2 Gotoh, T., Nishimura, T., Kuchida, K. & Mannen, H. The Japanese Wagyu beef industry: current situation and future prospects - A review. *Asian-Australasian journal of animal sciences* **31**, 933-950, doi:10.5713/ajas.18.0333 (2018).

3 Connolly, S., Dona, A., Hamblin, D., D'Occhio, M. J. & González, L. A. Changes in the blood metabolome of Wagyu crossbred steers with time in the feedlot and relationships with marbling. *Scientific Reports* **10**, 18987, doi:10.1038/s41598-020-76101-6 (2020).

4 Connolly, S. *et al.* Relationship of the blood metabolome to subsequent carcass traits at slaughter in feedlot Wagyu crossbred steers. *Scientific Reports* **9**, 15139, doi:10.1038/s41598-019-51655-2 (2019).

5 Nakahashi, Y., Maruyama, S., Seki, S., Hidaka, S. & Kuchida, K. Relationships between monounsaturated fatty acids of marbling flecks and image analysis traits in longissimus muscle for Japanese Black steers. *J Anim Sci* **86**, 3551-3556 (2008).

6 Maltecca, C. *et al.* Predicting Growth and Carcass Traits in Swine Using Microbiome Data and Machine Learning Algorithms. *Scientific Reports* **9**, 6574, doi:10.1038/s41598-019-43031-x (2019).

7 Shahinfar, S., Al-Mamun, H. A., Park, B., Kim, S. & Gondro, C. Prediction of marbling score and carcass traits in Korean Hanwoo beef cattle using machine learning methods and synthetic minority oversampling technique. *Meat science* **161**, 107997, doi:https://doi.org/10.1016/j.meatsci.2019.107997 (2020).

8 Shahinfar, S., Kelman, K. & Kahn, L. Prediction of sheep carcass traits from early-life records using machine learning. *Computers and Electronics in Agriculture* **156**, 159-177, doi:https://doi.org/10.1016/j.compag.2018.11.021 (2019).

9 Neethirajan, S. The role of sensors, big data and machine learning in modern animal farming. *Sensing and Bio-Sensing Research* **29**, 100367, doi:https://doi.org/10.1016/j.sbsr.2020.100367 (2020).

10 Morota, G., Ventura, R. V., Silva, F. F., Koyama, M. & Fernando, S. C. BIG DATA ANALYTICS AND PRECISION ANIMAL AGRICULTURE SYMPOSIUM: Machine learning and data mining advance predictive big data analysis in precision animal agriculture1. *Journal of Animal Science* **96**, 1540-1550, doi:10.1093/jas/sky014 (2018).

11 Shahinfar, S. *et al.* Prediction of insemination outcomes in Holstein dairy cattle using alternative machine learning algorithms. *Journal of Dairy Science* **97**, 731-742, doi:10.3168/jds.2013-6693 (2014).

12 van der Heide, E. M. M. *et al.* Comparing regression, naive Bayes, and random forest methods in the prediction of individual survival to second lactation in Holstein cattle. *Journal of Dairy Science* **102**, 9409-9421, doi:https://doi.org/10.3168/jds.2019-16295 (2019).

13 Becker, C. A., Aghalari, A., Marufuzzaman, M. & Stone, A. E. Predicting dairy cattle heat stress using machine learning techniques. *Journal of Dairy Science*, doi:https://doi.org/10.3168/jds.2020-18653 (2020).

14 Grzesiak, W. & Zaborski, D. *Examples of the Use of Data Mining Methods in Animal Breeding.*, (2012).

15 Aus-Meat. *Australian Beef Carcass Evaluation - Beef and Veal Chiller Assessment Language*, <https://www.ausmeat.com.au/WebDocuments/Chiller_Assessment_Language.pdf> (2017).
16 Nicholson, J. K., Foxall, P. J. D., Spraul, M., Farrant, R. D. & Lindon, J. C. 750 MHz 1H and 1H-13C NMR Spectroscopy of Human Blood Plasma. *Analytical Chemistry* **67**, 793-811, doi:10.1021/ac00101a004 (1995).
17 R Core Team. R: A language and environment for statistical computing. (R Foundation for Statistical Computing, Vienna, Austria, 2020).
18 Majka, M. naivebayes: High performance implementation of the Naive Bayes algorithm. R package version 0.9. 2. (2018).
19 Breiman, L. Random Forests. *Machine Learning* **45**, 5-32, doi:10.1023/A:1010933404324 (2001).
20 Kuhn, M. in *Pfizer Global and R&D* (Groton, CT, 2013).
21 Narkhede, S. Understanding AUC-ROC Curve. *Towards Data Science* **26** (2018).
22 de las Heras-Saldana, S. *et al.* Use of gene expression and whole-genome sequence information to improve the accuracy of genomic prediction for carcass traits in Hanwoo cattle. *Genetics Selection Evolution* **52**, 54, doi:10.1186/s12711-020-00574-2 (2020).
23 Lee, D.-H. *et al.* Estimation of carcass weight of Hanwoo (Korean native cattle) as a function of body measurements using statistical models and a neural network. *Asian-Australas J Anim Sci* **33**, 1633-1641, doi:10.5713/ajas.19.0748 (2020).
24 Kalaiselvi, G., Tirumurugaan, K. & Vijayarani, K. Metabolomics–A overview". *International Journal of Current Re Key Words*.
25 Li, J. *et al.* Genomic Heritability and Genome-Wide Association Studies of Plasma Metabolites in Crossbred Beef Cattle. *Frontiers in genetics* **11**, 538600-538600, doi:10.3389/fgene.2020.538600 (2020).

Chapter 5: Effect of adjusting feed efficiency for carcase fat on the relationship with the plasma metabolome in Wagyu crossbred steers

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Overview

This chapter examined the relationship between the plasma metabolome and various measures of feed efficiency adjusted or not for carcase fat. The relationship between RFI and carcase fat has been shown to be positive which is not desirable in Wagyu cattle because intramuscular fat is a very important trait. Potential applications include biomarker discovery for the selection of efficient animals with desirable carcase traits.

5.1 Abstract

The aim of the present study was to evaluate the relationship between the plasma metabolome and Residual Feed Intake (RFI) of crossbred Wagyu steers adjusting for carcase fat such as intramuscular fat. A secondary objective was to determine if these relationships were also found later in the feedlot. Blood samples were collected from 140 steers at 78 and 313 days on feed (DOF). The RFI was adjusted for subcutaneous and intramuscular fat measured at slaughter. ^{1}H-NMR spectroscopy identified 36 metabolites in the plasma samples with methionine, phenylalanine, serine, and histidine being negatively correlated ($P < 0.05$) with all measures of RFI at 78 DOF whereas glucose only reached significance when RFI was adjusted for P8 fat and intramuscular fat ($P < 0.05$). At 313 DOF, methionine and phenylalanine were the only metabolites that were also correlated with RFI ($P < 0.05$) but metabolites from lipid metabolism appeared with positive correlations with RFI ($P < 0.05$) including choline, glycoprotein acetyls, and lipids. Alternative measures of feed efficiency such as residual gain and gain to feed showed stronger and more correlations with plasma metabolites ($P < 0.05$). In conclusion, blood biomarkers of feed efficiency are not severely affected by the adjustment of RFI for carcase fat, but alternative measures have a larger effect. There is a shift in the correlation between metabolites and feed efficiency from a larger influence of protein to lipid metabolism as the animals mature. This information could help understanding the underlying metabolic process of tissue deposition and assist with genetic selection for RFI while avoiding undesirable effects on economically important carcase attributes such as intramuscular fat in Wagyu feedlot steers.

5.2 Introduction

Wagyu beef occupies premium niche markets because of the eating quality obtained from the amount and composition of intramuscular fat (IMF) [1]. However, Wagyu production is costly as animals require a relatively long period in a feedlot to achieve a high-value carcase[2]. In addition, the cost of production in a feedlot increases with time because feed efficiency declines as cattle deposit additional fat [1]. Improving feed efficiency is, therefore, central to the optimization of Wagyu beef production.

A common measure of feed efficiency is the gain to feed ratio (GF) which is the ratio of body growth to dry matter intake (DMI) [3]. Another measure of feed efficiency is residual feed

intake (RFI), calculated as the difference between actual intake and the expected intake for BW and growth rate [4]. The measurement of RFI is expensive and impractical on a commercial scale [5]. Hence, the ability to predict RFI using metabolic biomarkers would have large commercial benefits. An antagonistic relationship exists between RFI and body fat [6,7] and it is critical that RFI is corrected for carcase fat. Duff, et al. [8] proposed the use of RFI whilst accounting for marbling in beef breeding programs. The RFI can be adjusted to consider multiple factors such as average daily gain (ADG) and mean metabolic body weight (MMBW), and could also include marbling, subcutaneous P8 fat, and rib fat.

McGee, et al. [9] examined the relationship of feed efficiency to growth, and marbling in Wagyu bulls. RFI was negatively correlated with marbling measured by ultrasound on day 0 and 70, instead of actual carcase marbling. The negative relationship with MARBLING indicates that the selection of cattle for low RFI could affect carcase quality. The McGee, et al. [9] study highlighted the need for further studies on RFI in Wagyu cattle to improve the efficiency of production. The selection of efficient cattle whilst maintaining distinctive carcase attributes is an important goal in Wagyu beef production.

The relationship of the blood and tissue metabolomes to RFI has attracted recent interest in both European (*Bos taurus*) and Zebu (*Bos indicus*) beef cattle [10]. In Angus crossbred beef steers (*Bos taurus*), three metabolites (carnitine, creatine, hippurate) explained 32% of the genetic variation in RFI and 11 metabolites explained up to 52% of the genetic variation in RFI [9]. The ability to use the metabolome to reliably predict RFI has major application, as it would remove the need to measure individual feed intake in cattle. Hence, the present study looked at the relationship of the plasma metabolome to RFI in Wagyu crossbred steers maintained in a feedlot for 421 days. Previous studies had shown that different groups of metabolites were related to RFI [11] and carcase traits [12] at different times before slaughter. In the present study, the metabolome was characterized after 78 days in a feedlot when steers were undergoing RFI measurement, and at 313 days, which was 108 days before slaughter. The unique feature of the present study was that the metabolome was related to RFI adjusted for carcase traits such as marbling, rib fat and subcutaneous P8 fat and, additionally, residual gain and gain to feed.

5.3 Materials and Methods

The present study had approval from the institutional animal ethics committee of The University of Sydney (approval #1125).

5.3.1 Animals and management

The present study utilized 140 crossbred Wagyu steers with Brahman, Shorthorn and Angus the predominant dam breeds mated to Wagyu bulls. The animals were housed in a commercial feedlot and fed to allow for ad-libitum consumption of four diets for a total of 421 days (Table 5.1). The diet was changed on day 4, 10 and 93 after induction. For the first 28 days animals were acclimatized to electronic feeders (GrowSafe, Calgary, Alberta, Canada). Feed intake was measured from day 29 to 184 and animals were weighed at the start and end of this period. At day 185, the steers were transferred to a conventional feedlot pen with standard in-line concrete feed bunks and open dirt floor. Animals were weighed fortnightly for the remainder of the study. Feed was provided twice daily before 10:00 AM and after 3:00 PM. A timeline of events is shown in Figure 5.1. Complete sets of data (metabolome, carcase, marbling, RFI) were available for 123 steers.

5.3.2 Blood sampling

Blood samples were obtained at day 78 when the steers were undergoing feed intake measurement and at day 313. For blood sampling, steers were moved to a central handling facility at 1000h and blood was collected between 11:00 and 14:15 h. An 18G needle and evacuated lithium heparin tube was used (Vacutainer BD, Becton Dickinson, Frankland Lakes, NJ) to obtain blood. Samples were kept on ice for up to 20 min and centrifuged at 10,000 x g for 15 min. Plasma was stored at -80 ^{0}C until analysis.

Figure 5.1: Timeline of events throughout experimental days since the start of the trial with Wagyu crossbred steers to measure the plasma metabolome.

