Investigating Blood Biomarkers of Economic Traits in Sheep Using Metabolomics

by

Seyed Ali Goldansaz

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Departments of Agricultural, Food and Nutritional Science and Biological Sciences
University of Alberta

Abstract

Livestock metabolomics is an emerging field of metabolomics that is growing at a fast rate. The ability to rapidly detect and quantify hundreds or even thousands of metabolites within a single sample is helping livestock scientists paint a far more complete picture of animal metabolism and physiology. It is also helping livestock researchers identify robust biomarkers of health and disease. In this thesis, I applied livestock metabolomics techniques to study domestic sheep with the overarching objective of identifying blood biomarkers for important economic traits in sheep farming. In particular, I focused on several key traits including feed efficiency (the major pillar of cost of production), carcass merit and pregnancy (traits with substantial contribution to farm income). To lay the groundwork for these studies, I first conducted a systematic review of livestock metabolomics (chapter two of this thesis). The intent was to assess the status of livestock metabolomics in general, and to identify important gaps and trends relative to other fields of metabolomics research. As part of this review, I compiled all the known livestock metabolome data published until 2017 and made it publicly available in the Livestock Metabolome Database (LMDB; available at www.lmdb.ca). Using the knowledge gained from this review, I then conducted a study that looked at how metabolomics could assess residual feed intake (RFI; a measure of feed efficiency) and carcass merit in sheep (chapter three of this thesis). Direct measurement of these traits is labor-intensive and expensive. Therefore, finding or developing easily measured metabolite markers for these traits would be expected to reduce operator costs and encourage their widespread measurement. Using a combination of quantitative metabolomic methods, I assessed the serum metabolome and identified 161 unique metabolites. I also identified a panel of candidate serum biomarkers consisting of three metabolites for predicting sheep RFI (with an area-under-the-receiver-operating-characteristic-curve [AU-ROC]=0.80), and two panels

of candidate serum biomarkers for predicting sheep carcass merit, including seven metabolites for carcass yield grade (AU-ROC=0.77) and one metabolite for carcass muscle-to-bone ratio (AU-ROC=0.74). In chapter four, I used quantitative metabolomic techniques to identify and validate blood biomarkers of sheep pregnancy and litter size (PLS). Early detection of pregnant ewes and the number of lambs expected allows producers to adjust their management practices and feed rations based on ewe pregnancy requirements. I employed a longitudinal experimental design with separate discovery and validation phases aimed at identifying candidate blood biomarkers of sheep PLS. In doing so, I identified and quantified 107 metabolites associated with ewe pregnancy, and validated three panels of biomarkers (AU-ROC of 0.81-0.93) that can identify ewe PLS as early as 50 days post-breeding. These biomarkers are currently being translated into a handheld device that could be used as a low-cost, pen-side test for ewe PLS. It is hoped that the methods presented in this thesis would encourage more widespread application of metabolomics in livestock research, and the results presented here would provide added value for the sheep industry.

Preface

This thesis is an original work by Seyed Ali Goldansaz, supervised by Dr. David Wishart and Dr. Graham Plastow, both of whom are professors at the University of Alberta. The research project included in chapter 3 of this thesis received animal ethics approval from the University of Alberta Animal Care Committee, under the Project Name "Metabolomics analyses of sheep residual feed intake and carcass quality", No. 2016.006Wang, April 1, 2016. In addition, the research project included in chapter 4 of this thesis received animal ethics approval from the University of Alberta Animal Care Committee, under the Project Name "Improved accuracy in diagnosing pregnancy and predicting litter size at early ewe gestation", No. AUP00002510, September 13, 2018.

Chapter 2 of this thesis has been published in the journal *PLoS ONE* (Goldansaz, S. A., A. C. Guo, T. Sajed, M. A. Steele, G. S. Plastow, and D. S. Wishart. 2017. Livestock metabolomics and the livestock metabolome: A systematic review. PLoS ONE. 12:e0177675. https://doi.org/10.1371/journal.pone.0177675). I was responsible for the data collection and annotation of the data in the Livestock Metabolome Database (LMDB; www.lmdb.ca) as well as preparation of the initial version of the manuscript, and the subsequent revisions requested by the referees. Tanvir Sajed and An Chi Guo assembled the online version of the LMDB and assisted with data transfer. Dr. David Wishart was the supervisory author and was involved in the concept formation and led the manuscript development. All other coauthors provided feedback on the manuscript.

Chapter 3 of this thesis has been published in the *Journal of Animal Science* (Goldansaz, S. A., S. Markus, M. Berjanskii, M. Rout, A. C. Guo, Z. Wang, G. Plastow, and D. S. Wishart. 2020. Candidate serum metabolite biomarkers of residual feed intake and carcass merit in sheep. J. Anim. Sci. 98:skaa298. https://doi.org/10.1093/jas/skaa298). I was responsible for generating

the project idea, obtaining funding, experimental design, sample collection, laboratory analyses as well as the manuscript preparation. Dr. Susan Markus from Alberta Agriculture and Forestry was a critical collaborator on this project, supporting the fund-raising process. Dr. David Wishart and Dr. Graham Plastow were the supervisory authors. Dr. Zhiquan Wang was initially assigned as the principle investigator of the project which, after his departure from the University of Alberta, Dr. Plastow took over. Dr. Manoj Rout and Dr. Mark Berjanskii were instrumental in assisting with the data analyses. All other coauthors provided feedback on the manuscript.

Chapter 4 of this thesis is expected to be submitted to a scientific journal in the near future. I worked with Dr. Susan Markus to generate the initial research idea, and obtain funding. The Alberta Lamb Producers Executive Directorwas assigned as the principle investigator of this grant. I was responsible for assisting Dr. Markus with the study design, the management of the sample collection process, and components of the sample collection. I also conducted the NMR sample analysis, assisted with the MS-based analysis, performed the statistical analyses and prepared the manuscript. Dr. David Wishart and Dr. Graham Plastow were the supervisory authors.

Dedication

یوسف گم گشته بازآید به کنعان غم مخور
کلبه احزان شود روزی گلستان غم مخور

ای دل غمدیده حالت به شود دل بد مکن
وین سر شوریده باز آید به سامان غم مخور

گر بهار عمر باشد باز بر تخت چمن
چتر گل در سر کشی ای مرغ خوشخوان غم مخور

دور گردون گر دو روزی بر مراد ما نرفت
دائما یکسان نباشد حال دوران غم مخور

هان مشو نومید چون واقف نه‌ای از سر غیب
باشد اندر پرده بازیهای پنهان غم مخور

ای دل ار سیل فنا بنیاد هستی بر کند
چون تو را نوح است کشتیبان ز طوفان غم مخور

در بیابان گر به شوق کعبه خواهی زد قدم
سرزنشها گر کند خار مغیلان غم مخور

گر چه منزل بس خطرناک است و مقصد بس بعید
هیچ راهی نیست کان را نیست پایان غم مخور

حال ما در فرقت جانان و ابرام رقیب
جمله می‌داند خدای حال گردان غم مخور

حافظا در کنج فقر و خلوت شبهای تار
تا بود وردت دعا و درس قرآن غم مخور

Acknowledgement

It is with great joy and excitement to see this fruitful chapter of my life coming to a close. Living away and separate from my family for nearly a decade has had its challenges but nevertheless, it has shaped me into a better person. Going through all the ups and downs of thesis-based research has been very rewarding, yet not possible without the grace of God. I am grateful for all that He has put me through to shape who I am today. It is through Him that I see light, and try to be the best of me every day.

My family has also been integral to this journey and the greatest contributing factor to all the accomplishments I've achieved. Mom, Dad, I cannot begin to thank you for all that you've done for me; my being is rooted in you, and I am forever proud to be from your blood and grateful for having you as my parents.

I believe the best part of going through graduate studies has been working with two prominent researchers, Dr. Graham Plastow and Dr. David Wishart, who were also my thesis supervisors. I am most grateful for when they helped me navigate through the difficult times with their patience and detailed feedback. Not only did I benefit from their scientific integrity, but I also learned a lot for their academic character. Among the many useful takeaways from my supervisors, I mostly cherish tips on how to improve my writing. Dr. Wishart, Dr. Plastow, thank you! I also want to thank Dr. Michael Steele, the third member of my supervisory committee for his time and feedback.

I was also blessed to work with a wonderful group of individuals at The Metabolomics Innovation Center (TMIC) and Livestock Gentec that supported me and my research endeavors throughout my PhD program. I am most grateful to Dr. Mark Berjanskii for his ongoing support and empathy at times when I needed it the most. I want to thank Dr. Manoj Rout, Nazanin

Assempour, Dr. Rosa Vazquez Fresno, Fatemeh Shahin, Edison Dong, Jiamin Zheng, Dr. Rupasri Mandal, Allen Zhang, Janelle Jiminez, Yan Meng, Dr. Ghader Manafiazar, Dr. Reza Salehi, and Dr. Aidin Foroutan Naddafi for their friendship and support throughout my PhD program. I am also very much thankful to the long list of my friends and peers who were by my side and always willing to provide their wisdom and advice.

I also had the great pleasure of collaborating with Dr. Susan Markus over the last couple of years for the bulk of my thesis work. Her support for my ideas and initiatives has been most appreciated. Our collaboration has been very fruitful and I am happy to see that the journey continues. I am also very grateful to have interacted with the Canadian sheep industry, particularly the Alberta Lamb Producers, which helped me formulate many of the ideas presented in this thesis.

The accomplishments in this thesis would have not been possible without the financial support of a number of funding agencies. I am most grateful to the financial support of the Alberta Lamb Producers, Ontario Sheep Farmers, Canadian Sheep Breeders Association, Alberta Livestock and Meat Agency, Alberta Agriculture and Forestry, Genome Alberta, and Genome Canada.

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List of Abbreviations

AAA, aminoadipic acid

ACTH, adrenocorticotropic hormone

ADG, average daily gain

ADMA, asymmetric dimethylarginine

API, atmospheric pressure ionization

AUC, area under the curve

AU-ROC, areas-under-the-receiver-operating-characteristic-curve

AUS, Australia

BAR, whole barley with protein supplement

BHB, β-hydroxybutyrate

BF, back fat

CAD, Canadian dollar

CE, capillary electrophoresis

CIDR, controlled internal drug release

CL, corpus luteum

CNT, controls

DI/LC-MS/MS, direct injection liquid chromatography mass spectrometry/mass spectrometry

DI-MS, direct injection mass spectrometry

DFI, direct flow injection

DMI, dry matter intake

EI, electron ionization

EID, electronic identification device

ESI, electrospray ionization

FAO, Food and Agriculture Organization of the United Nations

FCR, feed conversion ratio

FF, follicular fluid

FID, free induction decay

FTIR, infrared spectroscopy

GC, gas chromatography

GIS, geographic information system

GPC, glycerophosphocholine

GPS, global positioning system

HPLC, high-performance liquid chromatography

ICP-MS, inductively coupled plasma mass spectrometry

IGF-1, insulin-like growth factor-1

LC, liquid chromatography

LC-MS/MS, liquid chromatography tandem mass spectrometry

LMDB, Livestock Metabolome Database

LysoPC, lysophosphatidylcholine

MALDI, matrix-assisted laser desorption ionization

MBR, muscle to bone ratio

MCH, mean corpuscular hemoglobin

MCV, mean corpuscular volume

mM, millimolar

MS, mass spectrometry

m/z, mass-to-charge ratio

N, nitrogen

NMR, nuclear magnetic resonance

NZ, New Zealand

P4, progesterone

PAG, pregnancy-associated glycoprotein

PC, phosphatidylcholines

PEL, barley-based pellet form

PFG, pulsed-field gradient

PLS, pregnancy and litter size

PLS-DA, partial least squares-discriminant analysis

PRG, pregnant ewes

PRISMA, preferred reporting items for systematic reviews and meta-analysis

PSPB, pregnancy specific protein B

RBC, red blood cells

RFI, residual feed intake

ROC, receiver operating characteristic

SCC, somatic cell count

SM, sphingomyelin

SM(OH), hydroxysphingomyelin

SNG, ewes that delivered a single lamb

T4, thyroxine

TMR, total mixed ration

TMIC, The Metabolomics Innovation Center

TWN, ewes that delivered a twin

TRP, ewes that delivered a triplet or more

μM, micromolar

UPLC, ultra performance liquid chromatography

VIP, variable importance plot

WBC, white blood cells

YG, yield grade

Chapter 1: Introduction

General Introduction

Feeding the world's population in the years to come is one of the great unmet challenges of the 21st century. According to FAO statistics (FAO, 2015), even today, one in every nine people is undernourished for at least one year of their lives. Lack of nourishment can arise from either the lack of sufficient amounts of food or the lack of sufficient amounts of nutrient-dense food. Red meat derived from livestock is, and will likely be, one of the main sources of nutrient-dense food needed to feed the world's growing population in the 21st century. Because of its high nutrient density, global demand for meat is expected to double by 2050 (Robinson et al., 2014), with the demand for red meat from ruminants expected to increase at a rate of 1.5% per year (FAO, 2017). Given these projections, it will be crucial for livestock farmers to provide a sufficient amount of animal protein not only for existing markets, but also for parts of the world where meat is lacking from the diet or its consumption is limited due to meat quality, or the requirement for certain processing procedures, such as halal and kosher rules (Rajaei Sharifabadi et al., 2012). Currently, meat production through livestock farming is a source of livelihood for more than a billion people worldwide. To meet the expected demand over the next three decades, livestock farmers will have to operate more efficiently, increasing their production by 70% while having to substantially reduce their land use and natural resource requirements to accommodate urban expansion and to limit the adverse environmental impacts of livestock farming (Cockrum et al., 2013). In addition to these large-scale, global challenges facing agriculture and food production, there are also significant domestic challenges facing the Canadian agricultural and livestock system.

One of the challenges facing the Canadian agricultural system, according to the Royal Bank of Canada (RBC, 2019), is the decreasing trend of Canadian agriculture exports relative to the total value of exported Canadian goods. Indeed, over the past 20 years the proportion has gone from

6.3% in 2000 to just 3.9% in 2019. Three factors have been identified to be impeding the growth of Canadian agriculture and livestock exports: (1) a declining workforce, (2) the need for highly qualified personnel, and (3) insufficient use of relevant technology and high-tech machinery on Canadian farms. The workforce in agriculture-related fields has shrunk by nearly 30% within the past two decades. The Canadian Agricultural Human Resources Council projects that more than 120,000 agricultural jobs will remain vacant in the upcoming decade (RBC, 2019). Canada's agricultural workforce will further decline due to the fact that nearly 40% of the existing farmers will retire within the next decade. Another 25% of Canadian farmers will be turning 65 years or older by 2025. On the contrary, the workforce educated in agricultural programs at Canadian academic institutions has expanded by almost 30% over the past decade. The RBC (2019) also reports that Canadian farmers are highly dependent on government support for the purchase of new technologies and integrated management systems. While Canada ranks among the top five global investors in agtech – with a share of 3.4% – there is still a huge gap between Canada and competitors from the USA, India and Brazil. One area of agtech investment that is gaining increasing attention is precision or "smart" agriculture. This is because smart agriculture technologies would increase the efficiency and accuracy of farming practices and could potentially stem the tide of the ever-shrinking agriculture workforce in Canada. At the time of writing this thesis, the COVID-19 pandemic has also shifted demand and attention to modify conventional agricultural practices towards remote and automated practices.

Smart agriculture or precision farming is driven by new technologies that modify both work culture and production efficiency. At the core of precision farming is the use of computational management and big datasets collected from smart, automated machinery that tailor farming practices to precise handling of individual agricultural units, i.e., an animal, a plant, or an

acre of land. Some of the emerging technologies include automated farming equipment, such as the autonomous DOT (named after the inventor's mother, Dorothy) system, pioneered in Saskatchewan, Canada. The DOT system allows heavy equipment to be self-driving, collects real-time data from the soil, and reports on the performance of the field. The use of GPS (Global Positioning System) and GIS (Geographic Information System) is also becoming more relevant than ever before, guiding precise applications of pesticides, movement and tracking of equipment, accurate irrigation of the land, livestock monitoring and activity assessment on pasture. Other examples of game-changing technologies include robotic milking machines, ultrasound diagnosis of pregnant animals, artificial insemination and embryo transfer, and automated feeding systems that measure individual feed consumption and feeding behavior of animals.

Among the most important smart agricultural practices to emerge over the past two decades are "omics" technologies. Omics technologies provide a holistic and comprehensive understanding of plants, animals or soils at all molecular levels, i.e., the genome, transcriptome, proteome, and metabolome. Omics platforms are also evolving, becoming smaller and more portable as hand-held and benchtop devices, improving their depth and breadth of detection, and becoming more user-friendly for widespread research application in the lab and on-farm use for farmers. One omics field in particular, metabolomics, has gained a lot of attention in recent years as it has been shown to have many useful applications in biomedical, veterinary, agriculture, and environmental research. The utility of metabolomics lies in the fact that it enables the measurement of subtle changes in the metabolome of plants and animals. In addition, metabolomics provides the opportunity to reveal biomarkers that represent alterations in the chemical phenotype of an organism. This chemical phenotype is often referred to as the "metabotype" (Fontanesi, 2016). While the application of metabolomics to plant breeding, crop science, food analysis and soil

science is thriving, the application of metabolomics in livestock farming and livestock research has just recently been gaining some attention. The metabotype can provide phenotypic data to inform on the outcome livestock genetic selection, and also to inform on the alterations in the underlying omics layers, i.e., proteomics. We discuss different aspects of livestock metabolomics further in this chapter (section 1.5.) and in chapter two of this thesis.