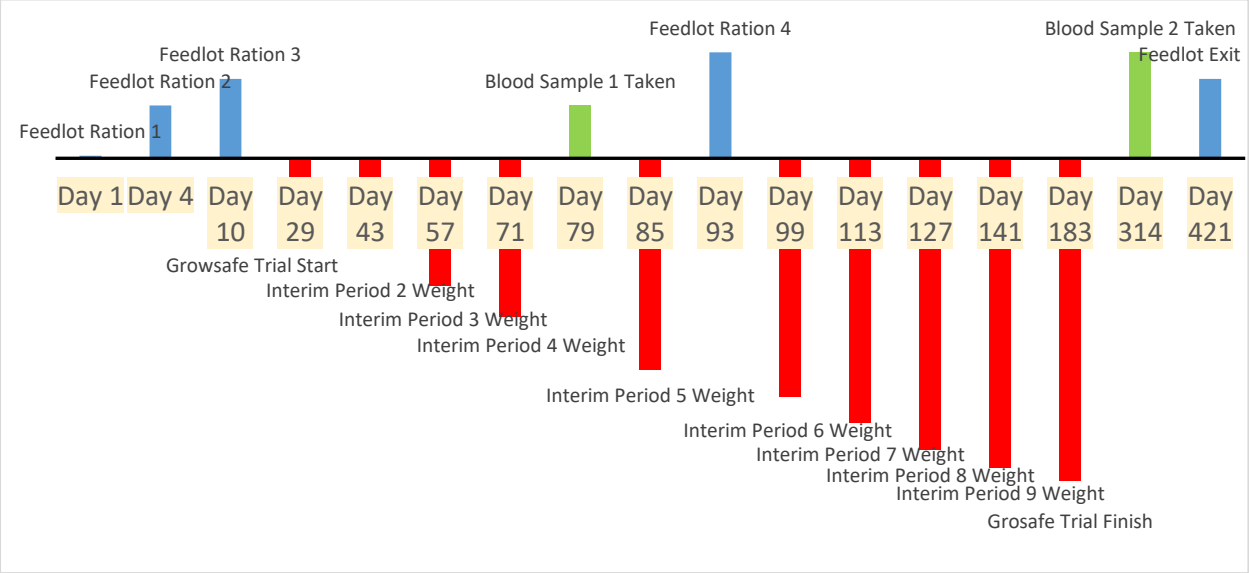


Table 5.1: Diet ingredients and composition.

Ingredient	Unit	Diet 1	Diet 2	Diet 3	Diet 4
Steam Flaked Barley	% as fed	21.5	28.5	37.5	47.0
Steam Flaked Wheat	% as fed	21.0	22.5	14.5	19.0
Finisher Supplement	% as fed	5.0	5.2	0.0	1.7
Growth Supplement	% as fed	0.0	0.0	5.0	3.5
Molasses	% as fed	12.0	10.1	4.8	5.0
Vegetable Oil	% as fed	0.0	1.2	1.4	1.5
Brewers Sweet Grain	% as fed	0.0	0.0	15.0	8.0
Sunflower Meal	% as fed	5.5	5.0	1.5	0.0
Corn Silage	% as fed	12.0	12.0	12.8	9.8
Barley Straw	% as fed	12.0	9.5	7.5	4.5
Lucerne Hay	% as fed	11.0	6.0	0.0	0.0
Chemical Composition					
Dry Matter	%	-	-	94.8	95.2
Moisture	%	-	-	5.2	4.9
Acid Detergent Fibre	% DM	-	-	9.0	7.0
Dry Matter Digestibility	% DM	-	-	81.0	85.0
Inorganic Ash	% DM	-	-	6.0	6.0
Organic Matter	% DM	-	-	94.0	94.0
Crude Fat	% DM	-	-	4.0	4.1
Crude Protein	% DM	14.1*	13.8*	12.5	12.4
Neutral Detergent Fibre	% DM	29.3*	25.6*	20.0	17.0
Metabolizable Energy	MJ/kg DM	10.9*	11.6*	13.0	13.5
Ionophore (monesin)	ppm	21.3*	22.2*	23.4*	23.9*
Net Energy of Gain	MJ/kg DM	4.4*	4.9*	5.5*	6.0*
Net Energy of Maintenance	MJ/kg DM	7.0*	7.6*	8.3*	8.9*

**Values are estimates from feed composition tables of the ingredients making up the diet.*

5.3.3 Carcase data

The grading of carcases was undertaken by accredited assessors using several methods including Aus-meat [11] and Meat Standards Australia (MSA) [12]. Marbling percentage was objectively measured using a hyperspectral camera (camera marbling, CM) (HK-333 camera; Hayasaka Rikoh Co. Ltd., Sapporo, Japan) [13]. The Aus-Meat grading measures marbling scores from 0 to 9+ and the MSA marbling score measures from 100 to 1100.

5.3.4 Metabolite profiling

Plasma samples were processed and analysed for metabolites in accordance with the protocol published in Dona, et al. [14] and using the exact method previously published in Connolly, et al. [15]. Spectra obtained from NMR (Bruker Advance III 600 MHz spectrometer equipped with a 5-mm TCI cryoprobe) were imported into Matlab 7.0 software for alignment, normalisation and automatic phasing, baseline correcting, and referencing of the dataset to the α-C1H-Glucose doublet (5.233 ppm) [16]. The residual water was removed and PCA was also undertaken to ensure the quality control samples were clustering as documented in Connolly, et al. [15]. The final step of the metabolite processing was to undertake statistical recoupling of variables (SRV) to determine the clusters or bucketing of the NMR spectra. The raw spectra was also imported in parallel into Chenomx® for the assignment of the metabolite name to the clusters using the spectral library of Chenomx® NMR Suite Professional (Chenomx Inc., Edmonton, AB, Canada) as well as using published literature and the livestock metabolite database [16-18].

5.3.5 Statistical analyses

Statistical analysis was undertaken using the statistical program SAS 9.4 (SAS Institute Inc., Cary, New Jersey, USA) with multiple databases that were merged including carcase data, feed intake data, phenotypic records, and metabolite data. Once the database was merged the RFI was calculated using the animals with complete records (n = 123). The RFI was then calculated as the residual from regressing observed feed intake against mid-trial $BW^{0.75}$ and ADG with further calculations adding P8 fat thickness (RFI P8), rib fat thickness (RFI Rib Fat), Aus-Meat marbling score (RFI Aus-meat), MSA marbling score (RFI IMF MSA), or camera marbling (RFI Camera Marbling). In addition, residual gain (RG) was calculated as the residual from regressing

ADG against feed intake and BW and Gain to Feed (GF) ratio diving average feed intake by ADG.

The RG indicates the difference between the actual gain and the predicted gain based on BW, intake and composition, where a positive RG indicates the animal gained more than the predicted values from the intake and BW [19]. The FCR indicates the ratio of dry matter intake to live weight gain; a lower FCR value is desirable if selection is for efficient animals as it indicates less feed is required per kg gained[19]. The GF ratio includes the average dry matter intake (kg) and the metabolic BW.

Pearson correlation coefficients were calculated for each trait in relation to all metabolites identified in Chenomx® and the RFI adjusted variables for sampling point 1 (78 DOF) and sampling point 2 (313 DOF). The final analysis included a principal component analysis (PCA) using all identified metabolites which were standardized using the z-score (mean = 0 and STD = 1). Only the principal components which had an eigenvalue >1 were included for the final PCA analysis. Loading plots were obtained to visualize potential clustering of metabolites depending and their influence on the principal components values. Finally, Pearson correlation coefficients were calculated between the PC and phenotypic traits measured at the feedlot and abattoir.

5.4 Results

5.4.1 Descriptive Statistics and Feedlot Data

Table 5.2 shows the descriptive statistics for the N=123 animals with the number of variables recorded for each trait including the carcase attributes, weight gains and the adjusted RFI variables. All measures of RFI were very similar and ranged by 4.34 to 4.67 kg/d (Table 5.2). The average Aus-meat marble score was 5.721 with a range from 2 through to 9, whereas the camera marbling was 23.7 ± 5.2% with the greatest being 36.5%. Measures of carcase fat showed the largest coefficient of variation (CV) between animals although CV was lowest for camera marbling and largest for Aus-Meat marble score.

Table 5.2: Descriptive statistics of N=123 Wagyu crossbred steers during the entire feedlot period and during the feed testing period to measure residual feed intake (RFI).

Variable	Minimum	Mean	Median	Maximum	Standard Deviation	Coefficient of Variation
Animal Data						
Wagyu percent (%)	48.44	68.48	75.00	100.00	16.25	23.74
Age At Induction (days)	586	721	766	835	91.23	12.65
Feedlot Induction Weight (kg)	316	384	382	478	31.00	8.072
Grow Safe Trial Data						
RFI Trial Start Weight (kg)	346	461	455	583	38.65	8.380
RFI Trial Finish Weight (kg)	519	640	636	815	56.24	8.791
ADG RFI Trial, (kg/d)	0.690	1.167	1.150	1.660	0.197	16.93
Dry matter intake, (kg/d)	7.01	9.95	9.96	12.750	1.085	10.91
Adjusted RFI Variables						
Mid-trial Metabolic Body Weight, $(kg)^{0.75}$	98	114	113	136	7.07	6.229
Dry matter intake (% of BW)	1.367	1.809	1.815	2.332	0.168	9.278
Feed Conversion Ratio, (kg DM/kg ADG)	6.49	8.68	8.60	12.70	1.231	14.20
Gain to Feed, (kg ADG/kg DMI)	0.079	0.117	0.116	0.154	0.016	13.30
RFI, (kg/d)	-2.130	0.000	-0.004	2.265	0.803	0.000
RFI RF, (kg/d)	-2.298	0.000	0.005	2.358	0.795	0.000
RFI P8, (kg/d)	-2.250	0.000	0.044	2.379	0.792	0.000
RFI Marbling, (kg/d)	-2.162	0.000	0.036	2.379	0.791	0.000
RFI Marbling MSA, (kg/d)	-2.167	0.000	0.028	2.172	0.789	0.000
RFI Marbling Aus-meat, (kg/d)	-2.197	0.000	0.013	2.147	0.791	0.000
Residual Gain, (kg/d)	-0.378	0.000	0.002	0.494	0.143	0.000
Carcase Data						
Hot carcase weight (kg)	344	462	460	615	48.8	10.57
Rib Fat, Log(mm)	1.386	2.290	2.303	3.258	0.427	18.63
P8 Fat, (mm)	10.00	18.14	16.0	41.0	6.178	34.05
Aus-meat Marble Score	2.00	5.721	5.00	9.00	2.311	40.39
MSA Marble Score	410	816	760	1170	229	28.09
Camera Marbling (%)	13.30	23.70	23.00	36.50	0.052	21.88

Table 5.3 includes the partial Pearson correlation coefficients between the feedlot and carcase data and the adjusted RFI variables. All the adjusted RFI variables were significant ($P \leq 0.01$) for the dry matter intake trait as well as the residual gain, gain to feed and feed conversion ratio. The Aus-meat marble, MSA marbling, and P8 fat, had tendencies ($P \leq 0.10$; table 5.3) for the original RFI trait. None of the other adjusted RFI traits were significant for the carcase traits such as camera marbling, HSCW, rib fat or eye muscle area.

Table 5.3 Pearson correlation table between different measures of feed efficiency and performance and carcase data for crossbred Wagyu steers.

Variable	RFI[1]	RFI[1] Rib Fat [2]	RFI[1] P8[3]	RFI[1] Camera Marbling[4]	RFI[1] Aus-meat[5]	RFI[1] Marbling MSA[6]	Residual Gain	Feed Conversion Ratio	Gain to Feed
Feedlot Induction Weight	-0.05	-0.05	-0.05	-0.05	-0.05	-0.05	-0.21*	-0.09	0.09
Average Daily Gain (Trial)	0.00	0.00	0.00	0.00	0.00	0.00	0.72***	-0.76***	0.76***
Dry Matter Intake (% BW)	0.87***	0.87***	0.86***	0.86***	0.86***	0.86***	0.01	0.26***	-0.27***
Camera Marbling	-0.13	-0.10	-0.10	0.00	0.01	-0.01	0.18*	-0.11	0.11
Aus-Meat Marble	-0.17†	-0.13	-0.13	-0.04	0.00	0.00	0.16†	-0.13	0.13
MSA Marbling	-0.15†	-0.11	-0.12	-0.05	0.02	0.00	0.16†	-0.13	0.14
Eye Muscle Area	0.08	0.11	0.08	0.11	0.12	0.12	0.05	-0.07	0.08
HSCW[7] (Kg)	0.12	0.10	0.09	0.10	0.10	0.10	0.04	-0.22**	0.24**
P8_Fat	0.16†	0.10	0.00	0.10	0.12	0.09	0.02	-0.07	0.05
Rib Fat	0.14	0.00	0.07	0.08	0.09	0.07	-0.13	0.09	-0.10
Metabolic Body Weight	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.29***	0.29***
Residual Gain	-0.43***	-0.42***	-0.42***	-0.42***	-0.42***	-0.42***		-0.91***	0.92***
Gain to Feed	-0.60***	-0.59***	-0.59***	-0.59***	-0.59***	-0.59***	0.92***	-0.98***	
Feed Conversion Ratio	0.58***	0.57***	0.57***	0.57***	0.57***	0.57***	-0.91***		-0.98***

***, **, *, † is for $P \leq 0.001$, $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.10$, respectively.

[1] *Residual Feed Intake*

[2] *Residual Feed Intake adjusted for camera marbling*

[3] *Residual Feed Intake adjusted for P8 fat*

[4] *Residual Feed Intake adjusted for Camera Marbling*

[5] *Residual Feed Intake adjusted for Aus-meat marbling*

[6] *Meat Standards Australia*

[7] *Hot Standard Carcase Weight*

5.4.2 The interaction of the 78 DOF metabolome with feed efficiency and marbling

Table 5.4 shows partial Pearson correlation coefficients between metabolites and the adjusted RFI variables at 78 DOF. All measurements of RFI had a high correlation between them ($r \geq 0.97$; data not shown). However, the correlation between the different measures of RFI and RG, FCR and GD were much lower ($r = -0.60$ to 0.58; data not shown). There were 36 metabolites identified using Chenomx. In general, the relationships between metabolites and measures of feed efficiency were few and low, ranging from -0.38 to 0.36 for methionine with FCR and GF, respectively ($P < 0.05$). Methionine, phenylalanine, histidine, and serine all had a negative relationship with the RFI traits ($P \leq 0.05$). Glucose showed a negative correlation with RFI RF, RFI Aus-meat, and RFI MSA (Meat Standards Australia) although it showed similar tendencies for the rest of the RFI measures this was also observed with creatine ($P \leq 0.10$). None of the RFI measures were positively correlated with the blood concentration of metabolites ($P \geq 0.05$) although proline tended to be correlated with RFI and RFI P8 and choline with RFI Aus-meat ($P \leq 0.10$; Table 5.4). The RG and GF were positively correlated with dimethyl sulfone, methionine, phenylalanine, glutamate, glutamine, carnosine, creatine, and acetone ($P \leq 0.05$; Table 5.4). In addition, GF also showed positive relationships ($P \leq 0.05$) with anserine, methyl-histidine, acetate, isobutyrate, glucose, allantoin and tyrosine ($P < 0.05$). The FCR produced similar results to RG and GF with the same metabolites but in the opposite direction with 13 negative relationships ($P \leq 0.05$).

Table 5.4: Heat map illustrating Pearson partial correlation coefficients of the relationship between the relative abundance of plasma metabolites at **78 days on feed** and different measurements of residual feed intake (RFI).

Variable	RFI[1]	RFI[1] Rib Fat[2]	RFI[1] P8[3]	RFI[1] Camera Marbling[4]	RFI[1] Aus-meat[5]	RFI[1] Marbling MSA[6]	Residual Gain	Feed Conversion Ratio	Gain to Feed
3 Hydroxybutyrate	-0.11	-0.10	-0.08	-0.08	-0.11	-0.10	0.08	-0.11	0.08
Acetate	-0.01	-0.03	0.00	0.02	0.00	0.01	0.16†	-0.21*	0.18*
Acetone	-0.10	-0.10	-0.10	-0.09	-0.11	-0.10	0.18*	-0.18*	0.18*
Allantoin	0.06	0.05	0.07	0.05	0.06	0.05	0.14	-0.19*	0.19*
Anserine	-0.14	-0.15†	-0.12	-0.15	-0.16†	-0.16†	0.16†	-0.16†	0.19*
Arginine	-0.06	-0.08	-0.05	-0.07	-0.09	-0.09	0.12	-0.10	0.14
Aspartate	-0.08	-0.08	-0.07	-0.11	-0.09	-0.11	0.04	-0.08	0.08
Carnosine	-0.13	-0.13	-0.11	-0.13	-0.12	-0.14	0.21*	-0.26**	0.27**
Choline	0.13	0.14	0.12	0.12	0.15†	0.13	-0.09	0.06	-0.08
Citrate	-0.02	-0.02	-0.01	0.00	0.01	0.00	-0.04	-0.03	0.02
Creatine	-0.16†	-0.16†	-0.12	-0.15†	-0.16†	-0.16†	0.18*	-0.21*	0.22**
Creatinine	-0.13	-0.12	-0.10	-0.13	-0.13	-0.13	0.03	-0.10	0.11
Dimethyl sulfone	-0.14	-0.14	-0.12	-0.15†	-0.13	-0.14	0.30***	-0.29***	0.29***
Formate	0.04	0.06	0.05	0.05	0.02	0.04	0.09	-0.06	0.06
Glucose	-0.16†	-0.18†	-0.15	-0.17†	-0.19*	-0.19*	0.15	-0.13	0.16†
Glutamate	-0.13	-0.15	-0.11	-0.13	-0.11	-0.13	0.22**	-0.27**	0.27**
Glutamine	-0.13	-0.14	-0.12	-0.14	-0.12	-0.14	0.19*	-0.21*	0.22*
Glycine	-0.14	-0.16	-0.13†	-0.15	-0.15†	-0.15	0.07	-0.11	0.13
Glycoprotein acetyls	0.01	0.03	0.02	0.03	0.04	0.04	-0.08	0.07	-0.09
Histidine	-0.19*	-0.18*	-0.15	-0.18*	-0.19*	-0.19*	0.05	-0.07	0.05
Isobutyrate	-0.15	-0.14	-0.11	-0.13	-0.15	-0.15	0.15†	-0.22**	0.20*
Isoleucine	0.06	0.05	0.04	0.05	0.05	0.03	-0.02	-0.03	0.06
Lactate	0.10	0.11	0.10	0.12	0.11	0.12	-0.10	0.13	-0.12
Leucine	0.00	-0.02	-0.03	-0.03	-0.04	-0.05	-0.01	-0.03	0.05
Lipid VLDL[8]	0.13	0.14	0.12	0.12	0.15	0.13	-0.14	0.11	-0.13
Lipid	0.11	0.11	0.09	0.09	0.12	0.11	-0.14	0.11	-0.13

Mannose	0.02	0.02	0.02	0.05	0.04	0.05	0.02	-0.02	0.00
Methionine	-0.23*	-0.23**	-0.21**	-0.21*	-0.21*	-0.21*	0.34***	-0.38***	0.36***
Methylhistidine	-0.15†	-0.17†	-0.15	-0.16†	-0.17†	-0.18*	0.16†	-0.16†	0.19*
Phenylalanine	-0.21*	-0.21*	-0.20*	-0.20*	-0.21*	-0.21*	0.25**	-0.29***	0.29***
Proline	0.15†	0.14	0.15†	0.10	0.10	0.09	-0.05	0.08	-0.04
Propionate	0.01	0.02	0.03	-0.01	-0.01	-0.01	0.02	0.01	0.01
Serine	-0.19*	-0.20*	-0.17*	-0.22†	-0.20*	-0.22**	0.07	-0.10	0.12
Tyrosine	-0.03	-0.05	-0.05	-0.04	-0.05	-0.06	0.12	-0.19*	0.18*
Unsaturated Lipid	-0.06	-0.05	-0.03	-0.06	-0.07	-0.06	-0.02	0.03	-0.02
Valine	-0.05	-0.06	-0.07	-0.06	-0.08	-0.08	0.07	-0.09	0.09

***, **, *, † *$P \leq 0.001$, $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.10$, respectively.*

[1] *Residual Feed Intake*
[2] *Residual Feed Intake adjusted for camera marbling*
[3] *Residual Feed Intake adjusted for P8 fat*
[4] *Residual Feed Intake adjusted for Camera Marbling*
[5] *Residual Feed Intake adjusted for Aus-meat marbling*
[6] *Meat Standards Australia*
[7] *Hot Standard Carcase Weight*
[8] *Very Low Density Lipid*

The PCA resulted in 5 PC selected which explained 68% of the variation were then used to draw correlation values with phenotypic variables of feed efficiency (Table 5.5). The PC1 was negatively correlated with RFI MARBLING MSA, RFI Aus-meat and FCR ($P < 0.05$), tended to be negatively correlated with RFI and RFI Camera Marbling ($P < 0.10$), and positively correlated ($P < 0.05$) with HSCW, induction and mid-trial metabolic BW, and ADG. The PC5 was negatively correlated with RFI, FCR, P8 fat, and rib fat ($P < 0.05$) and positively with marbling and RG ($P < 0.05$).

Table 5.5: Pearson correlation coefficients between five principal components obtained from plasma metabolites measured at 78 days on feed and different measures of residual feed intake (RFI), carcase and feedlot performance.

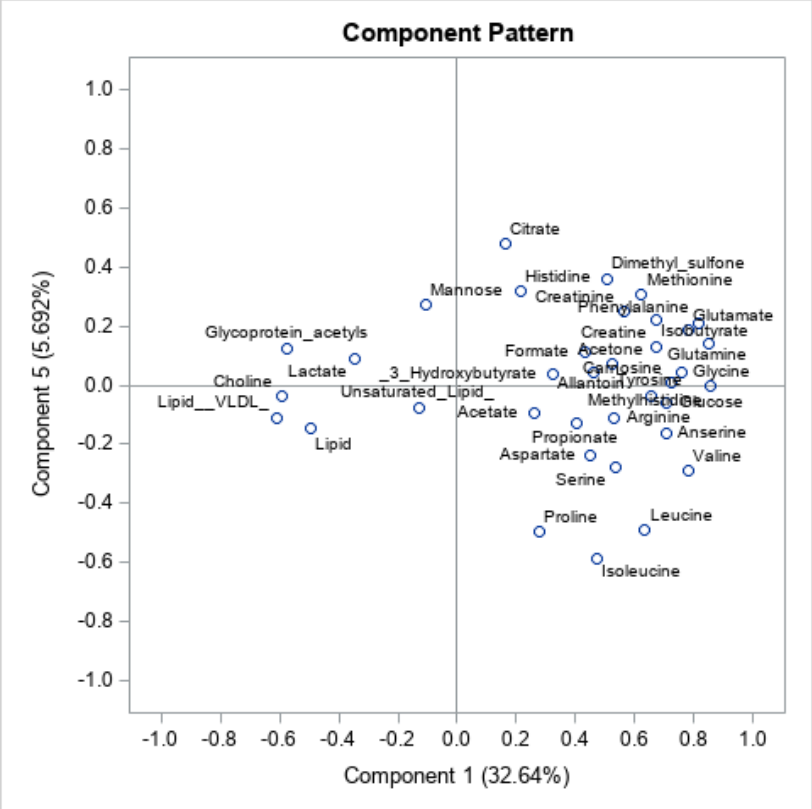
Variable	Prin1	Prin2	Prin3	Prin4	Prin5
Feedlot Induction Weight	0.20*	0.07	0.02	-0.07	-0.03
Average Daily Gain (Trial)	0.26**	0.09	0.04	-0.06	0.05
Dry Matter Intake (% BW)	-0.14	0.02	-0.03	-0.06	-0.09
Camera Marbling	0.01	0	0.05	0.02	0.23**
Aus-Meat Marble	-0.02	0.04	0.06	-0.01	0.30***
MSA Marbling	-0.01	0.03	0.08	-0.03	0.28***
Eye Muscle Area	0.07	0.05	0.07	-0.06	0
HSCW[1] (Kg)	0.17*	0.12	0.09	-0.14	-0.14†
P8 Fat	-0.05	0.05	-0.02	-0.22**	-0.22**
Rib Fat	0.03	0	0.08	-0.03	-0.17*
Metabolic Body Weight	0.22**	0.1	0.07	-0.12	-0.08
RFI[2]	-0.16†	0.02	-0.01	-0.1	-0.18*
RFI[2] Camera Marbling[3]	-0.17†	0.02	-0.02	-0.09	-0.13
RFI[2] Aus-meat[4]	-0.18*	0.04	-0.01	-0.1	-0.13
RFI[2] Marbling MSA[5]	-0.18*	0.02	-0.02	-0.09	-0.12
RFI[2] Rib Fat	-0.17†	0.03	-0.02	-0.09	-0.16†
RFI[2] P8	-0.15	0.02	-0.01	-0.06	-0.14
Residual Gain	0.21*	0.02	0	0.05	0.19*
Gain to Feed	0.26**	0.05	0.04	0.03	0.17†
Feed Conversion Ratio	-0.24**	-0.08	-0.02	-0.05	-0.20*