The Livestock Industry

Cattle, sheep, goats and pigs are the primary livestock species farmed for red meat production around the globe (FAO, 2018). Red meat is a rich source of essential nutrients, especially essential amino acids, which are biologically more compatible for absorption by our body than most other sources of nutrients (Wyness, 2016). For example, lean red meat is abundant in fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0; Daley et al., 2010). Red meat also provides the necessary amount of protein required for human growth in adolescence. In addition to macronutrients, such as fatty acids and amino acids, micronutrients such as iron are also concentrated in red meat and provide up to 30% of this micronutrient in its haem form (Wyness, 2016). This type of iron is critical for blood's capacity to transport oxygen. The main source of red meat production worldwide is from cattle and pigs, supplying 24% and 40% of the market demand, respectively (FAO, 2018). Additionally, sheep rearing is reported to be the second-largest live animal operation for red meat production (FAO, 2015). Sheep carcass weight has increased by 2% over the past decade, contributing to the steadily increasing global sheep meat production, reaching 9.7 million tons of meat produced from more than 573 million head in 2018 (FAO, 2018). A similar trend exists for goat meat production with more than 5.9 million tons of meat produced from 479 million head in 2018 (FAO, 2018). The most recent Canadian Agriculture Census (2016) indicates beef farming is still the main driver of red meat production in Canada, mainly in Alberta. However,

market demand for small ruminant meat, specifically sheep, is gaining momentum because of the large immigrant population across Canada and the reduced supply of imported meat. Canada's sheep industry has had a fairly consistent production of live sheep from 2016 to 2020 (Statistics Canada), and the amount of sheep meat produced has increased from an average of 9,000 tons in 1990 to more than 15,000 tons in 2016 (FAO, 2018).

Sheep Farming

Domestication of Sheep

Sheep were among the first animals to be domesticated by humans. Early signs of sheep domestication date to earlier than 8000 BC, as evidenced by Iranian statuary (Figure 1) portraying sheep farming (Zeder, 2008). Today, more than 1400 breeds of sheep exist across the globe (Scherf, 2000). The majority of the world's sheep production (Figure 2.a) is in Asia (>40%), led by China, India, Turkey and Iran, followed by Africa (>20%), Europe and Oceania (>10%; Zygoyiannis, 2006). North American producers own 0.7% of the world sheep population (Zygoyiannis, 2006). According to FAOSTAT (statistical data generated by FAO, available online for public access), the top five sheep producing countries are China (~150M head), Australia (~85M head), India (~65M head), Sudan (~50M head) and Iran (~50M head; Figure 2.b). Global sheep production in the 21st century has generally increased from 1.06 billion head in 2000 up to 1.20 billion head in 2018. Canada stands in 84th position worldwide in sheep production, with 0.82M head reported in 2018. Canadian sheep production surged in 2000 from 0.79M head to 0.97M in 2005. However, after the outbreak of mad cow disease in Canada, the livestock industry in general steadily declined including sheep production, which dropped to 0.82M head by 2018.

Sheep have been traditionally produced for three main purposes: meat, milk and wool. While some breeds, such as Suffolk, Canadian Arcott and Rideau Arcott, are generally destined

for meat production, others such as Lacaune and East Friesian are recognized for their milk production, and a few, such as Merino, are farmed for wool. Increasing demand for sheep meat has elevated the popularity of farming sheep for meat production (FAO, 2018). In North America, sheep farmers contribute slightly more than one percent of the worldwide sheep meat production compared to 50% in Asia, nearly 15% in Europe and Oceania, and more than 10% in Africa (Zygoyiannis, 2006). In Canada, the majority of sheep production is for meat, while a minority of producers (mainly in the Eastern provinces) rear sheep for milk production. Up to half of the sheep meat consumption in Canada is supplied by domestic producers and the remaining amount is imported mainly from Australia (AUS) and New Zealand (NZ). The per capita consumption of sheep meat is growing among Canadian consumers, and imports from AUS and NZ are declining because of the decreasing flock sizes in those countries. Therefore, there is more opportunity for Canadian producers to increase their market share of sheep meat.

Sheep Breeds Used in This Thesis

The breeds used in this thesis work include the Suffolk, Canadian Arcott and Rideau Arcott. Suffolk is a sheep breed that is predominantly used as a terminal sire (male Suffolk breeding with female of any breed to produce lambs for meat production) in North America due to its higher growth rate, higher meat yield, higher number of premium meat cuts, uniformity for easier slaughter, and higher market desirability (Leymaster, 1991; Pérez et al., 2002; Maierle, 2018). Canadian Arcott and Rideau Arcott are comparatively newer breeds that have been developed in Canada for meat production and prolificacy, respectively. Canadian Arcott rams and lambs have higher weights with greater lean meat and loin eye areas, while the Rideau have a moderate muscle content and are highly prolific (Shrestha and Heaney, 2003). Sheep producers in Canada traditionally use Rideau genetics to develop their maternal flock and Arcott rams as a terminal sire.

Sheep Meat Production in Canada

Sheep meat production is associated with relatively small profit margins for most Canadian producers. Implementing more efficient methods for sheep production is one way that producers can improve profitability and reduce their environmental footprint. In fact, livestock have the largest impact on agricultural land by overgrazing natural vegetation, and polluting soil, water and the air through manure production, urination and methane production (FAO, 2015). One approach to improving production efficiency is selecting for sheep that efficiently process feed to meat. Feed-efficient sheep consume less feed for a similar level of growth therefore, decreasing grazing pressure on pastures, and producing less manure, methane and urine. Having feed-efficient sheep on pasture also opens the opportunity to increase the stocking rate. This allows farmers to increase meat production using the same amount of resources. Another benefit of this approach is transmitting a positive message throughout the food chain, informing meat consumers of the lower environmental impact of sheep production. Improving production efficiency also favors producers by improving their profit margins. There are three traits that generally contribute to the profitability of livestock production: feed efficiency, carcass merit, and pregnancy (Norton, 2005; Morris, 2009; Spring, 2013; Farrell et al., 2020; Lockwood et al., 2020). These traits are investigated in this thesis and are explained in detail in the following sections and following chapters of this thesis.

Feed management is the major cost of production in sheep farming. A strategy to mitigate this cost is the selection of more feed-efficient sheep. While improving feed efficiency reduces farm costs, farm income is elevated by selecting lambs that have higher carcass merit. Farmers are paid a higher premium if they deliver carcasses with higher proportions of lean meat. Income and profitability also have a direct positive correlation with the number of lambs delivered per ewe. Early knowledge of the pregnancy status of an ewe and the adjustment of management practices

based on pregnancy requirements can reduce the chances of abortion and increase delivery of viable lambs (Heasman et al., 1998; Kenyon and Blair, 2014). Moreover, early identification of ewes that failed to breed during the breeding season would allow them to re-enter the breeding cycle, increasing the pregnancy rate of the flock. Another opportunity presented to sheep producers through early pregnancy detection is preventing the overfeeding of open ewes with rations balanced to meet pregnancy requirements, thus further saving on feed costs. A detailed description of these traits appears below. Currently, feed efficiency, carcass merit, and pregnancy status are measured in different ways on the farm. One of the relatively informative measures of feed efficiency is residual feed intake (RFI). As described below, the current practice of measuring RFI requires special equipment that collects feed data over a lengthy experimental trial. Carcass merit is also measured by different means, some of which require ultrasound measurements of live animals or post-mortem measurements after slaughtering the animal. Pregnancy and litter size in ewes can also be measured with ultrasound.

Residual Feed Intake

In livestock operations, where the cost of production is accurately quantified, animal feed is recognized as the single highest component of the costs. In sheep production, the cost of feeding animals accounts for 40-80% of the operational costs (Paisley and Cammack, 2010). The cost of feeding animals is affected by multiple factors such as the availability of feed resources, the duration of winter-feeding and grazing season, the geographical region and climate, labor costs and infrastructure (Kaliel, 2004). Another contributing factor to this cost is feeding all of the flock the same ration. Even though feeding all the flock with the same ration may seem to be a convenient option, assuming all animals have the same nutritional needs results in feeding some of the flock below/above their physiological requirement, thus increasing the cost of feeding.

Inherent features of certain animals also contribute to the cost of feeding depending on how efficiently they convert feed to meat. That is why a prevalent strategy to manage this cost is the selection of feed-efficient animals (Li et al., 2020a). In a world where producers pay a similar price for animal feed and sell their commodity (i.e., live animal or meat) for a similar price as most other producers, efficient animals will substantially contribute to the profitability of the farm, and give it a competitive advantage. Each individual animal has a different rate of feed conversion efficiency despite all animals receiving the same ration (Rajaei Sharifabadi et al., 2012). Feed-efficient sheep are expected to eat less while producing the same quality and quantity of products relative to the rest of the flock (Paula et al., 2013). Feed-efficient ruminants also have a lower environmental impact due to reduced feed intake, reduced stocking rate on pasture, and lower excretion of manure, urine and emission of methane (Basarab et al., 2003).

Measuring feed efficiency has evolved throughout the years with some examples including feed conversion ratio (FCR), average daily gain (ADG), and RFI. A discussion covering all measures of feed efficiency and the advantages and disadvantages of each is beyond the scope of this chapter. Here we will focus mostly on RFI with a brief discussion on FCR. The FCR is a trait that compares weight gain against feed intake of an animal. Animals with low FCR are favored because for each kilogram of weight gain, they consume less feed. The FCR trait is easily measured and often calculated for a group of animals in the pen rather than a single animal. Despite its advantages, FCR is correlated with multiple traits, such as feed intake, growth rate and mature body size, which makes genetic selection for FCR yield unfavorable responses in the correlated traits (Herd and Bishop, 2000; Crews, 2005; Kelly et al., 2010). Furthermore, FCR does not distinguish between maintenance and growth requirements of an animal because it measures gross feed intake. Moreover, the genetic correlation of FCR between different stages of maturity in the

same animal is very low (Arthur and Herd, 2005) suggesting that FCR is a constantly evolving measurement. Unlike the popularity of using FCR for measuring feed efficiency, RFI in sheep has not been widely explored and, to the best of our knowledge, no record of measuring RFI on Canadian sheep breeds exists. This is, in part, due to the lack of knowledge around RFI in the sheep industry and the cost of directly measuring this trait. Therefore, many of the inferences around sheep RFI are based on research conducted on other livestock species, specifically cattle. In this chapter, I have tried to reference sheep RFI research and in the case of it not being available, I have referred to RFI research on other ruminants.

Among the different feed efficiency measurements, RFI is defined as the difference between predicted and actual feed intake corrected for body weight and animal performance (Koch et al., 1963). Measurement of feed efficiency via RFI has become much more recognized in the study of ruminants because of its phenotypic independence from production level, age and weight (Karisa et al., 2014). The RFI value of an animal is the residual amount of feed it consumes above or below the level that has been predicted (from like-type animals and published standards; Figure 3). Therefore, sheep with lower RFI are desired because they consume less feed than expected and produce less waste, while not sacrificing body weight, size and productivity (Moore et al., 2009; Paula et al., 2013). Calculating RFI involves measuring and statistical processing of dry matter intake (DMI) and its energy content, ADG, metabolic body weight and ultrasound backfat for individual animals over a period of 40-100 days (Wang et al., 2006). It has been found that low-RFI Ghezel ram-lambs exhibited 12% lower FCR, further reflecting less consumption of DMI compared to high-RFI lambs (Rajaei Sharifabadi et al., 2012). Given the phenotypic independence of RFI, some have suggested that selection for this trait could improve the genetic performance of feed efficiency without affecting other carcass traits (Rajaei Sharifabadi et al., 2012). This

independence is also expected to reflect inherent differences in the metabolic pathways influencing RFI. Therefore, RFI is a desirable trait to study and select for feed efficiency, without negative or indirect effects on other production traits. Even though the physiological basis and molecular mechanisms underpinning RFI have not been fully described, various factors have been reported to affect this trait including feed intake, nutrient digestion and metabolism, body composition, physical activity and body temperature (Herd and Arthur, 2009).

Feed intake for an animal is, in part, driven by the maintenance requirements and energy expenditures of the digestive organs for nutrient metabolism. The more an animal eats, the larger its digestive organs will be, thus increasing the energy requirements of the organs (Herd and Arthur, 2009). Zhang et al. (2017) reported that organ weight and intestinal length have a positive correlation with lamb RFI. Low-RFI wethers (male castrated sheep) also have a tendency for greater weight of their spleen and pancreas (Meyer et al., 2015). This is important since the gastrointestinal tract and liver consume up to 50% of the ruminant's energy while accounting for only 6-13% of the body weight (Meyer et al., 2015). Nutrient digestion and metabolism are other sources of variation in RFI and are measured by the total disappearance of nutrients in the gastrointestinal tract. In relation to maintenance requirements, feed digestion and feed intake are negatively correlated, which means that as intake increases, digestion tends to decrease (Herd and Arthur, 2009). Sheep digestion varies up to 30% in DMI between the most and least feed-efficient animals (Muro-Reyes et al., 2011; Redden et al, 2014). This variability is in part due to the difference of metabolizable energy obtained by the feed, feeding duration (Nkrumah et al., 2005), feed particle size, processing and availability (Redden et al., 2013; Redden et al., 2014), and feed digestibility which varies at different stages of growth (Redden et al., 2011). Given the high

negative correlation of feed digestion with RFI (r =-0.44), nutrient digestibility is calculated to account for nearly 19% of the variation in RFI in ruminants (Richardson and Herd, 2004).

Carcass Merit

Carcass quality is one of the key factors contributing to farm income. The quantity of lean meat and fat content, in addition to the carcass conformation and meat cuts, are important factors that dictate the monetary value of each sheep sold (Scholz et al., 2015). Carcass evaluation is significant for breeding schemes and for the evaluation of the high-ranking seedstock to improve the proportion of carcass muscle mass (Silva, 2017; Ibrahim, 2019). Currently in Alberta, sheep producers are paid based on the carcass weight bracket (unpublished data from Alberta Lamb Producers). Conventional methods and current practices of determining the dressing percentage (i.e., the amount of lean meat in the carcass) and fat deposition are limited to pre-mortem ultrasonography and post-mortem carcass measurements (Scholz et al., 2015; Silva, 2017). Other emerging technologies, such as post-mortem X-ray computed tomography scans, have also been suggested for sheep carcass evaluation (Jones et al., 2002; Macfarlane et al., 2006); however, given the cost and limited availability of these technologies, their application on Canadian farms is very rare (Garza Hernandez et al., 2018). Indeed, the application of these emerging technologies are mostly limited to research purposes. Once these state-of-the-art technologies become more accessible/affordable and are coupled with smart technologies, they will no doubt serve the industry in the future for farm applications. On the other hand, carcass ultrasonography is a useful tool that offers real-time, on-farm, pre-mortem assessment of the carcass. However, application of this method is very limited mainly because most Albertan farmers are paid based on carcass weight and very few, if any, abattoirs offer premiums on the quality of meat cuts in sheep. When ultrasound is used to assess a sheep carcass, the thoracic and lumbar regions are scanned to

measure the longissimus thoracis and lumborum muscles, which are represented by the 3rd and 4th lumbar vertebra of the backbone and the 12th and 13th rib, respectively (Silva, 2017; Garza Hernandez et al., 2018). Post-hoc evaluation of the sheep carcass (Figure 4) generally includes measuring the carcass weight (hot and cold weights) and qualitative measurements of the meat content (Jones et al., 1996; Silva, 2017).

It has been speculated that carcass quality and RFI have some correlation, however the literature reports inconsistent results. The RFI trait is thought to be affected by, and in turn affects, body composition. Some of the evidence in the literature points out that feed-efficient animals expend most of their energy intake to muscle development. In fact, progeny of low-RFI cattle have lower total body fat and higher total body protein (Herd and Arthur, 2009). The underpinning biology that correlates carcass merit and RFI is not fully understood. However, a few molecular components, such as creatinine, urea and leptin, have been reported to be involved in both traits. The concentration of serum creatinine is negatively correlated with RFI and backfat (BF) and positively correlated with muscle mass (Paula et al., 2013). In addition, elevated concentrations of blood urea are correlated with higher fat deposition, lower muscle mass growth, and higher RFI scores in sheep and steers (Richardson et al., 2004; Herd et al., 2004). Moreover, leptin, which has historically been used as an indicator of fat deposition, is also positively correlated with RFI (Herd and Arthur, 2009). In particular, less feed-efficient cattle (higher RFI scores) have a higher blood content of leptin. Other reports (Richardson et al., 2001; Basarab et al., 2003; Schenkel et al., 2004) suggest a weak positive correlation between RFI and body fat, and a negative correlation between carcass lean and RFI (Herd and Bishop, 2000). Given the evidence correlating RFI and carcass quality, some suggest that RFI could be used not only as a measure to increase feed efficiency, but also an indirect means to increase muscle mass, thus increasing the sale value of the sheep carcass

(Paula et al., 2013). On the other hand, when measuring sheep DMI and feed efficiency, factoring in fat and muscle depth delivers a 10% improvement in the correlation coefficient for the prediction model (Knott et al., 2008; Redden et al., 2014). Zhang et al. (2017) reported a positive correlation between RFI and BF, with low-RFI lambs having less BF. A positive correlation between RFI and eye muscle area is also reported (Zhang et al., 2017).

While the evidence linking RFI to carcass merit is growing, some investigators reject the idea that any correlation exists between RFI and sheep carcass merit. For instance, a study conducted on fat-tailed Ghezel ram-lambs reports no difference in carcass traits, average daily gain and slaughter weight despite significant differences in the feed intake of low- and high-RFI groups (Rajaei Sharifabadi et al., 2012). The lack of solid evidence and biological knowledge of how RFI and carcass merit are associated may be due to the age and maturity level of the experimental animals, such that ruminants in growing stages have a higher rate of protein synthesis and turnover, while mature animals have greater fat deposition (Herd et al., 2004). Other factors differing between the experimental designs are breed, age, gender, feed type and access of the experimental units.