***, **, *, † *$P \leq 0.001$, $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.10$, respectively.*

[1] *Hot Standard Carcase Weight*
[2] *Residual Feed Intake*
[3] *Residual Feed Intake adjusted for camera marbling*
[4] *Residual Feed Intake adjusted for Aus-meat marbling*
[5] *Meat Standards Australia*

Figure 5.2 shows the loading plot for the PC 1 and PC5, which were selected because these showed the strongest correlations with RFI and carcase traits. The PC1 showed negative loading from those metabolites of the lipid metabolism and positive from amino acids. In contrast, positive loadings on PC5 were influenced by citrate, dimethyl sulfone, methionine, and histidine. Proline, leucine and isoleucine showed a negative loading in PC5.

Figure 5.2: Loading plot for principal components 1 and 5 from 36 identified metabolites of Wagyu crossbred steers sampled at 78 days on feed at the feedlot.



5.4.3 The interaction of the 313 DOF plasma metabolome with feed efficiency and marbling

Table 5.6 show the Pearson partial correlation coefficients between the metabolites and adjusted RFI variables at 313 DOF. There were 3 metabolites that had a negative relationship (acetone, dimethyl sulfone, and methionine), and 3 metabolites had a positive relationship (lipids, glycoprotein acetyls, and choline) with RFI adjusted values ($P < 0.05$). Citrate and phenylalanine tended to be negatively correlated with all FRI measures but glucose and methyl-histidine only with RFI ($P \leq 0.10$; Table 5.6). The GF and FCR were positively correlated with methionine, dimethyl sulfone, serine, and aspartate ($P < 0.05$), and glutamate ($p < 0.10$). The RG also resulted in similar trends to GF ($P < 0.10$).

T**able 5.6:** Heat map illustrating partial correlation coefficients of the relationship between adjusted RFI variables and the relative concentration of plasma metabolites at **313 days on feed (DOF)** in Wagyu crossbred steers.

Variable	RFI[1]	RFI[1] Rib Fat[2]	RFI[1] P8[3]	RFI[1] Camera Marbling[4]	RFI[1] Aus-meat[5]	RFI[1] Marbling MSA[6]	Residual Gain	Feed Conversion Ratio	Gain to Feed
3-Hydroxybutyrate	-0.03	-0.02	0.03	-0.01	-0.02	-0.01	0.13	-0.09	0.13
Acetate	0.04	0.04	0.07	0.05	0.05	0.05	0.11	-0.06	0.09
Acetone	-0.24**	-0.25**	-0.26**	-0.22**	-0.24**	-0.22**	0.01	-0.09	0.08
Allantoin	0.03	0.02	0.05	0.05	0.03	0.05	0.06	-0.09	0.11
Anserine	-0.14	-0.12	-0.09	-0.11	-0.13	-0.11	-0.01	-0.02	0.06
Arginine	-0.09	-0.07	-0.05	-0.07	-0.08	-0.07	-0.05	0.06	-0.02
Aspartate	-0.03	-0.04	-0.03	-0.02	-0.03	-0.03	0.17†	-0.18*	0.18*
Carnosine	-0.04	-0.05	-0.03	-0.04	-0.06	-0.05	0.10	-0.10	0.09
Choline	0.31***	0.29***	0.27**	0.28***	0.29***	0.27**	-0.08	0.17†	-0.18*
Citrate	-0.17†	-0.15†	-0.11	-0.15†	-0.15†	-0.14	-0.06	-0.01	0.01
Creatine	-0.12	-0.12	-0.07	-0.10	-0.11	-0.10	0.02	-0.08	0.09
Creatinine	-0.12	-0.10	-0.03	-0.10	-0.12	-0.10	0.04	-0.09	0.09
Dimethyl sulfone	-0.21*	-0.21*	-0.16†	-0.20*	-0.20*	-0.20*	0.14†	-0.21*	0.21*
Formate	-0.06	-0.05	-0.04	-0.04	-0.05	-0.04	-0.03	0.02	-0.04
Glucose	-0.15†	-0.13	-0.10	-0.12	-0.14	-0.12	-0.04	0.01	0.03
Glutamate	-0.07	-0.07	-0.02	-0.06	-0.06	-0.05	0.18*	-0.16†	0.16†
Glutamine	-0.06	-0.05	-0.02	-0.04	-0.05	-0.04	0.13	-0.13	0.13
Glycine	-0.09	-0.09	-0.05	-0.07	-0.09	-0.07	0.11	-0.14	0.13
Glycoprotein acetyls	0.25**	0.23**	0.20*	0.22**	0.23**	0.21*	-0.06	0.10	-0.13
Histidine	-0.06	-0.04	-0.01	-0.04	-0.05	-0.05	-0.14	0.10	-0.09
Isobutyrate	-0.06	-0.04	0.01	-0.03	-0.05	-0.04	0.12	-0.08	0.11
Isoleucine	0.07	0.06	0.07	0.08	0.07	0.08	-0.05	-0.01	-0.01
Lactate	-0.12	-0.11	-0.12	-0.12	-0.11	-0.11	0.04	-0.03	0.05
Leucine	0.04	0.03	0.02	0.04	0.03	0.04	-0.09	0.01	-0.03
Lipid VLDL[8]	0.16†	0.13	0.09	0.14	0.14	0.13	-0.12	0.12	-0.16†
Lipid	0.17*	0.14	0.10	0.15†	0.15†	0.14	-0.11	0.11	-0.14
Mannose	-0.07	-0.09	-0.10	-0.05	-0.06	-0.05	-0.17*	0.11	-0.11
Methionine	-0.20*	-0.19*	-0.19*	-0.18*	-0.18*	-0.17†	0.28***	-0.33***	0.30***

Methylhistidine	-0.15†	-0.13	-0.09	-0.12	-0.14	-0.12	0.00	-0.02	0.06
Phenylalanine	-0.16†	-0.15†	-0.13	-0.15†	-0.15†	-0.15†	0.06	-0.13	0.13
Proline	0.10	0.11	0.11	0.12	0.10	0.12	-0.12	0.05	-0.08
Propionate	-0.04	-0.02	0.03	-0.01	-0.03	-0.02	-0.10	0.05	-0.04
Serine	0.02	0.01	0.04	0.02	0.02	0.01	0.23**	-0.20*	0.21**
Tyrosine	-0.07	-0.09	-0.07	-0.07	-0.08	-0.08	-0.03	-0.05	0.03
Unsaturated Lipid	0.11	0.11	0.11	0.10	0.10	0.09	-0.15†	0.19*	-0.17**
Valine	-0.06	-0.06	-0.04	-0.04	-0.05	-0.04	0.03	-0.11	0.10

***, **, *, † *$P \leq 0.001$, $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.10$, respectively.*

[1] *Residual Feed Intake*
[2] *Residual Feed Intake adjusted for camera marbling*
[3] *Residual Feed Intake adjusted for P8 fat*
[4] *Residual Feed Intake adjusted for Camera Marbling*
[5] *Residual Feed Intake adjusted for Aus-meat marbling*
[6] *Meat Standards Australia*
[7] *Hot Standard Carcase Weight*
[8] *Very Low Density Lipid*

5.5 Discussion

The present study examined the relationship between RFI (feed efficiency) and the relative abundance of plasma metabolites, while adjusting RFI calculations for meat quality traits associated with carcase fat such as rib fat, P8 fat, and marbling. The production of beef for important premium markets requires balancing the apparent antagonistic relationship between feed efficiency, which reduces the cost of production and the amount of intramuscular fat (marbling) which is associated with eating quality [1-3]. This balance applies particularly to Wagyu beef cattle that require an extended period in a feedlot to achieve high marbling. In the present study, all measures of RFI adjusted for carcase fat were highly correlated and showed similar correlations with plasma metabolites. This suggested that BW and ADG are the main drivers of RFI and that adjusting for carcase fat may have little effect on the interpretation of metabolic data and the identification of biomarkers. The correlations between multiple measures of RFI and plasma metabolites were generally low (< 0.24). No strong biomarkers of RFI were found in the present study but instead there were a small number of metabolites with weak relationships with RFI.

Residual gain (RG), feed conversion ratio (FCR), and the gain to feed ratio (GF), showed positive correlations with several metabolites (methionine, phenylalanine, dimethyl sulfone, carnosine, creatine, glutamate, glutamine, creatine). This was interpreted to suggest that different measures of feed efficiency share some metabolic pathways, although there were some differences in the strength of the correlation amongst RG, FCR and GF. Some metabolites (allantoin, tyrosine, acetate, glutamate, carnosine) were positively correlated with GF and FCR but not with RFI. However, the PCA indicated that efficient animals with higher RG and GF were associated with higher PC1 values and thus greater concentration of these amino acids and glucose. These results suggested that traits such as RG, GF and FCR could be more easily predicted from blood biomarkers compared to RFI because of the stronger and larger number of correlations with metabolites. Methionine and phenylalanine seem to be good biomarkers for most measures of feed efficiency and others with weaker correlations with feed efficiency traits could be of value (creatine, anserine, methylhistidine, glucose). In a study with Jersey and Holstein dairy cows, four metabolites (leucine, ornithine, pentadecanoic acid, and valine) were marginally associated with RFI [21].

In Angus crossbred beef steers, different sets of metabolites were associated with RFI at 2 weeks (creatine, glycine), 6 weeks (hippurate, glutamate, betaine, citrate, lysine, phenylalanine,

creatine, acetate, carnitine, and threonine) and 10 weeks (hydroxyisobutyrate, tyrosine, and formate) in a feedlot [4]. Another study in Angus crossbred steers identified several metabolites (glycine, betaine, tyrosine, valine, and leucine) as potential biomarkers of RFI [5]. Phenylalanine, creatine, and glycine were also associated with RFI in the present study. Glycine was the only metabolite in the present study to show a weak tendency for a negative correlation with RFI Rib Fat and RFI Aus-meat. The differences between studies for metabolites associated with RFI could potentially be explained by breed differences, diet, and the time in a feedlot. This highlights the need for more research to identify biomarkers that are predictors of feed efficiency. It is an important field of scientific inquiry and additionally has major industry application to replace costly and time-consuming individual animal performance measurements.

In the present study, methionine was the only metabolite associated with RFI at both day 78 and day 313 in the feedlot, although glucose and phenylalanine showed similar trends. Glucose is an important molecule in the animal's metabolism and is related to insulin secretion which is involved in marbling deposition in Wagyu cattle. Insulin is proposed to increase lipogenesis and decrease lipolysis [6]. The results shown in the present study indicate that glucose resistance and sensitivity are also related to the feed intake measurements when adjusted for carcass traits. Glucose is an important molecule in the synthesis of fat in relation to intra-muscular fat deposition[7]. Methionine is used in many metabolic processes including protein synthesis, and it is often the first limiting amino acid in growing cattle [8,9]. Methionine and serine were also correlated with RG, FCR and GF. Higher concentrations of these amino acids in plasma may indicate greater synthesis of microbial protein in the rumen [10]. Serine is a non-essential amino acid that is an intermediary in glycolysis and methionine is involved in the synthesis of creatine by the liver [11]. Cantalapiedra-Hijar, et al. [5] reported that methionine supplementation improved ADG but not feed efficiency.

The strongest correlations with RFI at day 313 in the feedlot were for metabolites associated with lipid metabolism (choline, dimethyl sulfone, glycoprotein acetyls, lipids, lipid VLDL, and acetone). These metabolites were not associated with RFI at day 78. This finding suggested that metabolic processes linked to RFI change over time and may depend on the stage of body maturation and the rate of accretion of different tissues. For example, fat would be expected to have a faster accretion at day 313 compared with day 78 in the feedlot. Steers that had higher RFI early in the feedlot had higher relative abundance of lipids later when fat deposition is

expected to be faster. The relationship between marbling and RFI was weak ($r \leq -0.17$; $P < 0.10$) and P8 fat tended to be positively correlated with RFI ($r = 0.16$; $P = 0.09$). These observations were in agreement with previous studies in which animals with higher RFI had higher marbling at slaughter[12]. In a previous study we found that the negative correlation between lipids and marbling became stronger with increasing time in the feedlot and older animals [13,14] . It can be hypothesized that the greater rate of marbling accretion with maturity results in a faster uptake of circulating lipids. The present study builds on our previous studies and demonstrate that inefficient animals with high RFI adjusted for marbling have higher concentrations of lipids in blood as steers mature with time in a feedlot. It is proposed that lipid metabolism may be involved in feed efficiency though mechanisms in addition to fat deposition.

Consolo, et al. [4] have recently undertaken a study that examined muscle and liver signatures associated with RFI intake in Nellore cattle. The study aimed at examining tissue samples from the liver and *longissimus lumborum* after slaughter and identifying the metabolites within the samples using an NMR instrument. The study indicated there are underlying mechanisms in regards to the RFI trait that need to be investigated and understood further, however the study showed there might be novel predictors available to predict animals that are more efficient. Foroutan, et al. [15] have produced a study that claims to have identified serum metabolites that could be potentially used as biomarkers to predict the RFI of young Angus bulls. The study examined three techniques, including NMR, liquid chromatography-tandem mass spectrometry, and inductively coupled plasma mass spectrometry. The most interesting aspect was that within their study they identified that Formate and Leucine were two candidate biomarkers. This is not consistent with the results that were seen within the present study, however there were multiple factors that were different between the studies such as the breed of cattle, the country, feed ration and many other factors.

5.6 Conclusion

Residual Feed Intake (RFI) is an important trait that can increase the productivity within the beef production system significantly; however, it is important this does not happen at the cost of carcase attributes. However, adjusting RFI for carcase fat does seem to have relevant influence on potential metabolic biomarkers in blood or on the interpretation of metabolomic

pathways. Alternative measures of feed efficiency such as residual gain and gain to feed have a larger influence on the number and strength of the correlations with metabolites and therefore, these traits should consider alternative biomarkers and metabolic pathways compared to RFI. Furthermore, the stage or degree of physiological maturity when biomarkers are measured should also be considered because this has a large influence on the potential biomarkers for RFI. Biomarkers of RFI shifted from protein metabolism (amino acids) early in the feedlot to lipid metabolism (choline and lipids) late in the feedlot when animals are closer to slaughter and thus at a faster fat accretion rate. Methionine was the most consistent metabolite associated with RFI adjusted for carcase fat independently of sampling time and thus this could be a key metabolite participating in both muscle and fat accretion. Carcase fat should be considered to adjust RFI when selecting Wagyu cattle because the two traits are generally antagonistic although the effect on blood biomarkers are negligible.

5.7 References

1 Duff, C. J., van der Werf, J. H. J., Parnell, P. F. & Clark, S. A. Redefining residual feed intake to account for marbling fat in beef breeding programs. *Animal Production Science*, -, doi:https://doi.org/10.1071/AN21107 (2021).

2 Herd, R. & Pitchford, W. Residual feed intake selection makes cattle leaner and more efficient. *Recent Advances in Animal Nutrition* **18**, 45-58 (2011).

3 Kelly, D. N. *et al.* Feed efficiency and carcass metrics in growing cattle. *Journal of Animal Science* **97**, 4405-4417 (2019).

4 Consolo, N. *et al.* Muscle and liver metabolomic signatures associated with residual feed intake in Nellore cattle. *Animal Feed Science and Technology* **271**, 114757 (2021).

5 Cantalapiedra-Hijar, G. *et al.* Natural 15N abundance in specific amino acids indicates associations between transamination rates and residual feed intake in beef cattle. *Journal of Animal Science* **98**, doi:10.1093/jas/skaa171 (2020).

6 Rhoades, R. D. *et al.* Effect of dietary energy source on in vitro substrate utilization and insulin sensitivity of muscle and adipose tissues of Angus and Wagyu steers. *Journal of Animal Science* **85**, 1719, doi:10.2527/jas.2006-498 (2007).

7 Nafikov, R. A. & Beitz, D. C. Carbohydrate and Lipid Metabolism in Farm Animals. *The Journal of Nutrition* **137**, 702-705 (2007).

8 Richardson, C. R. & Hatfield, E. E. The limiting amino acids in growing cattle. *J Anim Sci* **46**, 740-745, doi:10.2527/jas1978.463740x (1978).

9 Titgemeyer, E. C. & Merchen, N. R. The effect of abomasal methionine supplementation on nitrogen retention of growing steers postruminally infused with casein or nonsulfur-containing amino acids. *Journal of animal science* **68 3**, 750-757 (1990).

10 Wu, G. *et al.* Amino Acid Nutrition in Animals: Protein Synthesis and Beyond. *Annual Review of Animal Biosciences* **2**, 387-417, doi:10.1146/annurev-animal-022513-114113 (2014).

11 Yanibada, B. *et al.* Inhibition of enteric methanogenesis in dairy cows induces changes in plasma metabolome highlighting metabolic shifts and potential markers of emission. *Scientific Reports* **10**, 15591, doi:10.1038/s41598-020-72145-w (2020).

12 Basarab, J. A. *et al.* Residual feed intake and body composition in young growing cattle. *Canadian journal of animal science* **83**, 189-204, doi:10.4141/A02-065 (2003).

13 Connolly, S., Dona, A., Hamblin, D., D'Occhio, M. J. & González, L. A. Changes in the blood metabolome of Wagyu crossbred steers with time in the feedlot and relationships with marbling. *Scientific Reports* **10**, 18987, doi:10.1038/s41598-020-76101-6 (2020).
14 Connolly, S. *et al.* Relationship of the blood metabolome to subsequent carcass traits at slaughter in feedlot Wagyu crossbred steers. *Scientific Reports* **9**, 15139, doi:10.1038/s41598-019-51655-2 (2019).
15 Foroutan, A., Fitzsimmons, C., Mandal, R., Berjanskii, M. V. & Wishart, D. S. Serum Metabolite Biomarkers for Predicting Residual Feed Intake (RFI) of Young Angus Bulls. *Metabolites* **10**, 491 (2020).

Chapter 6: General Discussion

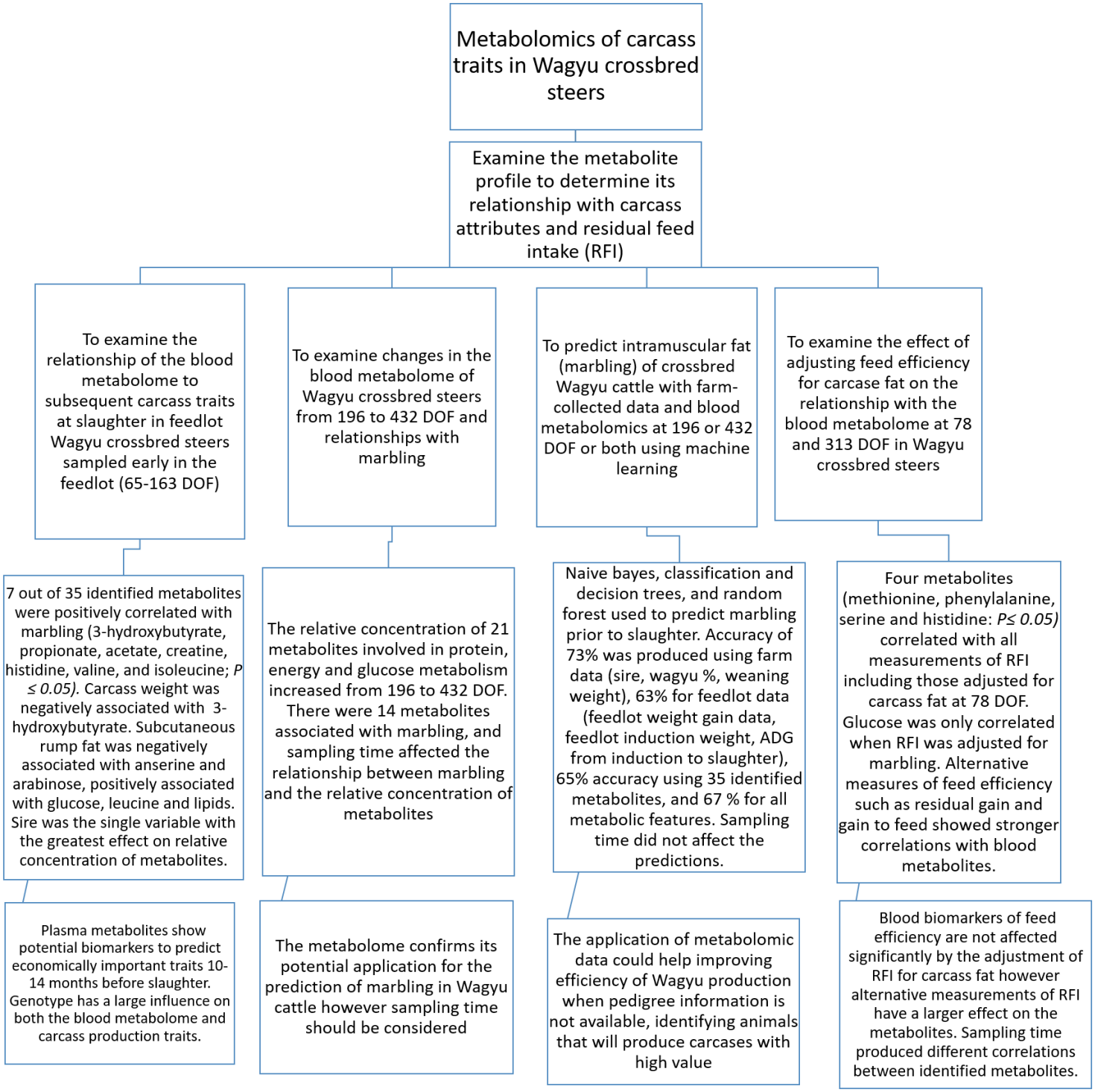
This chapter is an integration and summary of all main findings and conclusions from this thesis. Included within the discussion were the limitations and potential future research and applications arising from the findings shown in this thesis. The thesis attempted to investigate a systems biology approach to understanding the mechanisms involved in intramuscular fat or marbling deposition in Wagyu cattle by examining the metabolite profile, and a machine learning approach was then investigated to use the metabolomic information to predict how the animals would perform. The final approach was to examine the relationship between residual feed intake while adjusting the variable for carcase fat to ensure the animals that are selected are efficient as well as producing superior carcases.