Pregnancy and Litter Size

Another contributing factor to farm profitability and production efficiency is the rate of pregnancy and the number of lambs born per ewe. Sheep are seasonal breeders and usually deliver more than one litter during the lambing season. The sheep breeding season in Alberta is during the Fall (September to November). The breeding season is affected by daylight, as the amount of light regulates brain function and hormone production in ewes. In a conventional breeding management program, sheep are bred once a year. The breeding cycle (or estrus/heat cycle) in ewes varies depending on the breed and environmental conditions but usually follows a 14 to 18-day cycle.

Each estrus cycle is composed of four phases, including proestrus, estrus, metestrus, and diestrus. Successful breeding requires ewes to be in the second phase of the cycle. This is often referred to as being in estrus or in heat, when they are receptive to the rams and will stand to be bred. The duration in which ewes are in heat lasts approximately 24-36 hours. It is during this time when estrogen (a reproductive hormone) surges, causing release of the egg from the ovary (ovulation). Once ovulation happens, the corpus luteum (CL) forms in the ovary. The CL is a major source of progesterone production (P4; a reproductive hormone). Once the ewe is successfully bred, the CL is maintained to continue producing P4 throughout gestation. Otherwise, the CL regresses and P4 production drops, allowing estrogen to increase and the estrus cycle to restart. Once pregnant, the gestation period lasts approximately 140-160 days.

Detecting pregnancy and the number of fetuses a ewe is carrying at the earliest timepoint of gestation has a profound impact on farm management options. This is because it affects the health and performance of the dam and the progeny. For example, the nutrition of pregnant ewes significantly affects biological programming of amino acid concentrations needed for protein synthesis, skeletal muscle growth, and metabolism of their developing fetus (Torres and Fernanda, 2019). Nutrition of the dam during gestation also yields epigenetic alterations of the fetus, which is yet another determining factor that influences the phenotype of the progeny. Epigenetics is a branch of genomics research which explores heritable mechanisms, such as DNA/RNA methylation, that naturally alter gene expression within the cell or as a result of environmental factors (Barrera-Redondo et al., 2020). The mechanisms that alter gene expression involve chemical compounds and proteins that directly connect to the DNA and which switch the genes on/off. Maternal nutrition affects the nutrient profile of the body, providing substances that trigger

epigenetic changes in the fetus by altering placental efficiency of nutrient transport and directly impacting the weight of the lambs (Song et al., 2020).

Efficient pregnancy management boils down to two major areas: 1) managing pregnant ewes based on their litter size, and 2) identifying ewes that failed to get pregnant, i.e., open ewes. Identifying the latter group as early as possible will allow for separation of open ewes to prevent over-feeding with richer rations formulated for pregnant animals. Early detection of open ewes will also enable the animal to be re-entered into the breeding program, therefore increasing the chances of in-season breeding (during the fall season in the northern hemisphere) due to having higher fertility rates during the season. In addition, pregnant ewes have different dietary requirements based on the number of fetuses they carry (NRC, 2007). Offering feed that meets the nutrient requirements of a pregnancy enables maintenance of maternal health and a reduction of adverse epigenetic alterations on the fetus. This can directly impact maternal pregnancy performance, lamb viability and productivity of the lambs after birth (Wallace, 2011; Benítez et al., 2017).

The gold standard for pregnancy detection in sheep is ultrasonography (Jones and Reed, 2017). Ultrasound detection can also identify the number of fetuses each pregnant ewe carries, but this depends on the experience of the operator and the timepoint in the gestation cycle when the scan is conducted. The optimal time-range for scanning pregnant ewes is after 45 days and before 90 days into gestation (Ishwar, 1995). However, later timepoints are better for litter size detection. Despite its application for pregnancy detection, ultrasonography has limitations, which prevent its widespread use. For example, the number of licensed and skilled ultrasound professionals in the province of Alberta is well below demand, creating a burden on service providers during the breeding season and ultimately limiting the quality and quantity of service they can provide. The

cost of the service ($5-8/ewe in Alberta) is another limiting factor; it would only be a reasonable expense for a larger flock rather than for medium or smaller sized operations. The geographical proximity of the flock and the feasibility of accessing it are also factors limiting the application of pregnancy ultrasound.

Indirect Measures of RFI, Carcass Merit and Pregnancy Status

As described above, the direct measurement of RFI, carcass merit and pregnancy status all require expensive equipment, lengthy trials, scientific knowledge or technical experience to collect and process the data. These issues limit direct measurements of these traits on most sheep farms. However, indirect measurement of these traits is an alternative option that is relatively more feasible and less expensive. One method for indirect measurement involves measuring metabolic or metabolomic biomarkers via metabolomics. Biomarkers are cellular or molecular (gene, protein or metabolite) proxies of a trait that can be objectively and accurately measured. Metabolite biomarker detection in readily accessible biofluids, such as blood, offers a simple and potentially inexpensive route for the early measurement of these performance traits. Furthermore, studies with other livestock species have shown that metabolite markers do exist that ascertain RFI, carcass quality and pregnancy status (Wang and Kadarmideen, 2019; Connolly et al., 2019; Guo and Tao, 2018; Gómez et al., 2020). Unfortunately, in the field of sheep biology, there appears to be no published research using metabolomics to predict or characterize RFI in sheep. Likewise, there appears to be no published research using metabolite markers to predict or quantify sheep carcass quality. On the other hand, there are a few molecular biomarker options for pregnancy detection in sheep. Test kits are commercially available to identify blood hormones (i.e. metabolites) or macromolecules (i.e. proteins) associated with sheep pregnancy (Steckeler et al., 2019). Most of these require samples to be sent to a centralized lab which can lead to days or weeks of delays.

Some of the blood-associated biomarkers that are tested include a pregnancy-associated glycoprotein that can be measured in the blood by day 30 of pregnancy (Khan et al., 2020). The pregnancy specific protein B is another breed-specific blood molecule that is indicative of pregnancy and litter size (Pickworth et al., 2020). A range of small molecule hormones, such as P4 and estradiol, have also been used to make inferences about sheep pregnancy after day 50 of gestation (Sumaryadi and Manalu, 1999; Roberts et al., 2017). Outside of these steroid hormones, other small molecule metabolites have never been identified as proxies of sheep pregnancy. Given the paucity of metabolomic studies on sheep to measure feed efficiency, carcass merit, and pregnancy status, we decided that this would be a potentially fruitful area of research. Another reason why we sought to evaluate these traits was because of valuable feedback from our network of sheep farmers. These include the Alberta Lamb Producers, the Alberta Sheep Breeders' Association, the Ontario Sheep Farmers, and the Canadian Sheep Breeders' Association–all of whom guided us towards the needs and priorities for the industry. A detailed description of metabolomics and its applications follows in section 1.4. and in chapter 2 of this thesis.

Metabolomics

Application of Metabolomics in Other Fields

Metabolomics is an emerging field of systems biology which focuses on characterizing small molecule metabolites (with a molecular weight of <1500 Da) in biological samples or organisms (Wishart et al., 2007). These small molecules, or metabolites, include a wide range of compounds such as amino acids, fatty acids, minerals and vitamins. The collection of all metabolites in a biological sample or a given organism is referred to as "the metabolome" such as the blood metabolome (Psychogios et al., 2011). Metabolites are sometimes referred to as the "canaries" of the genome; just as canaries would alarm the coalminers of environmental dangers in the mine,

metabolites offer early warning signals or serve to amplify the signals arising from alterations in the genome, by the environment (Wishart, 2012).

Over the past two decades metabolomics has gained a significant amount of attention through its applications to biomedical research and clinical testing (Watkins and German, 2002; Vinayavekhin et al., 2010; Jalali et al., 2016). Newborn screening (which involves the detection by mass spectrometry of up to 30 different biomarker metabolites in the blood of newborn babies) is perhaps the best example of how metabolomics technologies have been adopted into routine clinical testing (López-Hernández et al., 2020). The glucose test kit, which uses a few drops of blood to target glucose for diabetes detection, or the pregnancy dip-stick test, which uses urine to detect pregnancy are other well-known examples of clinical application of metabolomics (Trivedi et al., 2017). Human reproductive performance has also benefited from metabolomics through the identification of potential male infertility biomarkers for clinical diagnosis (Minai-Tehrani et al., 2015) and by improving assisted reproductive therapy in women of different ages (Dogan et al., 2020; Zhang et al., 2020). Comprehensive characterizations of maternal biofluids to correlate with pregnancy outcome and postpartum health of infants is another active realm of metabolomics research (McKeating et al., 2019; Yang et al., 2020).

Extensive metabolomics research is also developing around nutrition and food science (Rådjursöga et al., 2018; Hosking et al., 2019; Kirchberg et al., 2019). Metabolite biomarkers of food or food metabolism are becoming more relevant to evaluate the consumption of different grains, sugars, animal proteins and alcoholic beverages (Bertram and Jakobsen, 2018; Mung and Li, 2019; Clarke et al., 2020). Other areas of agricultural sciences are also adopting metabolomics, particularly in environmental monitoring and crop plant assessments (Wishart, 2008; Kim et al., 2016). Multiple metabolomics technologies have been used to assess the diverse environmental

conditions for optimizing the growth and quality of plant products (Abreu et al., 2018; Saia et al., 2019; Deng et al., 2020). Plant breeders have used metabolomics technologies to screen for crop yield and measure metabolic alterations in response to environmental stress (Razzaq et al. 2019). For example, different cultivars of maize were exposed to various abiotic stressors, such as drought and heat, to identify significant metabolites and biological pathways that were altered due to these stresses (Obata et al., 2015). Metabolomics has also been used to understand plant pathology and how plant pathogens initiate plant resistance and alter various secondary metabolic pathways and secondary metabolites (Warth et al., 2015). For instance, different tomato cultivars were evaluated using mass spectrometry to investigate how the fruit and different parts of the plant are susceptible to infections by *Salmonella enterica* (Han and Micallef, 2016). In recent years, the metabolomes of a number of different grains have been investigated to identify predictive biomarkers of plant production and plant/seed quality (Abbiss et al., 2020). Metabolomics has also been extended to environmental screening for the identification and quantification of pollutants such as lead (Luo et al., 2020) and other heavy metals, especially in aquatic environments (Yanagihara et al., 2018).

The metabolomes of many different human and other mammalian biofluids and tissues have been analyzed by a number of groups. Some interesting samples explored to date include tear or lachrymal fluid (Yazdani et al., 2019), sweat (Harshman et al., 2019), cerebropsinal fluid (Wishart et al., 2008), breath (Maniscalco et al., 2018) and various body tissues (Foroutan et al. 2020). Biofluids such as blood, urine, and saliva seem to be among the best explored sample types (Zhang et al., 2012; Goldansaz et al., 2017; Giskeødegård et al., 2018). The blood metabolome is a superb indicator of most physiological changes in the body because it permeates all organs and therefore collects informative compounds from all tissues (Psychogios et al., 2011). Blood and its derivatives, i.e., serum and plasma, are among the most commonly measured samples in

metabolomics as these biofluids are particularly rich in metabolites. The mammalian serum/plasma metabolome contains more than 4200 metabolites (Psychogios et al., 2011; Goldansaz et al., 2017). The serum/plasma metabolome is often investigated in many mammalian species to detect various diseases and to identify metabolites that can serve as predictive biomarkers (Wikoff et al., 2009; Reinehr et al., 2014). Another important feature of blood is that it can be relatively easily drawn in adequate quantities, especially from livestock species.

Metabolomics Technologies

The two most common chemical analysis methods used in metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. In the following sections I will briefly describe the general principles behind NMR spectroscopy, MS, inductively coupled plasma-MS and how they are used in metabolomic studies. For each of these platforms I will also describe their particular strengths and weaknesses.

NMR for Metabolomics

Historically, NMR was the first chemical analysis technique used in metabolomics/metabonomics, however, MS (especially LC-MS) has become the predominant technique over the past 10 years. An NMR spectrometer consists of a large magnet (usually a superconducting magnet) with a radio-frequency generator/receiver on the outside of the magnet connected to a radio-frequency transceiver (called a probe) mounted inside the magnet. Samples are placed in thin glass tubes that are dropped (carefully) inside the magnet and onto the probe. When an organic substance is placed in a strong magnetic field, it becomes susceptible to the absorption of radio frequency radiation. In modern NMR spectrometers computers control the transmission and collection of the radio frequency radiation and the conversion of those signals into readable NMR spectra. Simply stated, NMR measures the response of atomic nuclei to radio-frequency perturbations under strong

magnetic fields. These lead to detectable absorptions (resonances) that occur in certain NMR-susceptible nuclei such as hydrogen (^{1}H), carbon (^{13}C), nitrogen (^{15}N) and phosphorus (^{31}P). These absorption bands appear as peaks in an NMR spectrum and their position is called a chemical shift. The peak positions (chemical shifts), intensity (number of nuclei) and peak splitting patterns (coupling constants) are often sufficient to fully determine the structure of a molecule (Figure 6). In NMR-based metabolomics, the separation of peaks due to chemical shift differences among different molecules means that the analysis of chemical mixtures by NMR does not require chromatographic or electrophoretic separation or chemical derivatization. This makes NMR-based metabolomics somewhat faster and easier than MS-based metabolomics. Once acquired, an NMR spectrum needs to be calibrated (chemical shift referencing, phasing and baseline correction) relative to an internal chemical shift standard. These internal chemical shift standards are also added to the sample as concentration references for quantification purposes (Foroutan et al., 2019). In NMR-based metabolomics, the identity and concentration of each metabolite in a biofluid or extract is determined by matching the chemical shifts (X-axis position) and the intensity (Y-axis position) of the observed spectral peaks against those of pure reference chemical standards (found in a computer library). This NMR spectral deconvolution process can be done automatically or semi-automatically through a number of commercial or open source programs (Wishart, 2008b; Emwas et al., 2019).

Identification of metabolites with NMR has both advantages and disadvantages relative to other analytical platforms. A disadvantage of NMR is its low sensitivity compared to MS-based platforms. NMR can typically detect metabolites in the micromolar range with the lower limit of detection being 1-5 μM (Pinu et al., 2019). This low sensitivity means that an NMR spectrometer is only able to detect and quantify between 40 to 70 metabolites, depending on the type of sample

analyzed and the method of spectrum collection used (Foroutan et al., 2019). Another drawback of NMR is the higher sample volume requirement. Typically, a sample of 100-500 µl is required for analysis (Wishart, 2009; Foroutan et al., 2019). On the other hand, NMR is very reliable and yields consistent and reproducible results when running the same sample multiple times (Pinu et al., 2019). With NMR, it is much easier to quantify metabolites than other analytical platforms, and sample preparation and analysis requires no chemical derivatization (Wishart, 2009). Among other features, NMR is suitable for the identification of organic acids, alcohols, amines, and sugars that are not easily detected or ionized by MS-based instruments (Foroutan et al., 2019).

MS for Metabolomics

Mass spectrometry is a chemical analysis method that has been used for more than a century to measure the mass-to-charge ratio (or m/z) of molecules or atoms. In MS, the molecules or atoms of interest must be ionized and these ions may exist in positive and/or negative states (this is done by switching the solvent type or ions in the sample between acid and base). In many cases the molecules of interest will acquire extra protons (H+), or metal ions (Na+ or K+) or will lose protons (H-) as part of the ionization process. The main molecular ionization methods used in metabolomics are electron ionization (EI), electrospray ionization (ESI), atmospheric pressure ionization (API), matrix-assisted laser desorption ionization (MALDI), although many other ionization methods are now emerging. Hard ionization techniques (such as EI) lead to extensive fragmentation of the molecules. Soft ionization techniques (such as ESI, API and MALDI) lead to the ionization of intact molecular ions. These molecular ions may be further fragmented by coupling another mass spectrometer to the first MS instrument to produce a tandem mass (MS/MS) spectrometer system. Detailed information about a molecule (such as molecular formula and molecular structure) can be acquired both from the (accurate) measured mass of molecular ion as

well as the m/z of the fragment ions. Indeed, it is through the detailed analysis of EI-MS or MS/MS spectra that compounds in complex mixtures can be identified. While it is possible to collect and analyze MS spectra of complex biological samples via direct injection mass spectrometry (called DI-MS), most MS methods used in metabolomics incorporate at least one or several chemical separation steps prior to injecting the sample into the mass spectrometer. This is done to reduce the complexity of the mixture while at the same time increasing the sensitivity and enhancing the ability of the MS instrument to detect individual metabolites. The most common chemical separation methods are gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE). Each of these methods separates molecules over time and space on the basis of their physiochemical properties. Separation of molecules by GC is based on their boiling point, mass, polarizability and molecular shape. Separation of molecules by LC is based on hydrophobicity, charge and size while CE separates molecules primarily on the basis of charge. The basic principle of chromatography (and electrophoresis) is that different chemicals move at different speeds through a column or tube under specific conditions. As a result, different molecules have distinct and uniquely characteristic retention times (the time it takes for a molecule to reach the detector from the chromatographic system entrance; Figure 7).

The sensitivity of metabolite detection is greatly improved when using MS-based analytical platforms such as LC-MS (Figure 4), relative to other techniques such as NMR (Pinu et al., 2019). Using LC-MS/MS platforms, one can expect up to a thousand-fold increase in the sensitivity of metabolite detection over NMR. On the other hand, due to the wide variety of separation, columns used LC-MS, metabolite identification using specific retention times/indices for each metabolite is practically impossible (Dunn, 2008). Moreover, LC-MS does a relatively poor job of detecting many polar compounds present in biofluid samples (Halket et al., 2005). Despite their increased

sensitivity, MS-based instruments have lower reproducibility of results and rely heavily on the precision of a locally maintained (lab-specific) MS library to accurately identify the metabolites (Kind et al., 2018).