6.1 Introduction

This thesis aimed at investigating the relationships between plasma metabolite profiles and carcase attributes, mainly marbling, residual feed intake (RFI) in Wagyu crossbred cattle. The ability to determine which animals are going to perform well early in the feedlotting process for both carcase traits and feed efficiency would increase the efficiency of Wagyu production significantly. This is due to the long time the animals spend in the feedlot (between 350-600 days). Chapter 2 examined the relationship between the plasma metabolome and carcase traits such as marbling, subcutaneous fat, carcase weight, growth rate and eye muscle area. Chapter 3 looked at changes in the plasma metabolome from early (196 DOF) compared to late (432 DOF) in the feedlotting process. The fourth chapter examined the potential of predicting marbling using multiple models and datasets with machine learning, such as including animal farm data, metabolomic data, feedlot data and then a combination of all data recorded to predict the marbling group of the animals. The fifth chapter examined the effect of adjusting the RFI trait for carcase fat on the relationship with plasma metabolites measured at two time points, early (78 DOF) and late (313 DOF) in the feedlot. A summary of the aims, objectives and findings for each paper is included in Figure 6.1.

6.2 Summary

Figure 6. 1. The main objectives and findings of experimental chapters 2 to 5.


Metabolomics of carcass traits in Wagyu crossbred steers
Examine the metabolite profile to determine its relationship with carcass attributes and residual feed intake (RFI)
To examine the relationship of the blood metabolome to subsequent carcass traits at slaughter in feedlot Wagyu crossbred steers sampled early in the feedlot (65-163 DOF)
To examine changes in the blood metabolome of Wagyu crossbred steers from 196 to 432 DOF and relationships with marbling
To predict intramuscular fat (marbling) of crossbred Wagyu cattle with farm-collected data and blood metabolomics at 196 or 432 DOF or both using machine learning
To examine the effect of adjusting feed efficiency for carcase fat on the relationship with the blood metabolome at 78 and 313 DOF in Wagyu crossbred steers
7 out of 35 identified metabolites were positively correlated with marbling (3-hydroxybutyrate, propionate, acetate, creatine, histidine, valine, and isoleucine; P ≤ 0.05). Carcass weight was negatively associated with 3-hydroxybutyrate. Subcutaneous rump fat was negatively associated with anserine and arabinose, positively associated with glucose, leucine and lipids. Sire was the single variable with the greatest effect on relative concentration of metabolites.
The relative concentration of 21 metabolites involved in protein, energy and glucose metabolism increased from 196 to 432 DOF. There were 14 metabolites associated with marbling, and sampling time affected the relationship between marbling and the relative concentration of metabolites
Naive bayes, classification and decision trees, and random forest used to predict marbling prior to slaughter. Accuracy of 73% was produced using farm data (sire, wagyu %, weaning weight), 63% for feedlot data (feedlot weight gain data, feedlot induction weight, ADG from induction to slaughter), 65% accuracy using 35 identified metabolites, and 67 % for all metabolic features. Sampling time did not affect the predictions.
Four metabolites (methionine, phenylalanine, serine and histidine: P≤ 0.05) correlated with all measurements of RFI including those adjusted for carcass fat at 78 DOF. Glucose was only correlated when RFI was adjusted for marbling. Alternative measures of feed efficiency such as residual gain and gain to feed showed stronger correlations with blood metabolites.
Plasma metabolites show potential biomarkers to predict economically important traits 10-14 months before slaughter. Genotype has a large influence on both the blood metabolome and carcass production traits.
The metabolome confirms its potential application for the prediction of marbling in Wagyu cattle however sampling time should be considered
The application of metabolomic data could help improving efficiency of Wagyu production when pedigree information is not available, identifying animals that will produce carcases with high value
Blood biomarkers of feed efficiency are not affected significantly by the adjustment of RFI for carcass fat however alternative measurements of RFI have a larger effect on the metabolites. Sampling time produced different correlations between identified metabolites.

6.3 Main Findings

The present thesis has highlighted several factors that should be considered to further advance the field of metabolomics to both understand the biology of fat deposition and muscle growth and continue developing the technique for the prediction of carcase traits and feed efficiency that could see applications for animal management and genetic improvement. This section discusses some of the most important factors to consider including the effect of sampling time, effects of animal genetics, and the integration of biological mechanisms involved in fat and muscle deposition.

6.3.1 Effect of sampling time

One of the key aims of this thesis was to evaluate the effect of time in the feedlot to sample Wagyu crossbred steers on the accuracy of the prediction of carcase traits such as marbling, and on the relationship between metabolites, marbling and RFI adjusted for carcase fat. There was a research gap in both topics and to the author's best knowledge there have not been any studies undertaken in Wagyu cattle to investigate the relationships between metabolomics and carcase traits or RFI as shown in this thesis. It was important to investigate the effect of sampling time in the feedlot because the earlier the animals are identified as likely to produce a superior carcase the more economic gains that could be achieved. Interestingly, glucose had a positive and lipids a negative relationship with marbling at the latest sampling time of 163 DOF but not earlier but the PCA demonstrated no significant separation of the metabolome of the 3 DOF groups. Wagyu feedlot production in Australia normally starts with the feeder animal inducted in the feedlot with approximately 350 kg of BW after these have been backgrounded to develop the frame and muscle but before they mature and deposit fat. Therefore, Wagyu feedlot cattle first develop the frame and then reach physiological maturity when fat deposition increases late in the feedlot. The metabolism of these animals is therefore changing as the animals reach maturity, with bone and muscle growth being predominant in the early period at the feedlot and fat deposition gaining importance later in the feedlot period [1]. Therefore, the animal is expected to change metabolism throughout the feedlot process, which may be reflected in the metabolome. The analysis undertaken in chapter 2 investigated the relationships between the relative abundance of metabolites early in the feedlot period (65 to 163 DOF) and frame size measured through HSCW, muscling measured through EMA, and carcase fatness measured through marbling and subcutaneous fat. The conclusion from this study was that the time of sampling was not a critical factor concerning the plasma metabolome

but the three groups of animals were relatively early in the feedlotting process. However, glucose and lipids had a strong positive and negative correlation with marbling at 163 DOF but not earlier. These results may support the hypothesis that the metabolome is changing with time in the feedlot. It is hypothesized that these findings are due to animals depositing more fat the later they are sampled with glucose being used for lipogenesis and lipids being uptake for fat deposition in tissues. In contrast to Chapter 2, Chapter 3 examined the effect of sampling a cohort of animals in the middle (196 DoF) and late (432 DoF) of the feedlotting process. There were significant changes in the plasma metabolite profile between these sampling times as demonstrated in the PCA with a decrease in lipids and an increase in metabolites of carbohydrate energy metabolism such as glucose, propionate, creatine, creatinine, lactate and β-hydroxybutyrate. In addition, marbling was correlated with more metabolites in Chapter 3 compared to Chapter 2 (21 vs 7 metabolites) and there were many more metabolites that showed a significant interaction between sampling time and marbling (9 metabolites with $P \leq 0.05$ and 8 metabolites with $P \leq 0.10$) including glucose and lipids which also became stronger later in the feeding period. These results demonstrate changes in the metabolism as the animal matures and deposit more fat late in the feeding period. However, it is also worth mentioning that predicting marbling of animals later in the feedlotting process would be less beneficial concerning improving the economics of Wagyu production as the animals have already consumed a large amount of feed. The ideal time to sample animals to predict the carcase traits would be early in the feedlotting process although results of the present thesis suggest this may be less accurate. The changes in the plasma metabolome were expected due to the changes in the animal's metabolism requiring different metabolites that are precursors of the tissue experiencing faster growth at a given point in time such as depositing fat within the muscle towards the end of the lot feeding process.

The relationship between glucose and lipids and marbling was shown to have a significant interaction in chapters 2, 3 and 5. Chapter 2 showed there was a significant relationship ($P < 0.05$) between marbling and DOF, chapter 3 indicated the molecules glucose and VLDL lipid had a correlation coefficient of 0.54 and 0.48 respectively. In chapter 5, glucose showed a tendency or a significant relationship with all of the RFI traits adjusted for carcass traits such as marbling and HSCW. The re-occurring significance of glucose and marbling relationships could be related to the insulin resistance or altered insulin sensitivity. Insulin resistance refers to the decreased amino acid use for protein synthesis and increased fat deopsition[2]. . Gotoh, et

al. [3] indicated there is little information detailing the relationship between insulin secretion and marbling in Wagyu cattle and the amount of insulin secreted is increased by plasma propionate, butyrate, or glucose in steers. The plasma insulin concentration and carcass fat proportion was greater in Wagyu (600 kg slaughter weight) than in Holstein cattle (700 kg slaughter weight)[4], this result indicates that Wagyu steers have greater plasma insulin levels during the fattening period than Holstein steers. Insulin is a molecule that is associated with the glucose metabolism, regulating many aspects of growth and development by signalling nutritional conditions to growing tissues. Wagyu cattle have a greater propensity to deposit intramuscular fat which highlights that there needs to be further research to examine the relationship between the endocrine system and carcass attributes such as marbling. Shingu, et al. [5] have also shown greater concentration of insulin in Japanese black heifers compared to Holstein heifers at 18 months of age. Each of the above studies indicate that there is a relationship between the plasma insulin secretion and intramuscular fat production in Wagyu cattle however there needs to be further study and investigation to be able to fully understand the mechanisms and relationships between them.

Chapter 5 also demonstrated a significant change in the metabolome of cattle sampled at 78 and 313 DOF (data not shown). More importantly, the relationship between the relative abundance of metabolites and the multiple measures of feed efficiency also changed between sampling times. The results demonstrated that RFI was correlated with compounds involved muscle protein, energy and glucose metabolism at 78 but not at 313 DOF (e.g., glucose, histidine, phenylalanine and serine), whereas those involved in lipid metabolism such as choline and glycoprotein acetyls were correlated with RFI at 313 DOF but not at 78 DOF. Interestingly, 9 amino acids were significantly correlated with residual gain and gain to feed at 78 DOF but only 3 were correlated with these at 313 DOF. The results indicate that the metabolic processes underlying RFI change over time as animals mature, with protein and energy metabolism being more prevalent early and lipid metabolism being more prevalent late in the feedlot. It is important to point out that this is assuming that RFI does not change over time and animals are still ranking the same for feed efficiency measured until 183 DOF as it would be at 313 DOF. However, it is important to note that RFI was not measured at 313 DOF but only till 183 DOF and therefore, results should be interpreted with caution. Some metabolites such as methionine were correlated with all measures of feed efficiency and both early and late in the feedlot. More research is needed to fully understand the role of methionine

on feed efficiency of long-fed feedlot Wagyu cattle including the effects on muscle and fat metabolism. Sampling animals earlier in the feedlotting process to determine which animals are going to be more efficient that others is the ideal time.

In addition to sampling time regarding the feedlotting period, sampling time within the day could also influence the metabolome of cattle although no research seems to exist demonstrating changes in the metabolome within a day. The animals in Chapter 5 were sampled later in the day (afternoon) compared to animals in the other chapters which were sampled early in the day before feed delivery due to other cohorts of animals being processed before them on the day of sampling. This factor seemed to influence the relationship with carcase attributes. Only proline had a significant relationship ($P \leq 0.05$) with camera marbling at sample point 1 (early) and citrate and glutamate tended to be correlated with marbling ($P \leq 0.10$). At sampling point 2, there were four metabolites that tended to be correlated with marbling (isobutyrate, methionine, glutamine, and citrate; $P \leq 0.10$). These findings may suggest that sampling of animals later in the afternoon is not ideal to predict carcase traits. However, more research is needed to confirm this observation where animals are sampled both in the morning before feeding and in the afternoon after feeding. It is proposed the ideal time to sample animals would be in the morning prior feed delivery even if the animals have ad libitum access to feed. The two cohorts of animals examined in Chapters 2 and 3 were also different groups of animals however, they were sampled using the same method, moving the animals to the central holding facilities in the morning and sampling them before the feed truck had arrived.