ICP-MS for Metabolomics

Inductively Coupled Plasma (ICP)-MS is a specialized mass spectrometry instrument designed for the detection and quantification of non-organic molecules, specifically metals, trace elements and their corresponding isotopes (Godfrey and Glass, 2011). Similar to an LC-MS, the ICP-MS instrument has two main components: an ICP component, which ionizes the compounds in the sample, and an MS component which detects the masses, as previously described. The ICP system consists of an electromagnetic coil and a source of gas (helium and argon) which is used to heat the sample to a very high temperature. The samples used for ICP-MS must initially be in a liquid form, which is then vaporized and converted to a plasma by heating the sample up to 10,000 ºC. This leads to the formation of positively charged ions (Singh, 2016). These ions are then passed into a vacuum tube (via standard MS electrodes) and detected in the MS component of the instrument based on their m/z ratio. The resulting spectrum is relatively straightforward to interpret as there are relatively few ion types (typically less than 30) that are produced and detected, and most have very distinct, non-overlapping m/z ratios. For quantification of each metal ion detected, a calibration curve must be generated for each element being measured. The calibration curve is constructed based on standard solutions of pure metal ion salts that cover the concentration range expected for each given element. Internal standards are another core component of the sample preparation, which assist in the calculation of a more accurate concentration by correcting for possible matrix effects and instrument drift.

Even though ICP-MS is relatively new to metabolomics, it provides great opportunities to detect and quantify many important metal ions, as well trace elements and their associated isotopes (Beauchemin, 2010) in different biological samples. Similar to other MS-based analytical platforms (Figure 5), ICP-MS offers very high sensitivity and can detect concentrations of metals in the femtogram level. These characteristics have made ICP-MS the go-to instrument for the identification and quantification of metal elements and their isotopes. Spectral analysis and quantification in ICP-MS is also easier than most other MS instruments. On the other hand, sample analysis with ICP-MS can be skewed due to matrix effects, meaning that diluted samples are preferred. Low concentration samples also assist with some of the hardware imperfections in ICP-MS by preventing clogging of its different components, such as the nebulizer and the cones.

Metabolomic Studies of Sheep

The application of metabolomics to sheep research has been relatively absent until the past five years. Published sheep metabolomics studies during this timeframe have investigated 12 different sheep breeds and the influence of metabolism on sheep products like meat quality (Wang et al., 2020; Li et al., 2020c) and milk composition (Caboni et al., 2017; Caboni et al., 2019), nutritional requirements and feed management (Palma et al., 2016; Zhang et al., 2019; Li et al., 2020b), metabolism during pregnancy (Guo et al., 2020; Sun et al., 2017), and animal disease (De Moraes Pontes et al., 2017). A handful of metabolomics studies used sheep as an animal model to study human diseases such as Huntington's disease (Skene et al., 2017) and myelomeningocele (Ceccarelli et al., 2015). The samples evaluated in these studies include urine (Guo et al., 2020), serum/plasma (Sun et al., 2017; De Moraes Pontes et al., 2017; Zhang et al., 2019), ruminal fluid (Li et al., 2020b), milk (Caboni et al., 2017; Caboni et al., 2019), and different tissues such as liver and muscle (Palma et al., 2016; Wang et al., 2020). The majority of sheep metabolomic studies

have been done on MS-based platforms and a few have used NMR instruments. Metabolomics studies on sheep meat have identified hundreds of metabolites that can differentiate meat quality based on animal management (Li et al., 2020c) and the origin of feed provided to the animals (Wang et al., 2020). Metabolomics coupled with other omics platforms have also been used to identify how the rumen microbiome impacts essential amino acids required for microbial metabolism. This study identified the most stable class of metabolites in the rumen (short chain fatty acids) and determined how animal age affects the rumen metabolome (Li et al., 2020b). Metabolomics of ewe pregnancy is another field that has gained some recent attention. One study reported that feed restriction (leading to malnutrition during the third trimester of gestation) can alter more than a dozen urine metabolites in pregnant ewes (Guo et al., 2020). Another study researched the kinetics of the serum metabolome in the first half of gestation and identified 13 metabolites associated with amino acid and lipid metabolism significantly involved in meeting the nutritional requirements of pregnant ewes (Sun et al., 2017). Sheep feed has been another focus of metabolomics research. In one recent study, researchers identified 15 significant metabolites involved in fatty acid oxidation, bile acid biosynthesis, purine and protein metabolism that were associated with reduced metabolism and immunity in overgrazing sheep (Zhang et al., 2019). In undernourished sheep however, metabolomics revealed that malnutrition tolerance of different breeds involves significant alterations in their amino acid and energy metabolism pathways (Palma et al., 2016).

Metabolomics offers a plethora of opportunities to complement methods used in sheep research. In human health research, a similar approach has been used to implement metabolomics to unravel complex clinical phenotypes associated with critical diseases such as prostate (Sreekumar et al., 2009) and ovarian cancer (Denkert et al., 2006). Similarly, in sheep research,

metabolomics can assist by measuring the metabotype of animals that possess genetic superiority for important economic traits. In concept, expression of any trait is a result of complex intersections between genetics and the environment; metabolomics can be used to quantify the resulting outcome of this intersection in the progeny. For example, low RFI dairy cows have reduced somatic cell count (SCC) and lower concentration of β-hydroxybutyrate (BHB) compared to high RFI animals (Hailemariam et al., 2020). Conventional practices for measuring the inheritance of RFI requires data collection and genotyping of the daughters (VandeHaar et al., 2016). Phenotypic recording of the progeny requires a relatively extensive timeframe and data collection, and the accuracy of information provided improves as the number of daughters increases (Manafiazar et al., 2016). Progeny genotyping also provides valuable information on the transfer of single nucleotide polymorphisms associated with RFI (Salleh et al., 2018). Concurrently, supplementing the genomics and phenotype data from the daughters with metabolomics data relevant to SCC and BHB would likely improve the accuracy of measuring heritability of RFI, performance of the daughters for RFI, and identify underpinning biological pathways associated with SCC and BHB.

Unfortunately, in the field of sheep biology, there appears to be no published research using metabolomics to predict or characterize economic traits that are the backbone to sheep production, such as RFI (as a measure of feed efficiency), carcass quality or pregnancy status. A number of small molecule hormones such as P4 and estradiol have been used to assess sheep pregnancy (Sumaryadi and Manalu, 1999; Roberts et al., 2017). Outside of these steroid hormones, other small molecule metabolites have never been identified as proxies of sheep pregnancy or litter size. Given the paucity of sheep metabolomic studies measuring feed efficiency, carcass merit, and pregnancy status, and given the need to find cheaper, faster and less time-consuming “indirect”

methods for detecting these traits in sheep, I decided to explore the possibility of using metabolomics to enable these measurements. Specifically, I decided to use metabolomics to identify and validate candidate blood biomarkers of sheep feed efficiency, carcass merit, and reproductive performance.

Thesis Hypotheses

My central hypothesis in this thesis was that the sheep serum metabolome can be used to identify candidate biomarkers of key economic traits in sheep. The specific hypotheses for each research chapter presented in this thesis are:

1) Serum metabolite biomarkers can be identified to categorize growing ram-lambs based on their residual feed intake (high versus low RFI) and carcass merit.
2) Serum metabolite biomarkers can be identified to determine ewe pregnancy and litter size at early stages of gestation.

Thesis Outline

The research presented in this thesis work is composed of five chapters. The first chapter (the current chapter) provides some historical context and additional background regarding sheep livestock research, backbone traits that are essential to sheep production and farming profitability. This chapter also provides a general background on metabolomics by covering different analytical platforms used in my thesis research. I have bridged these two fields by discussing the previous literature on sheep metabolomics.

The second chapter covers the application of metabolomics to bovine, ovine, caprine, equine and porcine settings. In this chapter, I discuss how livestock metabolomics has evolved and also identify a number of trends and gaps, as well as some new opportunities for implementing metabolomics in livestock research. The relevant data were compiled in a publicly accessible

database, the Livestock Metabolome Database (LMDB; www.lmdb.ca), which was designed to serve as a hub to facilitate livestock metabolomic studies. This chapter was published in PloS One and has gained nearly 100 citations since its publication (Goldansaz, S. A., A. C. Guo, T. Sajed, M. A. Steele, G. S. Plastow, and D. S. Wishart. 2017. Livestock metabolomics and the livestock metabolome: A systematic review. PLoS ONE. 12:e0177675. https://doi.org/10.1371/journal.pone.0177675). To the best of our knowledge, this is the first publication to systematically summarize the state of livestock metabolomics and assemble the relevant metabolomics data into a comprehensive database.

The third chapter describes the use of metabolomics to detect biomarkers of sheep RFI. This project used a comparative design study where direct RFI measurements were performed in parallel with serum metabolomic measurements to identify relevant candidate biomarkers for determining RFI. In this project, we collected blood from 165 ram-lambs at a single timepoint and used three analytical platforms (NMR, DI/LC-MS/MS and ICP-MS) to measure 161 unique serum metabolites. One hundred of these metabolites were never previously reported in the sheep metabolome. We also identified three candidate biomarkers associated with variation in RFI in sheep (isopropyl alcohol, aminoadipic acid and acetone). The other component of this chapter explores the use of serum biomarkers to assess sheep carcass quality. There are no reports on marker-assisted pre-mortem evaluation of sheep carcass traits using metabolomics, therefore we sought to see if this was possible. We also followed a comparative design in this component where post-hoc measurements of carcass yield gain and muscle to bone ratio were performed to compare with the serum metabolome to identify candidate biomarkers of carcass merit. Indeed, we revealed seven candidate biomarkers of carcass yield grade (total dimethylarginine, citric acid, hypoxanthine, hippuric acid, asymmetric dimethylarginine, L-phenylalanine, and SM C16:1), and

one candidate biomarker for muscle to bone ratio (lysoPC a C26:1) in sheep. This chapter has been published in the Journal of Animal Science (Goldansaz, S. A., S. Markus, M. Berjanskii, M. Rout, A. C. Guo, Z. Wang, G. Plastow, and D. S. Wishart. 2020. Candidate serum metabolite biomarkers of residual feed intake and carcass merit in sheep. J. Anim. Sci. 98:skaa298. https://doi.org/10.1093/jas/skaa298).

The fourth chapter describes the identification of serum biomarkers of sheep pregnancy and litter size. This chapter used a longitudinal experimental design in two phases (discovery and validation) with samples collected from sheep farms in Alberta and Ontario (total of 486 ewes throughout the project). In the discovery phase, blood was drawn at five timepoints during the pregnancy period from non-pregnant ewes, ewes that delivered a single lamb and those that delivered multiplets (twins, triplets, quadreplets). The discovery phase of this project revealed metabolite trends in the serum metabolome of pregnant ewes in the first 70 days of gestation. Through this process we profiled (identified and quantified) a total of 132 serum metabolites using two analytical platforms (NMR and DI/LC-MS/MS). We also identified four panels of serum candidate biomarkers that could detect sheep pregnancy and litter size as early as 50 days into gestation. The second phase of this project involved validating the candidate biomarkers using the discovery data as the reference (for both the metabolites and the optimal detection timepoint). We were able to replicate initial results and confirm the biomarkers and confirm that the day 50 timepoint is the best date to reveal if an ewe is pregnant and to determine the number of lambs carried. This research will be submitted to a scientific journal in the near future.

The fifth chapter concludes the thesis by summarizing its achievements and suggesting a roadmap for future research in this field. I have also shared my thoughts on how to design a robust experiment for the successful execution of livestock metabolomics research. Moreover, based on

my observations and experience with the Canadian and Albertan sheep industry, I have identified some of the gaps in the production system that could potentially be addressed with metabolomics research.

Literature Cited

1. Abbiss, H., J. P. A. Gummer, M. Francki, and R. D. Trengove. 2020. Untargeted liquid chromatography-mass spectrometry-based metabolomics analysis of wheat grain. J. Vis. Exp. 157:10.3791/60851. doi:10.3791/60851.
2. Abreu, A. C., L. M. Aguilera-Sáez, A. Peña, M. García-Valverde, P. Marín, D. L. Valera, and I. Fernández. 2018. NMR-based metabolomics approach to study the influence of different conditions of water irrigation and greenhouse ventilation on zucchini crops. J. Agricul. Food. Chem. 66:8422-8432. DOI: 10.1021/acs.jafc.8b02590.
3. Arthur, P. F., and R. M. Herd. 2005. Efficiency of feed utilization by livestock — Implications and benefits of genetic improvement. Can. J. Anim. Sci. 85(3): 281-290. https://doi.org/10.4141/A04-062.
4. Beauchemin, K. A. 2009. Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture. Vet. Sci. Nutr. Nat. Res. 4:35. doi: 10.1079/PAVSNNR20094035.
5. Barrera-Redondo, J., D. Piñero, and L. E. Eguiarte. 2020. Genomic, transcriptomic and epigenomic tools to study the domestication of plants and animals: a field guide for beginners. Fron. Gen. 11:742. https://doi.org/10.3389/fgene.2020.00742.
6. Basarab, J. A., M. A. Price, J. L. Aalhus, E. K. Okine, W. M. Snelling, and K. L. Lyle. 2003. Residual feed intake and body composition in young growing cattle. Can. J. Anim. Sci. 83:189-204. https://doi.org/10.4141/A02-065.
7. Benítez, R., Y. Núñez, and C. Óvilo. 2017. Nutrigenomics in farm animals. J. Invest. Genomics. 4:00059. DOI:10.15406/jig.2017.04.00059.

8. Bertram, H. C., and L. M. A. Jakobsen. 2018. Nutrimetabolomics: integrating metabolomics in nutrition to disentangle intake of animal-based foods. Metabolomics. 14:34. https://doi.org/10.1007/s11306-018-1322-3.
9. Caboni, P., C. Manis, I. Ibba, M. Contu, V. Coroneo, and P. Scano. 2017. Compositional profile of ovine milk with a high somatic cell count: A metabolomics approach. Int. Dairy J. 69:33-39. https://doi.org/10.1016/j.idairyj.2017.02.001.
10. Caboni, P., A. Murgia, A. Porcu, C. Manis, I. Ibba, M. Contu, P. Scano. 2019. A metabolomics comparison between sheep's and goat's milk. Food Res. Int. 119:869-875. https://doi.org/10.1016/j.foodres.2018.10.071.
11. Ceccarelli, G., E. Pozzo, F. Scorletti, L. Benedetti, G. Cusella, et al. 2015. Molecular signature of amniotic fluid derived stem cells in the fetal sheep model of myelomeningocele. J. Pedia. Surg. 50:1521-1527, ISSN 0022-3468, https://doi.org/10.1016/j.jpedsurg.2015.04.014.
12. Clarke, E. D., M. E. Rollo, K. Pezdirc, C. E. Collins, and R. L. Haslam. 2020. Urinary biomarkers of dietary intake: a review. Nutr. Rev. 78:364-381. doi:10.1093/nutrit/nuz048.
13. Cockrum, R. R., R. H. Stobart, S. L. Lake, and K. M. Cammack. 2013. Phenotypic variation in residual feed intake and performance traits in rams. Sm. Rumin. Res. 113:313-322. https://doi.org/10.1016/j.smallrumres.2013.05.001.
14. Connolly, S., A. Dona, L. Wilkinson-White, D. Hamblin, M. D'Occhio, and L. A. González. 2019. Relationship of the blood metabolome to subsequent carcass traits at slaughter in feedlot Wagyu crossbred steers. Sci. Rep. 9:15139. https://doi.org/10.1038/s41598-019-51655-2.
15. Crews, D. H. 2005. Genetics of efficient feed utilization and national cattle evaluation: a review. Genet. Mol. Res. 4:152-165. https://europepmc.org/article/med/16110437.

16. Daley, C. A., A. Abbott, P. S. Doyle, G. A. Nader, and S. Larson. 2010. A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. Nutr. J. 9:10. https://doi.org/10.1186/1475-2891-9-10.

17. De Moraes Pontes, J. G., F. B. De Santana, R. W. Portela, V. Azevedo, R. J. Poppi, and L. Tasic. 2017. Biomarkers of the Caseous Lymphadenitis in sheep by NMR-based metabolomics. Metabolomics. 7:2. DOI: 10.4172/2153-0769.1000190.

18. Deng, M., X. Zhang, J. Luo, H. Liu, W. Wen, H. Luo, J. Yan, and Y. Xiao. 2020. Metabolomics analysis reveals differences in evolution between maize and rice. Plant J. 103:1710-1722. doi:10.1111/tpj.14856.

19. Denkert, C., J. Budczies, T. Kind, W. Weichert, P. Tablack, J. Sehouli, S. Niesporek, D. Konsgen, M. Dietel, and O. Fiehn. 2006. Mass spectrometry-based metabolic profiling reveals different metabolite patterns in invasive ovarian carcinomas and ovarian borderline tumors. Cancer Res. 66:10795–10804. DOI: 10.1158/0008-5472.CAN-06-0755.

20. Dogan, B., A. Karaer, G. Tuncay, N. Tecellioglu, and A. Mumcu. 2020. High-resolution 1H-NMR spectroscopy indicates variations in metabolomics profile of follicular fluid from women with advanced maternal age. J. Assist. Reprod. Genet. 37:321–330. https://doi.org/10.1007/s10815-020-01693-x

21. Dunn, W. B. 2008. Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes. Phys. Biol. 5:011001. doi:10.1088/1478-3975/5/1/011001.

22. Emwas, A.-H., R. Roy, R.T. McKay, L. Tenori, E. Saccenti, et al. 2019. NMR spectroscopy for metabolomics research. Metabolites. 9:123. https://doi.org/10.3390/metabo9070123.

23. FAO statistical pocketbook: World food and agriculture. 2015. ww.fao.org/publications.

24. FAO. 2017. The future of food and agriculture – trends and challenges. Rome, Italy: FAO. http://www.fao.org/3/y4252e/y4252e07.htm.

25. FAO. 2018. http://www.fao.org/faostat/en/#data/QL.

26. FAOSTAT. http://www.fao.org/faostat/en/#data/QA/visualize.

27. Farrell, L. J., P. R. Kenyon, P. R. Tozer, T. Ramilan, and L. M. Cranston. 2020. Quantifying sheep enterprise profitability with varying flock replacement rates, lambing rates, and breeding strategies in New Zealand. Agri. Sys. 184:102888. https://doi.org/10.1016/j.agsy.2020.102888.

28. Fontanesi, L. 2016. Metabolomics and livestock genomics: Insights into a phenotyping frontier and its applications in animal breeding. Anim. Front. 6:73-79. https://doi.org/10.2527/af.2016-0011.

29. Foroutan, A., C. Fitzsimmons, R. Mandal, H. Piri-Moghadam, J. Zheng, A. Guo, C. Li, L. L. Guan, and D. S. Wishart. 2020. The Bovine Metabolome. Metabolites. 10:233. https://doi.org/10.3390/metabo10060233.

30. Foroutan, A., S. A. Goldansaz, M. Lipfert, and D. S. Wishart. 2019. Protocols for NMR analysis in livestock metabolomics. In: Bhattacharya S. (eds) Metabolomics. Methods in Molecular Biology, vol 1996. Humana, New York, NY. https://doi.org/10.1007/978-1-4939-9488-5_23.

31. Garza Hernandez, D., S. Mucha, G. Banos, K. Kaseja, K. Moore, N. Lambe, J. Yates, and L. Bunger. 2018. Analysis of single nucleotide polymorphisms variation associated with important economic and computed tomography measured traits in Texel sheep. Animal. 12: 915-922. doi:10.1017/S1751731117002488.

32. Giskeødegård, G. F., T. S. Madssen, L. R. Euceda, M-B. Tessem, S. A. Moestue, and T. F. Bathen. 2018. NMR-based metabolomics of biofluids in cancer. NMR in Biom. 32:e3927. https://doi.org/10.1002/nbm.3927.

33. Godfrey, L. V., and J. B. Glass. 2011. Research on nitrification and related processes, Part A. Methods in Enzymology. 486:608 page.

34. Goldansaz, S. A., A. C. Guo, T. Sajed, M. A. Steele, G. S. Plastow, and D. S. Wishart. 2017. Livestock metabolomics and the livestock metabolome: A systematic review. PLoS ONE. 12:e0177675. https://doi.org/10.1371/journal.pone.0177675.

35. Gómez, E., P. Salvetti, J. Gatien, S. Carrocera, D. Martín-González, and M. Muñoz. 2020. Blood plasma metabolomics predicts pregnancy in Holstein cattle transferred with fresh and vitrified/warmed embryos produced in vitro. J. Prote. Res. 19:1169-1182. https://doi.org/10.1021/acs.jproteome.9b00688.

36. Guo, Y. S., and J. Z. Tao. 2018. Metabolomics and pathway analyses to characterize metabolic alterations in pregnant dairy cows on D 17 and D 45 after AI. Sci. Rep. 8:5973. https://doi.org/10.1038/s41598-018-23983-2.

37. Guo, C., Y. Xue, Y. Yin, H. Xuan, F. Hu, and S. Mao. 2020. Urine metabolome alterations in malnutrition and the impact of glycerol or rumen-protected choline chloride supplementation in advanced pregnant ewes. Brit. J. Nutri. 123:1258-1268. doi:10.1017/S000711452000063X.

38. Hailemariam, D., G. Manafiazar, J. A. Basarab, P. Stothard, F. Miglior, G. Plastow, and Z. Wang. 2020. Comparative analyses of enteric methane emissions, dry matter intake and milk somatic cell count in different residual feed intake categories of dairy cows. Can. J. Anim. Sci. https://doi.org/10.1139/CJAS-2019-0085.

39. Halket, J. M., D. Waterman, A. M. Przyborowska, R. K. P. Patel, P. D. Fraser, and P. M. Bramley. 2005. Chemical derivatization and mass spectral libraries in metabolite profiling by GC–MS and LC– MS/MS. J. Exp. Botany. 56:219-243. https://doi.org/10.1093/jxb/eri069.

40. Harshman, S. W., R. L. Pitsch, N. M. Schaeublin, Z. K. Smith, K. E. Strayer, M. S. Phelps, A. V. Qualley, D. W. Cowan, S. D. Rose, M. L. O'Connor, J. J. Eckerle, T. Das, A. K. Barbey, A. J. Strang, and J. A. Martin. 2019. Metabolomic stability of exercise-induced sweat. J. Chrom. B. 1126–1127:121763. https://doi.org/10.1016/j.jchromb.2019.121763.

41. Heasman, L., L. Clarke, K. Firth, T. Stephenson, and M. E. Symonds. 1998. Influence of restricted maternal nutrition in early to mid gestation on placental and fetal development at term in Sheep. Pediatr. Res. 44:546–551. https://doi.org/10.1203/00006450-199810000-00013.

42. Herd, R. M., and S. C. Bishop. 2000. Genetic variation in residual feed intake and its association with other production traits in British Hereford cattle. Livest. Prod. Sci. 63:111-119. https://doi.org/10.1016/S0301-6226(99)00122-0.

43. Herd, R. M., V. H. Oddy, and E. C. Richardson. 2004. Biological basis for variation in residual feed intake in beef cattle. 1. Review of potential mechanisms. Aust. J. Exp. Agr. 44:423-430. https://doi.org/10.1071/EA02220.

44. Herd, R. M., and P. F. Arthur. 2009. Physiological basis of residual feed intake. J. Anim. Sci. 87:E64–E71. https://doi.org/10.2527/jas.2008-1345.

45. Hosking, J., J. Pinkney, A. Jeffery, O. Cominetti, L. Da Silva, S. Collino, M. Kussmann, J. Hager, and F. Martin. 2019. Insulin resistance during normal child growth and development is associated with a distinct blood metabolic phenotype (Earlybird 72). Pediatr. Diabetes. 20:832– 841. https://doi.org/10.1111/pedi.12884.

46. Ibrahim, A. H. M. 2019. Estimation of co-variance components, genetic parameters and genetic trends of VIA scan carcass traits of New Zealand Romney lambs. Sm. Rumin. Res. 178:130-136. https://doi.org/10.1016/j.smallrumres.2019.08.016.

47. Ishwar, A. K. 1995. Pregnancy diagnosis in sheep and goats: a review. Sm. Rumin. Res. 17:37-44. https://doi.org/10.1016/0921-4488(95)00644-Z.

48. Jalali, A., A. Hatamie, T. Safarpour, A. Khajeamiri, T. Safa, and F. Buazar. 2016. Impact of pharmaceutical impurities in Ecstasy tablets: gas chromatography-mass spectrometry study. Iran. J. Pharm. Res. 15:221-229. https://search.informit.org/doi/10.3316/informit.883492148347757.

49. Jones, H. E., R. M. Lewis, M. J. Young, and B. T. Wolf. 2002. The use of X-ray computer tomography for measuring the muscularity of live sheep. Anim. Sci. 75:387–399. DOI: https://doi.org/10.1017/S1357729800053169.

50. Jones, S. D. M., W. M. Robertson, M. A. Price, and T. Coupland. 1996. The prediction of saleable meat yield in lamb carcasses. Can. J. Anim. Sci. 76:49-53. https://doi.org/10.4141/cjas96-007.

51. Jones, A. K., and S. A. Reed. 2017. Benefits of ultrasound scanning during gestation in the small ruminant. Sm. Rumin. Res. 149:163-171. https://doi.org/10.1016/j.smallrumres.2017.02.008.

52. Kaliel, D. A. 2004. Insights into Managing Winter Feed Costs in Alberta Cow/calf Operations. URL: http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/econ9538/$FILE/winterfeed.pdf.

53. Karisa, B. K., J. Thomson, Z. Wang, C. Li, Y. R. Montanholi, S. P. Miller, S. S. Moore, and G. S. Plastow. 2014. Plasma metabolites associated with residual feed intake and other

productivity performance traits in beef cattle. Liv. Sci. 165:200–211. https://doi.org/10.1016/j.livsci.2014.03.002.

54. Kelly, A. K., M. McGee, D. H. Crews, A. G. Fahey, A. R. Wylie, and D. A. Kenny. 2010. Effect of divergence in residual feed intake on feeding behaviour, blood metabolic variables, and body composition traits in growing heifers. J. Anim. Sci. 88(1): 109-123. https://doi.org/10.2527/jas.2009-2196.
55. Kenyon, P. R., and H. T. Blair. 2014. Foetal programming in sheep – Effects on production. Sm. Rumin. Res. 118:16-30. https://doi.org/10.1016/j.smallrumres.2013.12.021.
56. Khan, D., H. Khan, A. Nazir, M. T. Tunio, M. Tahir, M. S. Khan, and R. U. Khan. 2020. Early pregnancy diagnosis using pregnancy-associated glycoproteins in the serum of pregnant ruminants. Pakistan J. Zool. 52:785-788. DOI: http://dx.doi.org/10.17582/journal.pjz/20190527080541.
57. Kim, S., J. Kim, E. J. Yun, and K. H. Kim. 2016. Food metabolomics: from farm to human. Curr. Opin. Biotechnol. 37:16-23. https://doi.org/10.1016/j.copbio.2015.09.004.
58. Kind, T., H. Tsugawa, T. Cajka, Y. Ma, Z. Lai, S. S. Mehta, G. Wohlgemuth, D. Barupal, M. Showalter, M. Arita, and O. Fiehn. 2018. Identification of small molecules using accurate mass MS/MS search. Mass. Spec. Rev. 37:513–532. https://doi.org/10.1002/mas.21535.
59. Kirchberg, F. F., V. Grote, D. Gruszfeld, P. Socha, R. Closa-Monasterolo, J. Escribano, E. Verduci, B. Mariani, J. Langhendries, P. Poncelet, B. Koletzko, C. Hellmuth, and European Childhood Obesity Trial Study Group. 2019. Are all breast-fed infants equal? Clustering metabolomics data to identify predictive risk clusters for childhood obesity. J. Pediatr. Gastroenterol. Nutr. 68:408-415. doi:10.1097/MPG.0000000000002184.

60. Knott, S. A., L. J. Cummins, F. R. Dunshea, and B. J. Leury. 2008. The use of different models for the estimation of residual feed intake (RFI) as a measure of feed efficiency in meat sheep. Anim. Feed Sci. Technol. 143:242–255. https://doi.org/10.1016/j.anifeedsci.2007.05.013.

61. Koch, R. M., L. A. Swiger, D. Chambers, and K. E. Gregory. 1963. Efficiency of feed use in beef cattle. J. Anim. Sci. 22:486–494. https://doi.org/10.2527/jas1963.222486x.

62. Leymaster, K. A. 1991. Straightbred comparison of a composite population and the Suffolk breed for performance traits of sheep. J. Anim. Sci. 69:993-999. https://doi.org/10.2527/1991.693993x.

63. Li, B., P. M. VanRaden, E. Guduk, J. R. O'Connell, D. J. Null, E. E. Connor, M. J. VandeHaar, R. J. Tempelman, K. A. Weigel, and J. B. Cole. 2020a. Genomic prediction of residual feed intake in US Holstein dairy cattle. J. Dairy Sci. 103:2477-2486. https://doi.org/10.3168/jds.2019-17332.

64. Li, H., Q. Yu, T. Li, L. Shao, M. Su, H. Zhou, and J. Qu. 2020b. Rumen microbiome and metabolome of Tibetan sheep (Ovis aries) reflect animal age and nutritional requirement. Front. Vet. Sci. 7:609. doi: 10.3389/fvets.2020.00609.

65. Li, J., C. Tang, Q. Zhao, Y. Yang, F. Li, Y. Qin, X. Liu, X. Yue, and J. Zhang. 2020c. Integrated lipidomics and targeted metabolomics analyses reveal changes in flavor precursors in psoas major muscle of castrated lambs. Food Chem. 333:127451. https://doi.org/10.1016/j.foodchem.2020.127451.

66. Lockwood, A., J. Trompf, S. Hancock, L. Kubeil, A. Thompson, and J. Young. 2020. Factors influencing the optimum mob size of ewes at lambing and the economic benefit of lambing ewes in smaller mobs to increase lamb survival across southern Australia. Agri. Sys. 184:102916. https://doi.org/10.1016/j.agsy.2020.102916.

67. López-Hernández, Y., J. J. Oropeza-Valdez, J. O. Blanco-Sandate, A. S. Herrera-Van Oostdam, J. Zheng, et al. 2020. The urinary metabolome of healthy newborns. Metabolites. 10:165. https://doi.org/10.3390/metabo10040165.

68. Luo, X., J. Meng, X. Chen, L. Cheng, S. Yan, L. Gao, M. Xue, and Y. Yang. 2020. Metabolomics-based study reveals the effect of lead (Pb) in the culture environment on Whitmania pigra. Sci. Rep. 10:4794. https://doi.org/10.1038/s41598-020-61745-1.

69. Macfarlane, J. M., R. M. Lewis, G. C. Emmans, M. J. Young, and G. Simm. 2006. Predicting carcass composition of terminal sire sheep using X-ray computed tomography. Anim. Sci. 82:289–300. DOI: 10.1079/ASC200647.

70. Manafiazar, G., L. Goonewardene, F. Miglior, D. Crews, J. Basarab, E. Okine, and Z. Wang. 2016. Genetic and phenotypic correlations among feed efficiency, production and selected conformation traits in dairy cows. Animal. 10:381-389. https://doi.org/10.1017/S1751731115002281.

71. Maniscalco, M., D. Paris, D. Melck, N. Chiariello, F. Di Napoli, M. Manno, I. Iavicoli, and A. Motta. 2018. Biomonitoring of workers using nuclear magnetic resonance-based metabolomics of exhaled breath condensate: A pilot study. Toxico. Let. 298:4-12. https://doi.org/10.1016/j.toxlet.2018.10.018.

72. Maierle, C. L. 2018. Effect of terminal sire breed on feed efficiency in Katahdin crossbred feedlot-finished lambs. J. Anim. Sci. 96:67-68. https://search.proquest.com/openview/695f811ee7e34c2f1cf45be78b9c914e/1?pq-origsite=gscholar&cbl=49113.

73. McKeating, D. R., J. J. Fisher, and A. V. Perkins. 2019. Elemental Metabolomics and Pregnancy Outcomes. Nutrients. 11:73. doi:10.3390/nu11010073.

74. Meyer, A. M., R. A.Vraspir, M. J. Ellison, and K. M. Cammack. 2015. The relationship of residual feed intake and visceral organ size in growing lambs fed a concentrate- or forage-based diet. Livest. Sci. 176:85-90. https://doi.org/10.1016/j.livsci.2015.03.019.

75. Minai-Tehrani, A., N. Jafarzadeh, and K. Gilany. 2015. Metabolomics: a state-of-the-art technology for better understanding of male infertility. Andrologia. 48: 609-616. doi:10.1111/and.12496.

76. Moore, S. S., F. D. Mujibi, and E. L. Sherman. 2009. Molecular basis for residual feed intake in beef cattle. J. Anim. Sci. 87:E41–E47. https://doi.org/10.2527/jas.2008-1418.

77. Morris, S. T. 2009. Economics of sheep production, Sm. Rumin. Res. 86:59-62. https://doi.org/10.1016/j.smallrumres.2009.09.019.

78. Mung, D., and L. Li. 2019. Chemical isotope labeling liquid chromatography mass spectrometry for investigating acute dietary effects of cow milk consumption on human urine metabolome. J. Food. Drug. Anal. 27:565-574. doi:10.1016/j.jfda.2018.10.007.

79. Muro-Reyes, A., H. Gutierrez-Banuelos, L. H. Diaz-Garcia, F. J. Gutierrez-Pina, L. M. Escareno-Sanchez, R. Banuelos-Valenzuela, C. A. Medina-Flores, and A. Corral Luna. 2011. Potential environmental benefits of residual feed intake as strategy to mitigate methane emissions in sheep. J. Anim. Vet. Adv. 10:1551-1556. https://www.cabdirect.org/cabdirect/abstract/20113185769.

80. Norton, M. 2005. Factors affecting beef and cattle producer prices movements. Mont. Lab. Rev. 128:32. https://heinonline.org/HOL/LandingPage?handle=hein.journals/month128&div=52&id=&page=.

81. NRC (National Research Council). 2007. Nutrient requirements of small ruminants: Sheep, goats, cervids and new world camelids. National Academy Press. Washington, D.C.

82. Nkrumah, J. D., E. K. Okine, G. W. Mathison, K. Schmid, C. Li, J. A. Basarab, M. A. Price, Z. Wang, and S. S. Moore. 2005. Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle. J. Anim. Sci. 84:145–153. https://doi.org/10.2527/2006.841145x.

83. Obata, T., S. Witt, J. Lisec, N. Palacios-Rojas, I. Florez-Sarasa, S. Yousfi, J. L. Araus, J. E. Cairns, and A. R. Fernie. 2015. Metabolite profiles of maize leaves in drought, heat, and combined stress field trials reveal the relationship between metabolism and grain yield. Plant Physiol. 169:2665–2683. DOI: https://doi.org/10.1104/pp.15.01164.

84. Pearson H. 2007. Meet the human metabolome. Nature. 446:8. https://doi.org/10.1038/446008a.

85. Pérez, P., M. Maino, G. Tomic, E. Mardones, and J. Pokniak. 2002. Carcass characteristics and meat quality of Suffolk Down suckling lambs. Sm. Rumin. Res. 44:233-240. https://doi.org/10.1016/S0921-4488(02)00076-7.

86. Paisley, S., and K. Cammack. 2010. How far will that pound of feed take you? UW examines feed efficiency. University of Wyoming. College of Agriculture and Natural Resources. Available: http://www.uwyo.edu/uwexpstn/publications/reflections/reflections-2010-web.pdf.