6.3.2 Effects of genetics

Sire had a strong effect on important carcase traits (such as marbling and carcase weight) as described in Chapters 2 and 3 demonstrating the genetic influence on the performance of the animals. Sire had a significant effect ($P \leq 0.05$) on the carcase traits of Aus-meat marbling, camera marbling, rump fat, growth rate, age at induction (feedlot), age at slaughter, induction weight, feedlot exit weight and Wagyu content. Furthermore, sire influenced the relative concentration ($P \leq 0.05$) of 13 out of the 35 identified metabolites and there were 6 metabolites that tended ($P \leq 0.10$) to be affected by sire. The results of Chapter 2 showed that genotype had a large impact on both the metabolome and carcase traits and meat quality. Although not published in Chapter 3, the results also showed the effect of sire on these carcase traits. Sire had a significant ($P \leq 0.05$) for the metabolites glycoprotein acetyls, unsaturated lipids,

arginine, choline, methylhistidine, lipid VLDL, isobutyrate, lipids, tyrosine, allantoin, propionate and 3-hydroxybutyrate in chapter 3 although these results were not published as it was not the objective of the study. Therefore, sire has a significant influence on profit due to the long feeding regimes of Wagyu cattle to ensure high marbling is achieved. This strongly supports the hypothesis that genetic mechanisms control the animals' metabolism, which is linked to intramuscular fat deposition. Park, et al. [6] have conducted a review and shown that marbling is influenced by multiple factors such as management, environmental, genetic and nutritional influences can all impact how well the animal produces marbling.

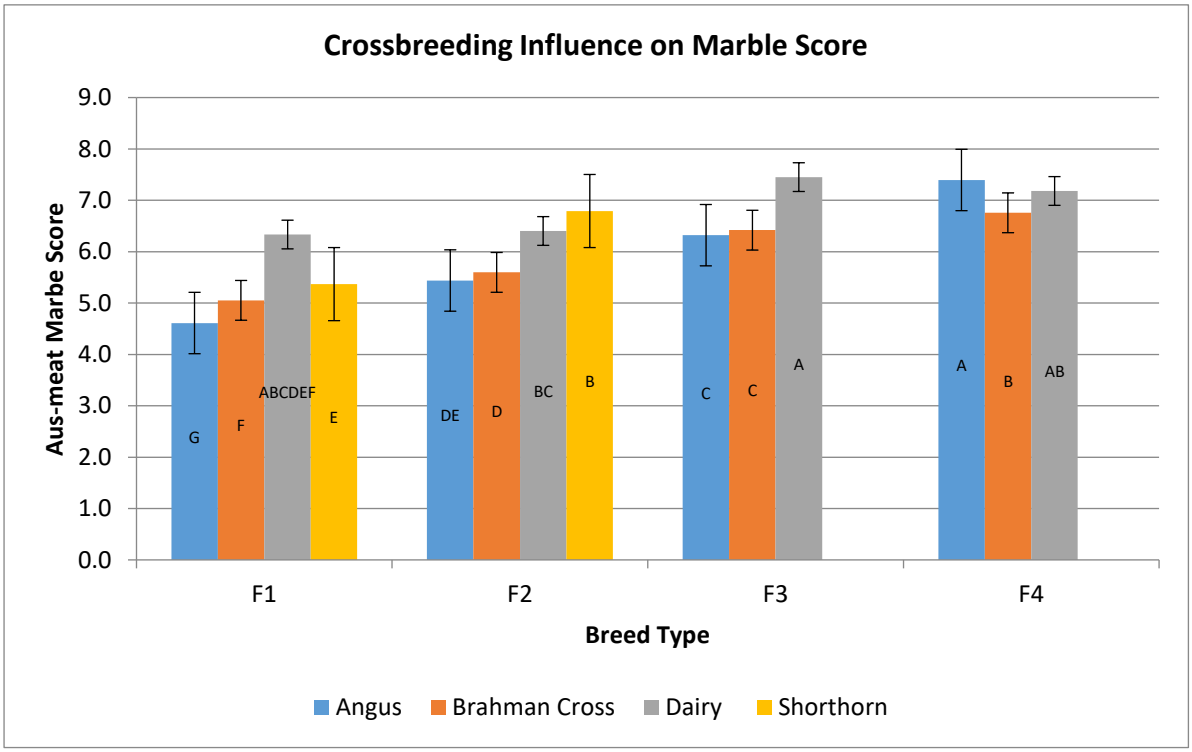
Another analysis that was undertaken as part of this thesis included a genome wide association study using 150K SNP data that was imputed, 895 animal genotypes were used. There was not enough time to complete the analysis within the present thesis however the table below shows the results that were produced for Aus-meat marbling, camera marbling, HSCW and the metabolite 3-hydroxybutyrate. This metabolite was chosen because it was correlated with marbling in both Chapters 2 and 3. The source of genetic variance (V (G)), environmental variance (V (E)), phenotypic variance (V (p)) and the overall heritability for each trait (V (G) / V (E)) are shown in Table 6.1. The heritability calculated for the animals in the present study are consistent with previous literature. Oyama [7] reviewed that the heritability ranged from 0.23-0.78 for carcase weight, 0.16-0.74 for marbling (on a 12-point scale). The heritability of the present thesis was 0.38 and 0.54 for Aus-meat and camera marbling, respectively (Table 6.1). The interesting aspect of this analysis was the heritability of the metabolite 3-hydroxybutyrate (Table 6.1), which was similar to that of marbling, and it was correlated with marbling in chapters 2 and 3. These results suggest that the use of metabolomics and genomics together could increase the accuracy of prediction of marbling in cattle.

Table 6.1: Genetic, environmental and phenotypic variance, and heritability of Aus-meat marbling, camera marbling, hot standard carcase weight, and the metabolite 3-hydroxybutyrate measured in 895 crossbred wagyu steers using 150K SNP profile.

Source	Aus-Meat Marbling	Camera Marbling	HSCW	3-hydroxybutyrate
Genetic Variance (V(G))	0.95 ± 0.216	13.37 ± 2.739	970.18 ± 152.392	15.25 ± 4.922
Environmental Variance (V(E))	1.57 ± 0.167	11.60 ± 1.938	487.13 ± 95.321	27.51 ± 3.722
Phenotypic Variance (V(p))	2.53 ± 0.136	24.98 ± 1.548	1457.31 ± 87.206	42.76 ± 2.635
Heritability V(G) / V (E)	0.38 ± 0.074	0.54 ± 0.089	0.67 ± 0.076	0.36 ± 0.102

Another factor that influences marbling deposition of Wagyu crossbred cattle includes the breed used in the crossbreeding program and the Wagyu content of the animals [8]. In Wagyu cattle, there is a trade-off between marbling and carcase weight because the price received per kg depends on the marbling score and animals that reach greater marbling tend to have lighter carcases as demonstrated in Chapter 2. Increasing Wagyu content of the animals increases marbling however there are breeds that can produce a highly desirable product in the first generation (F1). The current slaughter grid price is $16/kg, $15.30/kg, $14.40/kg, $13.20/kg, $12/kg, $10.50/kg and $9/kg for a marble score 9, 8, 7, 6, 5, 4 and 3 respectively for Wagyu steers that have been fed for greater than 350 days in a feedlot. Figure 6.2 illustrates the increasing marbling score as the Wagyu content increases. Interestingly, the progeny of Dairy dams produced carcases with high marbling from F1 whereas the other breeds increased marbling with the proportion of Wagyu.

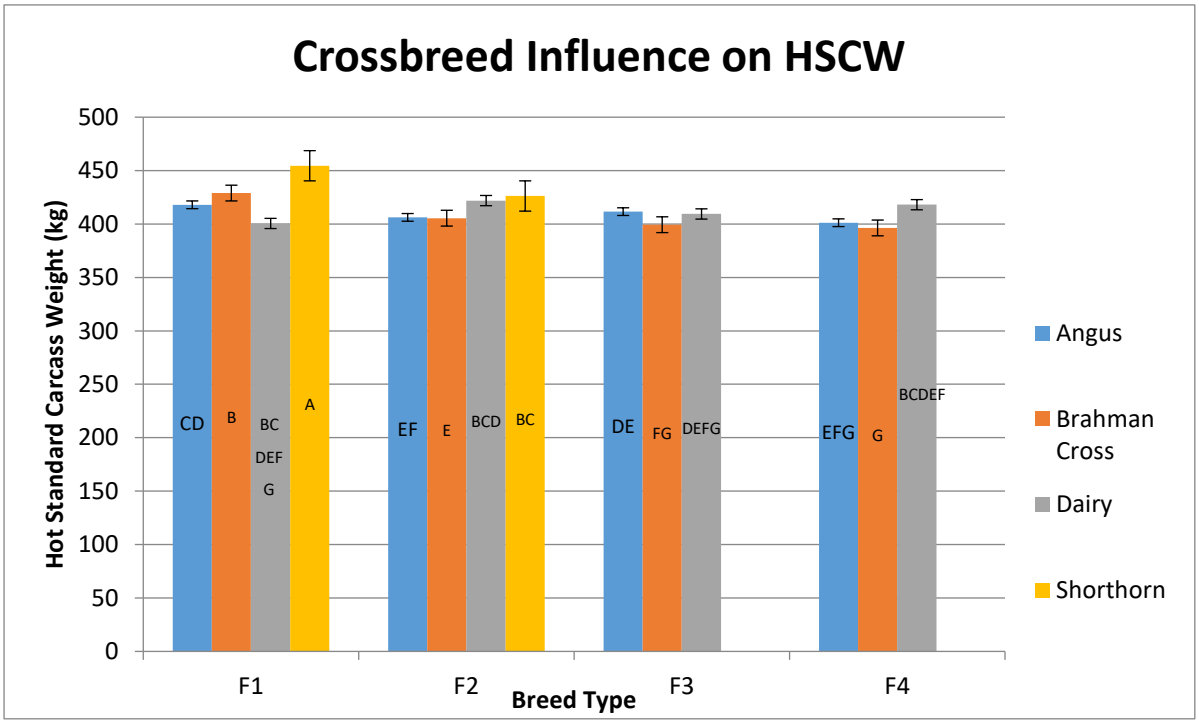
Figure 6.2: Average Aus-meat marbling score for F1, F2, F3 and F4 crossbred Wagyu steers illustrating the impact of the dam breed crossed with and Wagyu content.



A, B, C, D, E, F, G Means within rows without a common superscript differ (P < 0.05).

Similarly, Figure 6.3 shows the relationship between HSCW, dam breed, and Wagyu content. Results show that Shorthorn not only produce carcases with high marbling but also the heaviest carcases, and all breeds (except Dairy) produce lighter carcases with increasing Wagyu content.