87. Palma, M., T. Scanlon, T. Kilminster, J. Milton, C. Oldham, J. Greeff, M. Matzapetakis, and A. M. Almeida. 2016. The hepatic and skeletal muscle ovine metabolomes as affected by weight loss: a study in three sheep breeds using NMR-metabolomics. Sci. Rep. 6:39120. https://doi.org/10.1038/srep39120.

88. Paula, E. F. E. de, D. F. de Souza, A. L. G. Monteiro, M. H. de A. Santana, S. Gilaverte, P. Rossi Junior, and R. Locatelli Dittrich. 2013. Residual feed intake and hematological and metabolic blood profiles of Ile de France lambs. Rev. Bras. Zoot. 42:806-812. https://dx.doi.org/10.1590/S1516-35982013001100007.

89. Pickworth, C. L., J. Sheffield, and R. K. Poole. 2020. Pregnancy specific protein B concentration for the prediction of lambing rate in sheep. Sm. Rumin. Res. 84:106052. https://doi.org/10.1016/j.smallrumres.2020.106052.

90. Pinu, F. R., S. A. Goldansaz, and J. Jaine. 2019. Translational metabolomics: current challenges and future opportunities. Metabolites. 9:108. doi:10.3390/metabo9060108.

91. Psychogios, N., D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend, and D. S. Wishart. 2011. The human serum metabolome. PLoS ONE. 6:e16957. doi:10.1371/journal.pone.0016957.

92. Rådjursöga, M., H. M. Lindqvist, A. Pedersen, B. G. Karlsson, D. Malmodin, L. Ellegård, and A. Winkvist. 2018. Nutritional metabolomics: postprandial response of meals relating to vegan, lacto-ovo vegetarian, and omnivore diets. Nutrients. 10:1063. doi:10.3390/nu10081063.

93. Rajaei Sharifabadi, H., M. J. Zamiri, E. Rowghani, W. G. Bottje. 2012. Relationship between the activity of mitochondrial respiratory chain complexes and feed efficiency in fat-tailed Ghezel lambs. J. Anim. Sci. 90:1807–1815, https://doi.org/10.2527/jas.2011-4791.

94. Razzaq, A., B. Sadia, A. Raza, M. K. Hameed, and F. Saleem. 2019. Metabolomics: A way forward for crop improvement. Metabolites. 9:303. https://doi.org/10.3390/metabo9120303.

95. Redden, R. R., L. M. M. Surber, B. L. Roeder, B. M. Nichols, J. A. Paterson, and R. W. Kott. 2011. Residual feed efficiency established in a post-weaning growth test may not result in more efficient ewes on the range. Sm. Rumin. Res. 96:155–159. https://doi.org/10.1016/j.smallrumres.2010.12.007.

96. Redden, R. R., L. M. M. Surber, A. V. Grove, and R. W. Kott. 2013. Growth efficiency of ewe lambs classified into residual feed intake groups and pen fed a restricted amount of feed. Sm. Rum. Res. 114:214–219. https://doi.org/10.1016/j.smallrumres.2013.07.002.

97. Redden, R. R., L. M. M. Surber, A. V. Grove, and R. W. Kott. 2014. Effects of residual feed intake classification and method of alfalfa processing on ewe intake and growth. J. Anim. Sci. 92:830–835. https://doi.org/10.2527/jas.2013-6768.

98. Richardson, E. C., R. M. Herd, V. H. Oddy, J. M. Thompson, J. A. Archer and P. F. Arthur. 2001. Body composition and implications for heat production and Angus steer progeny of parents selected for and against residual feed intake. Anim. Pro. Sci. 41:1065–1072. https://doi.org/10.1071/EA00095.

99. Richardson, E. C., and R. M. Herd. 2004. Biological basis for variation in residual feed intake in beef cattle. 2. Synthesis of results following divergent selection. Aus. J. Exp. Ag. 44:431-440. https://doi.org/10.1071/EA02221.

100. Robinson, D. L., J. P. Goopy, A. J. Donaldson, R. T. Woodgate, V. H. Oddy, and R. S. Hegarty. 2014. Sire and liveweight affect feed intake and methane emissions of sheep confined in respiration chambers. Animal. 8:1935–1944. https://doi.org/10.1017/S1751731114001773.

101. Royal Bank of Canada. 2019. https://thoughtleadership.rbc.com/farmer-4-0-how-the-coming-skills-revolution-can-transform-agriculture/.

102. Reinehr, T., B. Wolters, C. Knop, N. Lass, C. Hellmuth, U. Harder, W. Peissner, S. Wahl, H. Grallert, J. Adamski, T. Illig, C. Prehn, Z. Yu, R. Wang-Sattler, and B. Koletzko. 2014. Changes in the serum metabolite profile in obese children with weight loss. Eur. J. Nutr. 54:173-181. https://doi.org/10.1007/s00394-014-0698-8.

103. Roberts, J. N., K. J. May, and A. Veiga-Lopez. 2017. Time-dependent changes in pregnancy-associated glycoproteins and progesterone in commercial crossbred sheep. Therio. 89:271-279. https://doi.org/10.1016/j.theriogenology.2016.10.029.

104. Saia, S., M. Fragasso, P. De Vita, and R. Beleggia. 2019. Metabolomics Provides Valuable Insight for the Study of Durum Wheat: A Review. J. Agr. Food Chem. 67:3069-3085. DOI: 10.1021/acs.jafc.8b07097.

105. Salleh, M. S., G. Mazzoni, M. O. Nielsen, P. Løvendahl, and H. N. Kadarmideen. 2018. Identification of expression QTLs targeting candidate genes for residual feed intake in dairy cattle using systems genomics. J. Genet. Genome Res. 5:035. DOI: 10.23937/2378-3648/1410035.

106. Schenkel, F. S., S. P. Miller, and J. W. Wilton. 2004. Genetic parameters and breed differences for feed efficiency, growth, and body composition traits of young beef bulls. Can. J. Anim. Sci. 84:177–185. https://doi.org/10.4141/A03-085.

107. Scherf, B. D. 2000. World watch list for domestic animal diversity. Ed. 3. Food and Agriculture Organization of the United Nations. Rome. https://www.cabdirect.org/cabdirect/abstract/20013016397.

108. Scholz, A., L. Bünger, J. Kongsro, U. Baulain, and A. Mitchell. 2015. Non-invasive methods for the determination of body and carcass composition in livestock: Dual-energy X-

ray absorptiometry, computed tomography, magnetic resonance imaging and ultrasound: Invited review. Animal. 9:1250-1264. doi:10.1017/S1751731115000336.

109. Shrestha, J. N. B., and D. P. Heaney. 2003. Review of Canadian, Outaouais and Rideau Arcott breeds of sheep: 1. Development and characterization. Sm. Rumin. Res. 49:79-96. https://doi.org/10.1016/S0921-4488(03)00070-1.

110. Silva, S. R. 2017. Use of ultrasonographic examination for in vivo evaluation of body composition and for prediction of carcass quality of sheep. Sm. Rumin. Res. 152:144-157. https://doi.org/10.1016/j.smallrumres.2016.12.020.

111. Singh, A. K. 2016. Experimental methodologies for the characterization of nanoparticles. Eng. Nano. pp.125-170. DOI: 10.1016/B978-0-12-801406-6.00004-2.

112. Skene, D., B. Middleton, C. Fraser, J. L. A. Pennings, T. R. Kuchel, S. R. Rudiger, C. S. Bawden, and A. J. Morton. 2017. Metabolic profiling of presymptomatic Huntington's disease sheep reveals novel biomarkers. Sci. Rep. 7:43030. https://doi.org/10.1038/srep43030.

113. Song, W., M. Puttabyatappa, L. Zeng, D. Vazquez, S. Pennathur, and V. Padmanabhan. 2020. Developmental programming: Prenatal bisphenol a treatment disrupts mediators of placental function in sheep. Chemosphere. 243:125301. https://doi.org/10.1016/j.chemosphere.2019.125301.

114. Sun, L., Yixuan Guo, Y. Fan, H. Nie, R. Wang, and F. Wang. 2017. Metabolic profiling of stages of healthy pregnancy in Hu sheep using nuclear magnetic resonance (NMR). Therio. 92:121-128. https://doi.org/10.1016/j.theriogenology.2017.01.025.

115. Spring, P. 2013. The challenge of cost effective poultry and animal nutrition: optimizing existing and applying novel concepts. Lohm. Info. 48:38-47. https://www.lohmann-information.com/content/l_i_48_artikel5.pdf.

116. Sreekumar, A., L. M. Poisson, T. M. Rajendiran, A. P. Khan, Q. Cao, J. Yu, B. Laxman, R. Mehra, R. J. Lonigro, Y. Li, M. K. Nyati, A. Ahsan, S. Kalyana-Sundaram, B. Han, X. Cao, J. Byun, G. S. Omenn, D. Ghosh, S. Pennathur, D. C. Alexander, A. Berger, J. R. Shuster, J. T. Wei, S. Varambally, C. Beecher, and A. M. Chinnaiyan. 2009. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature. 457:910–914.

117. Statistics Canada. Table 32-10-0425-01. Sheep and lambs on census day. https://doi.org/10.25318/3210042501-eng.

118. Statistics Canada. Table 32-10-0129-01. Number of sheep and lambs on farms (x 1,000). https://doi.org/10.25318/3210012901-eng.

119. Steckeler, P., F. Weber, H. Zerbe, A. Rieger, K. Voigt. 2019. Evaluation of a bovine visual pregnancy test for the detection of pregnancy-associated glycoproteins in sheep. Repro. Dom. Anim. 54:280–288. https://doi.org/10.1111/rda.13356.

120. Sumaryadi, M., and W. Manalu. 1999. Prediction of litter size based on hormones and blood metabolites concentrations during pregnancy in Javanese thin-tail ewes. Asian-Aus. J. Anim. Sci. 12:682-688. https://doi.org/10.5713/ajas.1999.682.

121. Torres, S., and C. Fernanda. 2019. Effect of maternal nutrient restriction on fetal skeletal muscle growth and metabolism in sheep. Doctoral dissertation, Texas A&M University. http://hdl.handle.net/1969.1/186572.

122. Trivedi, D. K., K. A. Hollywood, and R. Goodacre. 2017. Metabolomics for the masses: The future of metabolomics in a personalized world. New Hor. Trans. Med. 3:294-305. https://doi.org/10.1016/j.nhtm.2017.06.001.

123. VandeHaar, M. J., L. E. Armentano, K. Weigel, D. M. Spurlock, R. J. Tempelman, and R. Veerkamp. 2016. Harnessing the genetics of the modern dairy cow to continue improvements in feed efficiency. J. Dairy Sci. 99:4941-4954. https://doi.org/10.3168/jds.2015-10352.

124. Vinayavekhin, N., E. A. Homan, and A. Saghatelian. 2010. Exploring disease through metabolomics. ACS Chem. Bio. 5:91-103. DOI: 10.1021/cb900271r.

125. Wallace, J. M. 2011. Adaptive maternal, placental and fetal responses to nutritional extremes in the pregnant adolescent: lessons from sheep. in: C.G.N. Mascie-Taylor, L. Rosetta (Eds.) Reproduction and adaptation. University Press. Cambridge. 112–127. https://doi.org/10.1017/CBO9780511973642.007.

126. Wang, X., and H. N. Kadarmideen. 2019. Metabolomics analyses in high-low feed efficient dairy cows reveal novel biochemical mechanisms and predictive biomarkers. Metabolites. 9:151. https://doi.org/10.3390/metabo9070151.

127. Wang, Z., J. D. Nkrumah, C. Li, J. A. Basarab, L. A. Goonewardene, E. K. Okine, D. H. Crews, Jr, and S. S. Moore. 2006. Test duration for growth, feed intake, and feed efficiency in beef cattle using the GrowSafe system. J. Anim. Sci. 84:2289–2298. doi:10.2527/ jas.2005-715.

128. Wang, J., Z. Xu, H. Zhang, Y. Wang, X. Liu, Q. Wang, J. Xue, Y. Zhao, and S. Yang. 2020. Meat differentiation between pasture-fed and concentrate-fed sheep/goats by liquid chromatography quadrupole time-of-flight mass spectrometry combined with metabolomic and lipidomic profiling. Meat Sci. 108374. https://doi.org/10.1016/j.meatsci.2020.108374.

129. Warth, B., A. Parich, C. Bueschl, D. Schoefbeck, N. K. N. Neumann, B. Kluger, K. Schuster, R. Krska, G. A., M. Lemmens, and R. Schuhmacher. 2015. GC–MS based targeted

metabolic profiling identifies changes in the wheat metabolome following deoxynivalenol treatment. Metabolomics. 11:722–738. https://doi.org/10.1007/s11306-014-0731-1.

130. Watkins, S. M., and J. B. German. 2002. Metabolomics and biochemical profiling in drug discovery and development. Cur. Opin. Mol. Therap. 4:224-228. https://europepmc.org/article/med/12139307.

131. Wikoff, W. R., A. T. Anfora, J. Liu, P. G. Schultz, S. A. Lesley, E. C. Peters, G. Siuzdak. 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. PNAS. 106:3698-3703. DOI: 10.1073/pnas.0812874106.

132. Wishart, D. S., D. Tzur, C. Knox, R. Eisner, A. C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, and C. Fung. 2007. HMDB: the Human Metabolome Database. Nucleic Acids Res. 35:D521–526. https://doi.org/10.1093/nar/gkl923.

133. Wishart, D. S. 2008. Metabolomics: application to food science and nutrition research. Tren. Food. Sci. Tech. 19:482-493. https://doi.org/10.1016/j.tifs.2008.03.003.

134. Wishart, D. S. 2008b. Quantitative metabolomics using NMR. TrAC. Tre. Analy. Chem. 27:228-237. https://doi.org/10.1016/j.trac.2007.12.001.

135. Wishart, D. S., M. J. Lewis, J. A. Morrissey, M. D. Flegel, K. Jeroncic, Y. Xiong, D. Cheng, R. Eisner, B. Gautam, D. Tzur, and S. Sawhney. 2008. The human cerebrospinal fluid metabolome. J. Chrom. B. 871:164-173. https://doi.org/10.1016/j.jchromb.2008.05.001.

136. Wishart, D. S. 2009. Computational strategies for metabolite identification in metabolomics. Bioanalysis. 1:1579-1596. https://doi.org/10.4155/bio.09.138.

137. Wishart, D. S. 2012. Systems biology resources arising from the human metabolome project. In: Genetics meets metabolomics: from experiment to systems biology. Edited by Suhre K. New York: Springer. 157–175. DOI:10.1007/978-1-4614-1689-0_11.

138. Wyness, L. 2016. The role of red meat in the diet: nutrition and health benefits. Proc. Nutr. Soc. 75:227-32. doi: 10.1017/S0029665115004267.

139. Yanagihara, M., F. Nakajima, and T. Tobino. 2018. Metabolomic responses of an estuarine benthic amphipod to heavy metals at urban-runoff concentrations. Water Sci. Technol. 78:2349–2354. https://doi.org/10.2166/wst.2018.518.

140. Yang, X., M. Zhang, T. Lu, S. Chen, X. Sun, Y. Guan, Y. Zhang, T. Zhang, R. Sun, B. Hang, X. Wang, M. Chen, Y. Chen, and Y. Xia. 2020. Metabolomics study and meta-analysis on the association between maternal pesticide exposome and birth outcomes. Environ. Res. 182:109087. doi:10.1016/j.envres.2019.109087.

141. Yazdani, M., K. B. P. Elgstøen, H. Rootwelt, A. Shahdadfar, Ø. A. Utheim, and T. P. Utheim. Tear metabolomics in dry eye disease: A review. Int. J. Mol. Sci. 20:3755. doi:10.3390/ijms20153755

142. Zeder, M. A. 2008. Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion, and impact. PNAS. 105:11597-11604. doi: 10.1073/pnas.0801317105.

143. Zygoyiannis, D. 2006. Sheep production in the world and in Greece. Sm. Rum. Res. 62:143-147. https://doi.org/10.1016/j.smallrumres.2005.07.043.

144. Zhang, A., H. Sun, P. Wang, Y. Han, and X. Wang. 2012. Recent and potential developments of biofluid analyses in metabolomics. J. Proteo. 75:1079-1088. https://doi.org/10.1016/j.jprot.2011.10.027.

145. Zhang, X., W. Wang, F. Mo, Y. La, C. Li, and F. Li. 2017. Association of residual feed intake with growth and slaughtering performance, blood metabolism, and body composition in growing lambs. Sci. Rep. 7:12681. doi:10.1038/s41598-017-13042-7.

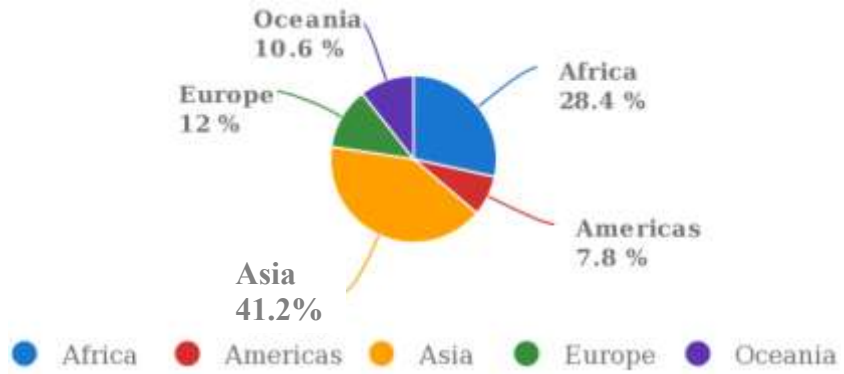
146. Zhang, J., Y. Gao, H. Guo, Y. Ding, and W. Ren. 2019. Comparative metabolome analysis of serum changes in sheep under overgrazing or light grazing conditions. BMC Vet. Res. 15:469. https://doi.org/10.1186/s12917-019-2218-9.

147. Zhang, X., T. Wang, J. Song, J. Deng, and Z. Sun. 2020. Study on follicular fluid metabolomics components at different ages based on lipid metabolism. Repro. Biol. Endocrinol. 18:42. https://doi.org/10.1186/s12958-020-00599-8.

Figures

Figure 1. Iranian statuary showing early sheep domestication. This limestone wall from the Persepolis Palace in Iran shows what appears to be a lamb (left) and sheep gifted to the Persian king by the Assyrians (right). Pictures taken by Osama Shukir Muhammed Amin (left; https://www.ancient.eu/image/7108/gift-bearer-holding-a-lamb-from-persepolis/) and Andrew Selkirk (right; http://www.travellingthepast.com/iran/persepolis/persepolis-the-sculptures/).

a.



b.

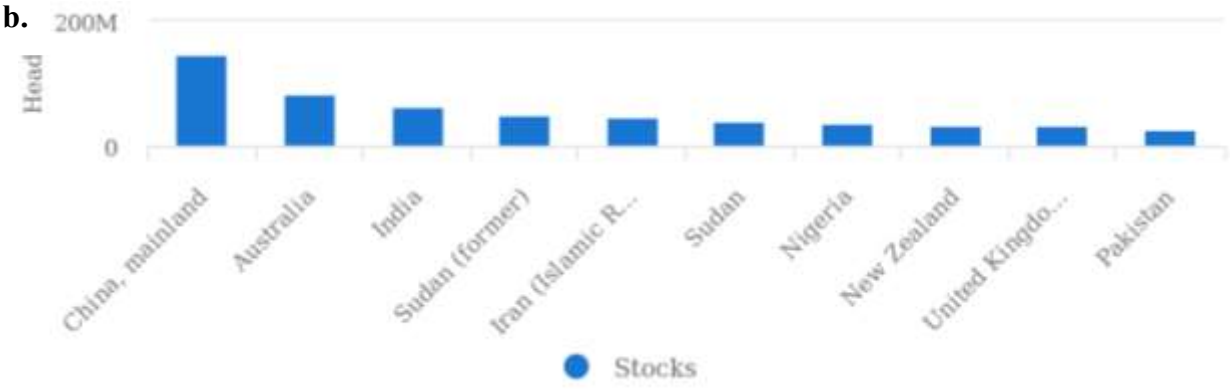


Figure 2. World sheep production. Data from FAOSTAT shows that the majority of global sheep production (a) in the 21st century is in Asia, followed by Africa, Europe and Oceania. The top five sheep producing countries since 2000 are China, Australia, India, Sudan and Iran (b). Graphs obtained from FAOSTAT.

Figure 3. GrowSafe Systems measuring sheep feed consumption. The GrowSafe Systems was used in our experiment (refer to chapter three) to measure individual sheep feed intake to calculate its RFI. Picture by courtesy of Dr. Susan Markus.

Figure 4. Post-hoc measurement of sheep carcass. Carcass measurements of muscle to bone ratio were conducted in my first experimental chapter on sheep RFI and carcass merit (please refer to chapter three for more details). Picture provided by Dr. Susan Markus.

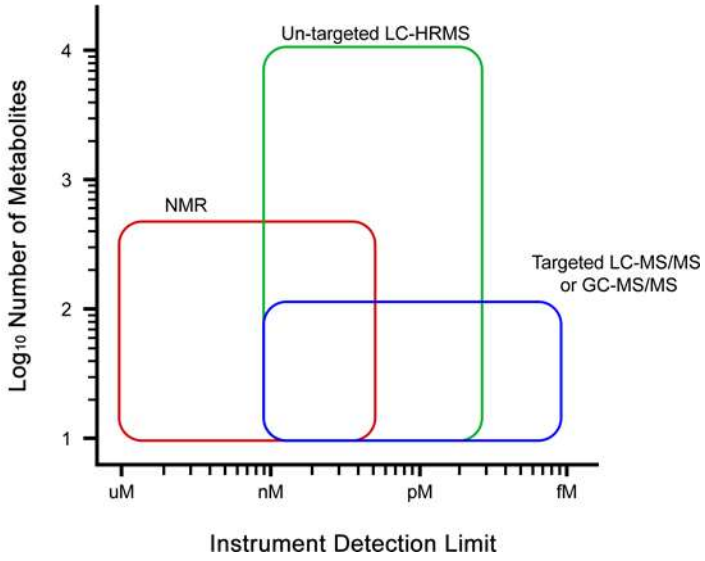


Figure 5. Detection limits of different metabolomics platforms. This scheme shows the sensitivity of each analytical platform used in metabolomics and the extent of its detection. Figure adapted from Pinu et al., 2019.

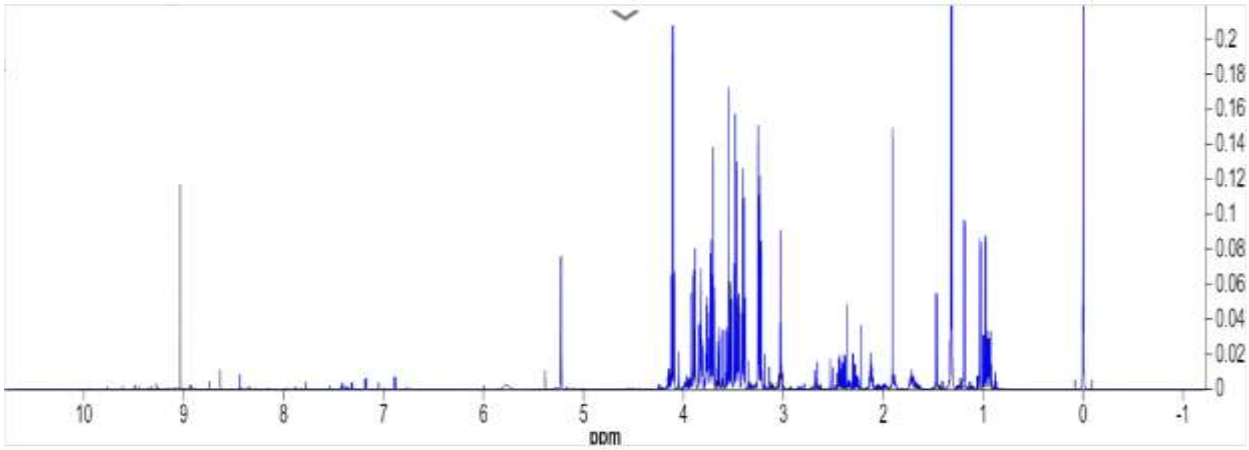


Figure 6. Typical NMR Spectrum. This 700 MHz ^{1}H NMR spectrum shows the peaks at different locations (i.e., chemical shifts) of approximately 50 different metabolites as seen in a typical serum sample as analyzed and deconvoluted using the Bayesil web server.

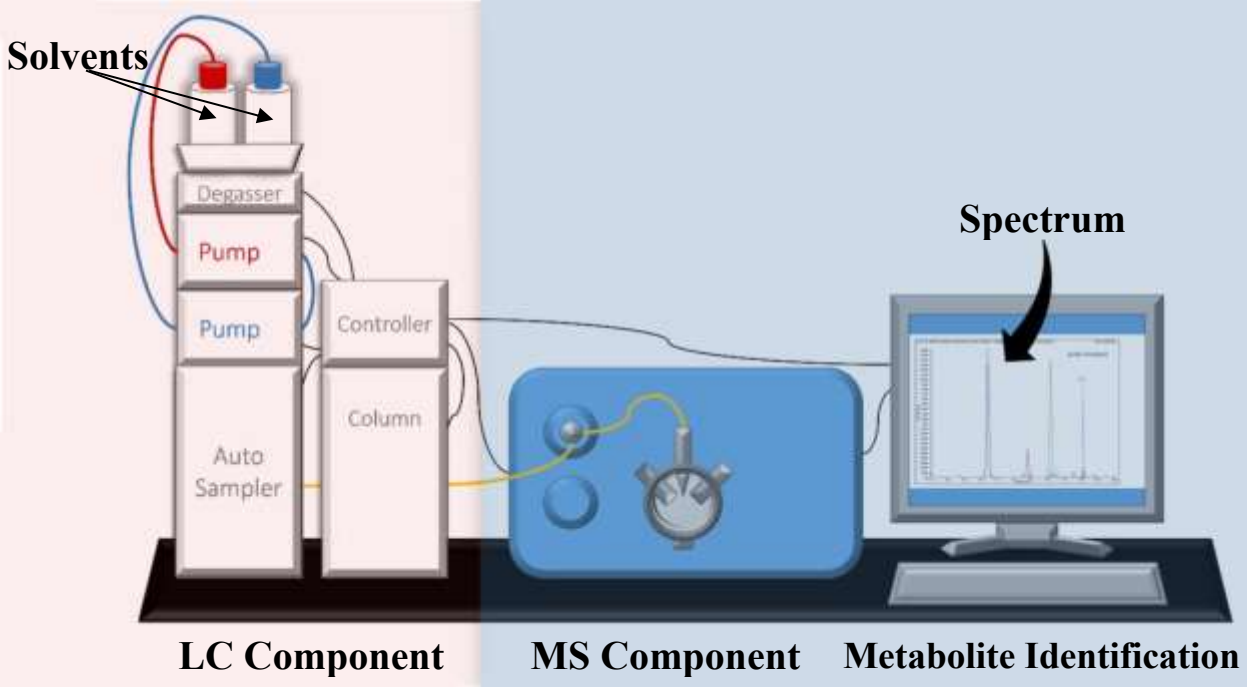


Figure 7. Schematic of LC-MS. This is a general overview of a LC-MS instrument consisting of an LC component, an MS component and the metabolite identification that is done through the computer.

Chapter 2: Livestock Metabolomics and the Livestock Metabolome: A Systematic Review

Seyed Ali Goldansaz [1,2], An Chi Guo[2,3], Tanvir Sajed[3], Michael A. Steele[1], Graham S. Plastow[1], David S. Wishart [2,3]

[1]Department of Agriculture, Food and Nutritional Sciences, University of Alberta, Edmonton, AB T6G 2P5, Canada

[2]Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada

[3]Department of Computing Science, University of Alberta, Edmonton, AB T6G 2E8, Canada

*This chapter has been reformatted from the originally published paper format to make it consistent with the formatting used throughout the thesis.

Abstract

Metabolomics uses advanced analytical chemistry techniques to comprehensively measure large numbers of small molecule metabolites in cells, tissues and biofluids. The ability to rapidly detect and quantify hundreds or even thousands of metabolites within a single sample is helping scientists paint a far more complete picture of system-wide metabolism and biology. Metabolomics is also allowing researchers to focus on measuring the end-products of complex, hard-to-decipher genetic, epigenetic and environmental interactions. As a result, metabolomics has become an increasingly popular "omics" approach to assist with the robust phenotypic characterization of humans, crop plants and model organisms. Indeed, metabolomics is now routinely used in biomedical, nutritional and crop research. It is also being increasingly used in livestock research and livestock monitoring. The purpose of this systematic review is to quantitatively and objectively summarize the current status of livestock metabolomics and to identify emerging trends, preferred technologies and important gaps in the field. In conducting this review we also critically assessed the applications of livestock metabolomics in key areas such as animal health assessment, disease diagnosis, bioproduct characterization and biomarker discovery for highly desirable economic traits (i.e., feed efficiency, growth potential and milk production). A secondary goal of this critical review was to compile data on the known composition of the livestock metabolome (for 5 of the most common livestock species namely cattle, sheep, goats, horses and pigs). These data have been made available through an open access, comprehensive livestock metabolome database (LMDB, available at www.lmdb.ca). The LMDB should enable livestock researchers and producers to conduct more targeted metabolomic studies and to identify where further metabolome coverage is needed.

Introduction

Metabolites are sometimes referred to as the "canaries" of the genome (Pearson, 2007). Just as canaries for coalminers served as sensitive indicators of problems in coal mines, metabolites can be exquisitely sensitive indicators of problems in the genome (as well as the transcriptome or proteome). Metabolites are effectively the end products of complex interactions occurring inside the cell (the genome) and events, exposures or phenomena occurring outside the cell or organism (the environment). As a result, the comprehensive measurement of metabolites (via metabolomics) allows one to determine interactions between genes and the environment. In other words, metabolomics allows researchers to obtain a highly sensitive and more complete description of the phenotype (Bouatra et al., 2013; Monteiro et al., 2013). This metabolic readout of the phenotype is often called the "metabotype" (Fontanesi, 2016). Recent advances in both analytical chemistry and metabolite data analysis techniques are now making metabolomics far more accessible to a wider range of research disciplines. Indeed, metabolomics is now routinely used in biomedical research (for biomarker discovery and disease mechanism research), food and nutritional analysis, crop characterization and environmental monitoring (Moore et al., 2007; Wishart, 2008; Kim et al., 2016; Jalali et al., 2016). As a result, the field of metabolomics has experienced very rapid growth with just two papers published on the subject in 1999 to more than 2400 in 2015.

However, unlike in other areas of agriculture research where metabolomics is widely used in crop trait selection, pesticide monitoring, crop breeding or crop evaluation (Simo et al., 2014; Summer et al., 2015; Mahdavi et al., 2015; Mahdavi et al., 2016), the application of metabolomics to livestock research is somewhat less widely used or appreciated. This is surprising given the potential of metabolomics to address many important questions in livestock and animal science. In particular, the power of metabolomics to non-invasively detect subtle phenotypic changes,

innate phenotypic propensities and dietary responses makes it an ideal tool for livestock research, breeding and assessment (Fiehn, 2002; Houle et al., 2010; Duggan et al., 2011; Jones et al., 2012; May et al., 2013; Gilany et al., 2014; Minai-Tehrani et al., 2015). Recently, there have been a number of papers in livestock metabolomics that have generated compelling results showing how metabolomics and metabolite-based phenotyping (metabotyping) can help farmers, veterinarians, livestock researchers and the livestock industry. These include papers demonstrating how metabolomics can be used to predict feed efficiency and residual feed intake (RFI; Karisa et al., 2014), ascertain disease propensity (Hailemariam et al., 2014; LeBlanc et al., 2005; Sundekilde et al., 2013), evaluate dietary responses to different feeds (Saleem et al., 2012; Abarghuei et al., 2014), assess carcass merit (Weikard et al., 2010; Karisa et al., 2013a; Kuhn et al., 2014), fertility (Chapinal et al., 2012), milk quality (Melzer et al., 2012; Melzer et al., 2013), determine bioproduct content (Castejón et al., 2015) and ascertain other important economic or breeding traits associated with livestock.

Fast, effective, and quantitative phenotyping is critical for farm trials dealing with animal selection and breeding. Many traditional phenotypic measurements such as those related to animal feed consumption and RFI are expensive, time consuming and require specific recording equipment (Karisa et al., 2014). Others, such as carcass trait evaluation, may require animal slaughter, which obviously eliminates the potential breeding value of the animal. Similarly for reproductive traits, animals have to reach a stage of maturity and sexual activity to allow measurement of related traits. Metabolomics allows many of these trait measurements to be conducted earlier, more routinely, non-invasively and often at a lower cost than current techniques (Zhang et al., 2012; Fontanesi, 2016). However, metabolomics is not without its challenges. Metabolomic experiments must be carefully designed as diet and other variables such as sex,

diurnal variations and sampling time can profoundly affect results. Likewise, metabolomic technologies, such as gas chromatography (GC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are not yet widely available in many livestock research facilities. Furthermore, there continues to be a significant shortage of data resources that could facilitate the interpretation of livestock metabolomic data.

Given the many applications of metabolomics in both the livestock industry and livestock research as well as the diversity of journals in which livestock metabolomics is often published, we felt it was important to conduct a thorough, systematic review of the field. By consolidating the results from diverse journals and different studies into a single review paper, we believed this content would provide a more complete picture of both the strengths and the weaknesses of livestock metabolomics. In conducting this review we sought answers to 4 key questions: 1) What are the most common applications of metabolomics in animal science and where are they trending?, 2) What are the preferred metabolomics technologies in livestock metabolomics and how are they evolving?, 3) What are the most obvious gaps or weaknesses in livestock metabolomics relative to other fields of metabolomics research? and 4) What are the known or measured metabolites for the 5 major livestock species (i.e., bovine, ovine, caprine, equine, and porcine) in different tissues and biofluids? This metabolite compilation, which we have called the livestock metabolome database or LMDB (available at www.lmdb.ca), is intended to help lay a more solid foundation in terms of data resources that would make livestock metabolomic studies much easier to perform, analyze and compare. The LMDB catalogues all metabolite compounds that have ever been identified and reported in the 5 livestock species (for multiple biofluids and tissues), along with concentration ranges, compound descriptions, chemical structures, reference

NMR and MS spectra and other information associated with each metabolite for both healthy and a variety of abnormal physiological conditions.

Materials and Methods

In compiling this review and assembling the livestock metabolome database, we used a combination of web-accessible data mining tools along with manual curation to survey 2313 peer reviewed journal articles covering the period from 1930 to 2015. From this initial set of articles, we reduced the number further to cover published livestock papers reporting the measurement or characterization of ≥8 metabolites for any of the 5 major livestock species (i.e., bovine, ovine, caprine, equine, and porcine). This reduced the target number of peer-reviewed manuscripts to a total of 149. The livestock species selected for this review were based on their global population, economic impact and use in agricultural systems (Robinson et al., 2014; Thornton, 2010). Details regarding the keyword selection, search engines and databases, journals and search strategy are given below and summarized in the preferred reporting items for systematic reviews and meta-analysis (PRISMA) checklist and flow chart (Figure 1).