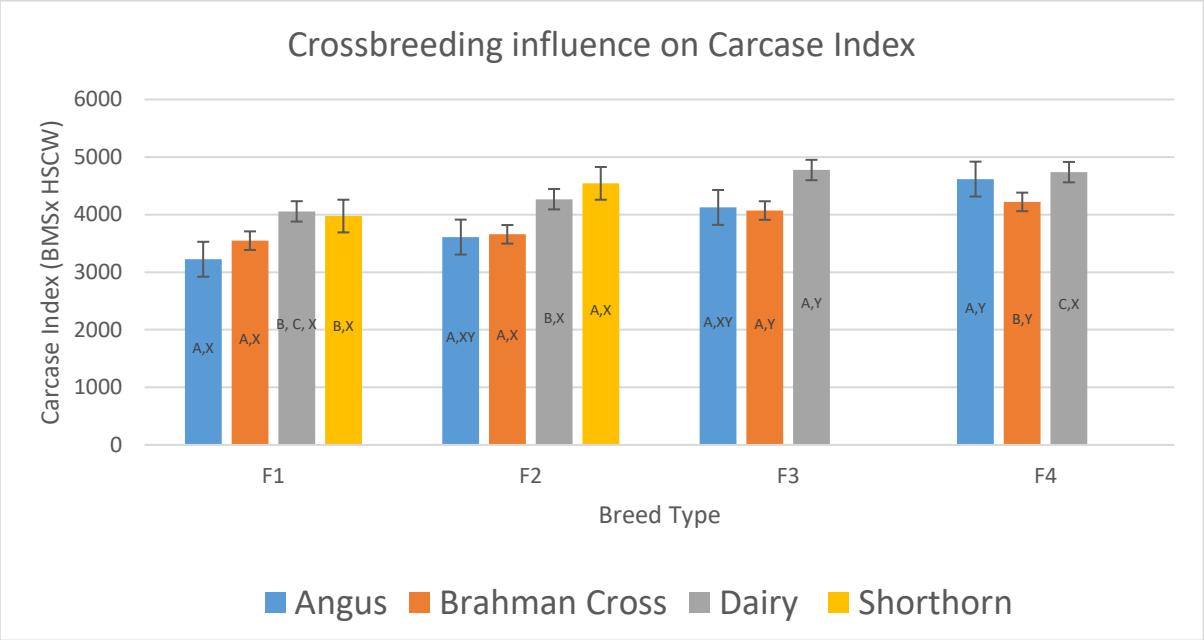
Figure 6.3: Average hot standard carcase weight for F1, F2, F3 and F4 crossbred Wagyu steers illustrating the impact of the dam breed crossed with and Wagyu content.



A, B, C, D, E, F, G Means within rows without a common superscript differ (P < 0.05).

Therefore, an index can be calculated to integrate both marbling and HSCW by multiplying both variables. Figure 6.4 illustrates the increase in carcase index (marble score x HSCW) with Wagyu content (%) or the generation repeatedly used in crossbreeding where the bull is always fullblood or purebred Wagyu. The F1 with 50% Wagyu had on average the lowest carcase index whereas F4 animals with 94% Wagyu have the highest carcase index because of the greater marbling achieved. This is also translated to increasing carcase value with increasing Wagyu content as shown in Chapter 1. The breed of the dam that the Wagyu bull is crossed with also has an impact on marbling with Shorthorn animals responding better to crossbreeding in F1 and F2 compared to Angus as reflected in higher carcase index (Figure 6.4). This is an important finding because demonstrates the impact of the crossbreeding program on marbling, carcase weight and potential economic returns. Furthermore, these results demonstrate that the lack of carcase feedback information to the calf producer or breeder would affect genetic progress to increase marble score.

Figure 6.4: Average carcase index calculated as marbling score by carcase weight for F1, F2, F3 and F4 crossbred animals illustrating the impact of the dam breed crossed with and Wagyu content.



A, B, C, X Means within rows without a common superscript differ (P < 0.05).

The discussion above demonstrates the importance of genetics on performance, carcase traits and animal metabolism, all of which are linked. The present thesis demonstrated proof-of-concept of these relationships and encourages further research integrating these datasets to advance genetic and production improvement in the Wagyu industry.

6.3.3 Integration of biological mechanisms involved in fat and muscle deposition

The present thesis also aimed to improve our understanding of the biological processes driving carcase traits using the metabolome. The identification of metabolites that had a significant relationship with carcase traits across different groups of animals was investigated to determine the potential repeatability between studies. Chapters 2 and 3 examined different mobs of cattle that were fed at different times of the year although they were sourced from the same producer and may have been genetically similar. The metabolic mechanisms involved in intramuscular fat deposition seem similar because there were some metabolites that showed a significant relationship with marbling in both Chapters 2 and 3. For example, 3-hydroxybutyrate,

propionate, glucose and lipids showed a significant relationship with marbling in both Chapters 2 and 3, which indicates these metabolites have an important function in the metabolism and deposition of marbling in Wagyu steers. Propionate and 3-hydroxybutyrate are molecules that are used in the tricarboxylic acid cycle (TCA); plasma propionate has been shown to increase the secretion of insulin, which activates lipogenic enzymes, which can accelerate the fatty acid synthesis needed for intramuscular fat [9]. Furthermore, 3-hydroxybutyrate was correlated with HSCW in Chapter 2 indicating it is an important molecule for muscle energy and metabolism as well. Glucose is another molecule that is also used in the TCA cycle, as it is one of the precursors for fatty acid synthesis and Duarte, et al. [10] have shown that glucose is preferred by intramuscular adipocytes.

The metabolites acetate, histidine, creatine, and isoleucine were correlated with marbling in Chapter 2 although these were not significant in Chapter 3. One potential explanation is that these metabolites are not critical for intramuscular fat deposition when the animals are maturing but they may benefit adipocytes in younger animals such as it was the case with animals in Chapter 2. However, evidence to support this speculation is lacking and further research is needed. Acetate is a key part of the TCA cycle, and it is converted to 3-hydroxybutyrate once absorbed from the rumen and then oxidised in the TCA cycle or used for fatty acid synthesis [11]. Creatine is also an important molecule although it was assumed that it was not used in fat tissue metabolism but in muscle and brain tissue metabolism aiding in the recycling of ATP [12] . However, the fact that creatine was correlated with marbling in both Chapters 2 ($P < 0.05$) and 3 ($P = 0.06$) suggest that it may play a direct or indirect role in intramuscular fat deposition. The findings from Chapters 2 and 3 highlight that the metabolites that were identified in the plasma of Wagyu steers are important in both muscle and fat metabolism. These are encouraging results opening new opportunities to select higher performing animals using metabolomics and understanding the relationships between the molecules and animals' metabolism.

6.4 Practical applications, limitations, and future directions

The studies within this thesis undertook a novel approach to understand muscle and fat metabolism, and feed efficiency in Wagyu cattle and search for potential new ways to select high performing animals using blood biomarkers. Further research is required to continue

developing the framework and address important challenges because the production system of Wagyu cattle is currently a time consuming and expensive process. Alternative ways to determine feed efficiency and future marbling of Wagyu cattle would enable the whole industry to improve. The application of metabolomics in this context was important and multiple aspects of the technology were investigated in the present thesis such as the effect of sampling time, machine learning and biological relationships between metabolic processes and economically important outcomes.

The current thesis examined samples collected in a commercial feedlot to ensure industry application and potential generalisation of results, and to determine if all the processes used could be applied efficiently to the current production systems. Although this is an important part of the process, it was also challenging to evaluate the relationships under commercial conditions with sampling of steers from different cohorts, times of the year, genetics, and diets. In addition, the animals were selected to enter the feedlot under commercial drivers and the time in which the animals could be sampled was determined by the operators and feedlot staff. The duration in which the animals were sampled couple also influence the results due to the core temperature increasing, the animals missing the morning feeding and being hungry and excited by the change of routine. All of these factors influence the plasma metabolome, moreover collaborating with a commercial feedlot, the researcher has limited control over the heterogeneity/homogeneity of the study cohorts. There were variables that were not ideal throughout the studies.

Therefore, there were questions arising from the findings in the present thesis that may need to be examined under more controlled conditions. For example, the methods used in the present thesis would need to be evaluated in other populations of cattle from different producers to ensure the results are repeatable at multiple locations across the country. Further research on the within-day changes in the metabolome of cattle with feeding and under different diets (e.g., high and low forage diets) is also needed. Research under more controlled conditions where liver or tissue biopsies can be obtained for further metabolomic analysis, gene expression, epi-genetic changes, or proteomics would also be of great value to link with changes in circulating metabolites.

The ability to select higher performing animals using a metabolite biomarker would be an ideal situation if the steers could be drafted at feedlot induction or a week or two into it. To enable

this process to happen efficiently, a crush side test would be the ideal way to identify which animals to put into a long-fed program and which animals should be sent into another feeding regime or production system. This would require evaluation of the biomarkers identified in different groups of cattle and at different locations, and then the development of an assay that can be used at the feedlot. Although this technology may be possible in the distant future, it is worth to continue evaluating the methods and approach developed in the present thesis with the aim to increase the productivity of the Wagyu production system.

The effect of sire on metabolites and carcase traits indicate there is a genetic factor that is influencing intramuscular fat deposition in Wagyu cattle. The present study used animals from a crossbreeding program with potentially 'unstable' or changing gene pool which may differ from using purebred or full blood Wagyu progeny. The genomic analysis and integration with metabolomics were beyond the scope of the present thesis but further research into the relationship between the genome and metabolites in Wagyu cattle is encouraged. The inclusion of metabolomic data may increase the accuracy of the genomic predictions to identify animals that are going to produce a superior carcase early in the feedlotting process.

6.5 Final Conclusions

This thesis has made a significant contribution to the understanding of marbling and residual feed intake in Wagyu crossbred cattle using a metabolomics approach. The results presented have shown that sampling within 160 days from arrival to the feedlot does not have a significant influence on the metabolome, but samples obtained further apart than approximately 200 days could imply significant changes in the metabolome and the interpretation of the results. Machine learning can help developing prediction algorithms of marbling and RFI traits adjusted for important carcase traits such as marbling using metabolomics data as predictors. This approach showed promise to increase knowledge of the biological mechanisms involved in the regulation of these traits and for selection purposes. Metabolomics is an important tool in the search for increasing efficiency in the Wagyu feedlotting sector. There is a requirement for further evaluation of the methods produced in the present thesis to new cohorts of animals as well as investigation into the relationships between genomics and metabolomics.

6.6 References

1 Berri, C. *et al.* Predicting the Quality of Meat: Myth or Reality? *Foods* **8**, 436 (2019).

2 Trenkle, A. & Topel, D. G. Relationship of some endocrine measurements to growth and carcass composition of cattle. *Journal of Animal Science* **46**, 1604-1609 (1978).

3 Gotoh, T., Takahashi, H., Nishimura, T., Kuchida, K. & Mannen, H. Meat produced by Japanese Black cattle and Wagyu. *Animal Frontiers* **4**, 46-54, doi:10.2527/af.2014-0033 (2014).

4 Matsuzaki, M., Takizawa, S. & Ogawa, M. Plasma insulin, metabolite concentrations, and carcass characteristics of Japanese Black, Japanese Brown, and Holstein steers. *Journal of animal science* **75**, 3287-3293 (1997).

5 Shingu, H. *et al.* Profiles of growth hormone and insulin secretion, and glucose response to insulin in growing Japanese Black heifers (beef type): comparison with Holstein heifers (dairy type). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **130**, 259-270 (2001).

6 Park, S. J. *et al.* Genetic, management, and nutritional factors affecting intramuscular fat deposition in beef cattle - A review. *Asian-Australasian journal of animal sciences* **31**, 1043-1061, doi:10.5713/ajas.18.0310 (2018).

7 Oyama, K. Genetic variability of Wagyu cattle estimated by statistical approaches. *Animal Science Journal* **82**, 367-373, doi:10.1111/j.1740-0929.2011.00895.x (2011).

8 Nguyen, D. V., Nguyen, O. C. & Malau-Aduli, A. E. O. Main regulatory factors of marbling level in beef cattle. *Veterinary and Animal Science*, 100219, doi:https://doi.org/10.1016/j.vas.2021.100219 (2021).

9 Ladeira, M. M. *et al.* Nutrigenomics and Beef Quality: A Review about Lipogenesis. *International Journal of Molecular Sciences* **17**, 918, doi:10.3390/ijms17060918 (2016).

10 Duarte, M. S. *et al.* Enhancement of adipogenesis and fibrogenesis in skeletal muscle of Wagyu compared with Angus cattle. *Journal of animal science* **91**, 2938-2946, doi:10.2527/jas.2012-5892 (2013).

11 Church, D. C. *The ruminant animal: digestive physiology and nutrition*. Rev. edn, (Waveland, 1993).

12 Hanset, R. & Michaux, C. 237-256 (1982).