Keyword Selection

As noted above, this review is focused on 5 main livestock species including cattle, sheep, goats, horses and pigs. Therefore, a combination of keywords was selected to target those specific animals and to identify the associated metabolomics studies. Keywords were divided into 3 main groups: 1) animal species, 2) sample types, and 3) metabolomic methods. Selected keywords for animal species included the name of the species and its various derivatives or synonyms, i.e., bovine, cattle, cow, calf, *Bos taurus*, etc. To target metabolomics papers in animal science, a broad range of metabolomics keywords were identified and used. These included different variations of the term “metabolomics” (such as metabolomics, metabonomics, metabolite profiling, metabolite

fingerprint, chemical profile, chemical analysis, chemical composition, etc.) to target publications prior to and after 1999, as well as the names of various analytical platforms (i.e., NMR, mass spectrometry, liquid chromatography, gas chromatography-MS, etc.). Moreover, a wide variety of sample types such as different body fluids (i.e., serum, blood, plasma, urine etc.) and different organs or tissues were selected to further enrich the keyword search.

Search Engines and Databases

An initial comparison among many open access search engines showed that most search results are similar regardless of the search engine used. Therefore, Google Scholar (https://scholar.google.ca/) was selected as the primary literature search engine. In addition, a number of agriculture-specific databases such as Agricola and AGRICULTUREnetBASE were also used. Other databases included Scopus, the Web of Science, ScienceDirect (http://www.sciencedirect.com/) and PubMed (www.ncbi.nlm.nih.gov/pubmed). Settings for all search engines and databases were adjusted to increase search efficiency and filter irrelevant results.

Search Methods and Selection Criteria

Different keywords were combined to target metabolomics papers in the field of animal science. For example, “cattle”, “cattle serum” or “cattle milk” was accompanied with “metabolomics”, “chemical composition” or “metabolite profiling”. Consequently, each combination of the keywords in the search engines generated a long list of results. These included various types of publications (full papers or abstracts) that contained any or all of the used keywords. A manual review was performed on all retrieved publications. Typically, the first 3-5 pages of the search results from the aforementioned search engines were manually reviewed to select for articles of interest. Among the papers identified as worth pursuing, research papers, abstracts or textbooks

that showed relevance in their title or abstract were selected. In addition to papers reporting experimental results, review articles that included specific metabolite data sets were also selected. Among the selected manuscripts, only those papers that reported ≥8 metabolites were chosen for this review. The threshold of 8 or more as the minimum number of metabolites was based on a *post hoc* analysis of the retrieved papers and the need to optimize both metabolite coverage and the time devoted to manual analysis. We also determined that this selection cut-off allowed us to cover most, if not all, of metabolites reported in papers with <8 metabolites. Based on these criteria, a total number of 149 manuscripts covering all 5 animal categories were selected for this review. Selected publications were carefully read to extract and annotate a set of 10 pieces of information including: 1) metabolite names; 2) tissue or biofluid origin; 3) quantified values (concentration) if any; 4) experimental conditions; 5) animal breed; 6) sample size; 7) analytical platform; 8) field of research, 9) physiological condition (disease or state of health), and 10) Pubmed/DOI references.

Compilation of the Livestock Metabolome Database

In compiling the data for this livestock metabolome database or LMDB (www.lmdb.ca), all reported concentrations were transformed into a standardized concentration unit (micromolar; μM) and each entry was associated with an abbreviated description of the experimental context, the sample type, and the methodologies used for the metabolomic analyses. In identifying a metabolite for inclusion in this study the compound had to: 1) have a molecular weight <1500 daltons; 2) it could not be a peptide, protein or oligonucleotide; 3) it had to correspond to a reasonably unique chemical entity (triglycerides and amino acids are not unique chemical entities, but LysoPC-16:2 is sufficiently unique) and 4) it had to be identified with a structurally interpretable name. This literature-based effort generated 1070 metabolites from 149 peer-reviewed papers, abstracts or

textbooks. Metabolites extracted from these manuscripts were systematically categorized into the LMDB. Nearly all metabolites extracted were linked to a standard Human Metabolome Database (HMDB) identifier (Wishart et al., 2007; Wishart et al., 2009; Wishart et al., 2013) which provides a freely-accessible comprehensive description of each metabolite. A brief description of experimental data for each metabolite was also extracted from the articles and included in the database including information on the analytical platform, experimental conditions and field of research. A PubMed and/or DOI id was also associated with each metabolite to provide a link to the article reporting that metabolite. Additional data on each metabolite, including structure, synonyms, chemical classifications, physicochemical data, reference NMR, GC-MS or LC-MS spectra and links to other databases were obtained through an in-house annotation tool called DataWrangler. All of this information was used to construct the on-line version of the LMDB (http://www.lmbd.ca). The LMDB was prepared using a Ruby-on-Rails (Viswanathan, 2008) framework, modeled after other on-line species-specific metabolomic databases prepared in our laboratory. Details regarding their construction, required operating systems, browser compatibility and hardware requirements can be found elsewhere (Wishart et al., 2007; Jewison et al., 2012; Sajed et al., 2016).

Results and Discussion

Growth and Trends in Livestock Metabolomics Research

Based on the data collected from our literature survey, it is clear that the majority of metabolomics studies among all livestock categories have been conducted in cattle (Figure 2) with a total of 76 articles (50% of the selected articles) focusing on various fields of bovine research and assessment. Metabolomics studies on pigs and sheep came second and third with 28% and 12% of the selected articles, respectively. The least studied group were horses with only 5 (3%) reported equine

metabolomic studies. As might be expected, most livestock metabolomic studies focused on issues related to animal health, nutrition and production (65%). These studies are obviously useful for characterizing bioproduct quality, identifying biomarkers or understanding animal responses to different stressors. However, we were surprised to see relatively few efforts focused on metabolomic characterization of healthy animals with the aim of identifying baseline values for different metabolites in different biofluids or tissues. In fact, only 16 studies (10%) of this kind were reported. These "referential surveys" are foundational and are often needed before biomarker studies could/should be undertaken or fully understood.

As noted earlier, most metabolomic studies of cows, sheep, goats and pigs appear to be directed towards disease detection, production and bioproduct assessment, feed efficiency determination and reproduction. In contrast, the primary focus for equine metabolomics has been on drug discovery and doping detection, specifically for thoroughbred horses (Peters et al., 2010). Given the large sums of money directed to horse racing, this is not unexpected. However, compared to the widespread applications of metabolomics in other livestock species for other purposes, it is clear that equine metabolomics is being under-utilized. Certainly, equine metabolomics could be used to select more desirable traits and higher value or higher performing animals, similar to what is being done for bovine metabolomics. Likewise, metabolomics could serve as a diagnostic or prognostic tool for improving equine health and disease resilience (as it has for essentially all other livestock species).

Temporal categorization of all 149 published studies showed that the majority of livestock metabolomics papers were published after 1999. Less than 9% (13 articles) of the selected papers were published prior to 1999 while, ~91% (136 articles) of the papers were published thereafter. The earliest paper in our collection dates from 1930. It is noteworthy that the term "metabolomics"

was not coined until 1998 (Tweeddale et al., 1998; Oliver et al., 1998) therefore, metabolomics studies prior to this date had to be identified using other keywords such as "chemical composition", "biochemical profiling", etc. Based on our observations, it is clear that interest in livestock metabolomics is growing rapidly, especially over the last couple of years. Our data indicates that from 2000-2010 just 29 articles (19%) were published in this field, while from 2011-2015 a total of 107 (72%) articles were published. In terms of percentage growth, the most rapidly expanding subfield appears to be caprine and equine metabolomics with a growth rate of 100% over the past 5 years. In terms of overall growth, the most significant changes were in bovine metabolomics with the number of papers growing from just 10 prior to 1999 to 49 in 2011-2015. The most recent additions to the field of livestock metabolomics are studies focused on goats (starting in 2014) and horses (starting in 2007).

Trends and gaps in livestock metabolomics applications

We found that livestock metabolomics studies can be categorized in 7 main areas (Table 1). These include animal health, animal nutrition, animal production, animal reproduction, animal physiology (mainly analysis of different biofluids), animal products (products originating from livestock such as milk, meat, yogurt, etc.), and human health (livestock models used for human health studies). This general categorization was based on a *post hoc* analysis of the types of articles where we manually assessed article keywords, subject headings, journal titles and the general focus of each article. Most of these categorizations (such as animal reproduction, human health and animal health) were relatively simple to make. For instance, the category "animal reproduction" obviously refers to articles using metabolomics to study reproduction in livestock. Likewise, the category "animal health" refers to articles using metabolomics to study livestock health or disease while "human health" refers to application of metabolomics to study human disease using various

livestock models. Other categories proved to be somewhat more ambiguous. For instance, the field of "animal products" typically contains metabolomics investigations related to food, nutrition and human consumption of animal products, such as meat and cheese. On the other hand, "animal production" is focused on investigating the associated biochemical profile with each animal product. In some cases, we had to be fairly strict with our definitions. For instance, we limited "animal physiology" to include only those articles focused on analyzing various biofluids or characterizing the metabolite composition of specific biofluids, organs and tissues.

Among the seven different categories, animal health (52) and animal production (40) had the most metabolomics articles published for the largest number of animal groups (Table 1). However, this varied depending on the livestock species being studied. In human health research, porcine metabolomic studies covered the majority of articles (14 articles) compared to all other livestock categories. This is not unexpected, given the comparable physiology of pigs to that of humans (Nielsen et al., 2014). In the category of animal products, bovine-based studies had the most articles published (16 articles) relative to all other groups. Some of the more interesting applications of metabolomics found in our survey include the use of metabolomics for quality control of animal products (Cevallos-Cevallos et al., 2011; Regal et al., 2011), evaluating nutritional value and impact of various feed sources on animal health and products (Abarghuei et al., 2014), investigating disease biology by using animal models of human disease (Merrifield et al., 2011; Mickiewicz et al., 2015), investigation of potential metabolite biomarkers of animal disease (LeBlanc et al., 2005; Sundekilde et al., 2013), assessment of production traits (Lu et al., 2013; Sun et al., 2015), reproductive performance (Chapinal et al., 2012), and general metabolome characterization (Saleem et al., 2013; Escalona et al., 2015).

In terms of gaps in the existing literature, it is perhaps most useful to use bovine metabolomic studies as the "gold standard" by which to compare other livestock species. While metabolomics is routinely being used to understand the biology or diagnose a few common bovine production diseases (including acidosis, mastitis, milk fever) we found no metabolomic studies looking at common diseases in sheep or goats (such as brucellosis, campylobacteriosis, pneumonia, Q fever), in horses (equine flu, equine herpes, equine sleeping sickness, anemia, laminitis, azoturia), or in pigs (respiratory diseases, swine dysentery, parvovirus). Indeed, we found only 22 metabolomic studies focused on the health of sheep, goats, pigs and horses, compared to 30 metabolomic studies for cattle alone. Of these 22 non-bovine studies, most were focused on metabolic, growth and neurodegenerative disorders.

Livestock metabolomics studies also appear to be missing a number of opportunities currently being pursued in human biomedical research. One of particular note is the use of metabolomics to predict (as opposed to diagnose) or detect subclinical forms of disease. While disease diagnosis is useful, often it is too late or too costly to perform useful veterinary interventions. Detecting diseases before they manifest or predicting them before they occur allows inexpensive prophylactic or preventative measures to be taken. In human metabolomic studies, the identification of disease prediction biomarkers is becoming increasingly common (Wang-Sattler et al., 2012; Reinehr et al., 2014; Kordalewska et al., 2015; Mirsaeidi et al., 2015; Shajahan-Haq et al., 2015). This is because metabolic changes appear to precede significant physiological changes, possibly because metabolites play an important signaling role to activate later stage (i.e. symptomatic) physiological responses (Mathew et al., 2014; Johnson et al., 2016). However, we could only find 2 papers (limited to cattle) that focused on disease diagnosis/prognosis or (sub)clinical detection of diseases (Hailemariam et al., 2014; Klein et al., 2012). A similar

approach could also be used towards the prediction of later-life production traits on the basis of early-life metabolic fingerprints. This, too, is an area of interest in the field of human metabolomics, where later-life health is being predicted on the basis of early-life metabolic fingerprints (Moco et al., 2013; Morrow et al., 2013; Mohamad et al., 2015). Obviously the reliable prediction of economically important traits is an important tool for livestock management and strategic planning.

Metabolomics is already being used in the evaluation and/or prediction of production traits such as residual feed intake (RFI), carcass merit, reproductive performance and metabolic disorders for cattle. However, there is a surprising dearth of similar studies regarding evaluation or prediction of production traits for sheep, goats and pigs. Metabolomics potentially offers a unique opportunity for indirect, inexpensive marker-assisted measurement of these economical traits. This can be achieved through non-invasive sample collection of readily accessible biofluids such as blood, urine, milk and saliva. In most cases, the standard measurement or prediction of some traits such as RFI and carcass merit requires labour intensive, invasive, costly and time consuming measurements (Moore et al., 2009). Metabolomic studies regarding the prediction of RFI in beef cattle have already been very promising with a reported initial prediction accuracy of 95% (Karisa et al., 2014; Widmann et al., 2015). Metabolomic data, when coupled with genomic data, appear to increase the accuracy of trait prediction (Karisa et al., 2013b). This combination potentially allows one to screen for individual animals with superior traits that could be used for breeding stock. Given the positive results already seen for cattle, the application of these metabolomic concepts to other livestock species is certainly worth investigating. Overall it appears that there is still a considerable body of useful metabolomic work that could be pursued with most

other livestock species by simply applying or extending what has already been done in bovine metabolomics.

Trends and Gaps in Sample Size

Nearly 50% of the selected articles for all animal species used ≤30 animals or samples (from an even smaller number of animals) to conduct their metabolomics analysis. Other sample size categories shown in Table 2 account for ~10% of the peer-reviewed livestock metabolomics literature. The maximum number of samples reported from the selected papers were: 1587 (bovine), 163 (ovine), 80 (caprine), 36 (equine), and 506 (porcine). It is noteworthy that sample size does not always reflect the total number of animals used in the study. For instance, longitudinal studies typically collect multiple samples from a relatively small number of animals over an extended period of time. Relative to many reported human metabolomic studies (Reinehr et al., 2014) or rodent model studies (Chen et al., 2015) the number of samples and the number of subjects (i.e. animals) used in most livestock metabolomics studies is generally quite small. Indeed, many human and rodent model studies routinely measure 100s to 1000s of samples. This difference in sample size likely reflects the relatively high cost of performing large animal studies as well as the somewhat limited funding available to agriculture research relative to medical research.

However, it is important to note that the smaller sample sizes in livestock metabolomics also mean that statistical significance and "power" of the published results is also somewhat less than many human-subject or model organism studies. This represents a significant gap for livestock metabolomics and requires either study sizes to be increased or more effort being directed to conducting validation studies on similar-to-largely sized cohorts for confirmation of previously reported results. Indeed, we found only one bovine metabolomic study reporting either independent cross validation (using a different animal cohort) or independent follow-up validation

of any newly identified biomarkers or interesting metabolite findings (Karisa et al., 2014). On the other hand, follow-up validation studies are becoming routine in human metabolomic studies (Alves et al., 2015; Bussche et al., 2015; Ganna et al., 2016). Clearly, this is a gap in livestock metabolomics that must be filled if metabolomic findings are going to be translated to practical pen-side or on-farm applications.

Another consistent problem detected in the published livestock metabolomics literature is incomplete reporting. We found that 13% of all published livestock metabolomics papers did not report the number of samples used in their research. Providing information on sample size is an essential scientific measurement and reflects on the quality and reliability of published papers. Failure to report sample sizes along with failure to provide information on the numbers of animals or animal replicates indicates a major flaw in manuscript preparation and scientific work.

Trends and Gaps in Biological Sample Types

As can be seen in Figure 3 and Table 3, a total of 30 different sample types have been used for livestock metabolomics analyses. The most commonly used sample types include milk, plasma, serum, urine and ruminal fluid. These biofluids account for 78% of the total sample types reported. Milk and plasma are the most commonly used samples in bovine metabolomics manuscripts. Among all other animal groups, plasma was the most widely examined sample type (Table 3), reflecting perhaps the ease of collection but also its potential utility as a proxy reporter for all of the organs in the body (Psychogios et al., 2011). Some of the least frequently used samples include cerebrospinal fluid, colostrum, semen, adipose tissue, kidney and kidney perfusate, feces, amniotic fluid, bile and liver (Table 3). The relatively low number of papers reporting data on tissue metabolomics likely reflects the challenges and costs of animal culling especially for larger livestock, sample collection, and the need to rapidly perform metabolic quenching via liquid

nitrogen (immediately after surgery or necropsy) to obtain useful tissue samples for metabolite analysis (Kosmides et al., 2013; Verma et al., 2013).

While studies on bovine milk are quite prevalent, there are essentially very few studies on sheep or goat milk (Table 3). Given the importance of goat and sheep milk in the global agrifood economy, it is surprising that only a total of 6 papers have been published on goat/sheep milk metabolites. One notable study, however, is that of Park and colleagues (Park et al., 2007) who used LC-MS to identify/quantify 82 metabolites in sheep and goat milk. This paper reports a number of other macronutrient milk constituents including fat, protein, minerals and vitamins. In another more recent study, the effect of a specific grazing patterns and their associated dietary effect on goat milk was evaluated (Steinshamn et al., 2014). These authors used GC-MS techniques to identify and quantify 25 milk metabolites.

Similar trends are also seen in other biofluid or sample types, with bovine samples or bovine-related papers dominating. For instance, there are a number of metabolomic studies on bovine ruminal fluid, plasma and urine, but very few studies on these biofluids for sheep, goat, horses or pigs (43 for all 4 species and 3 sample types). Likewise, metabolomics studies on colostrum and semen are limited to cattle only with one study each. Interestingly, some of the less-frequently used sample types such as cerebrospinal fluid, synovial fluid, amniotic fluid, bile and vitreous humor are limited to the less frequently studied livestock species (sheep, goat and pig). What is also quite striking is the dearth of fecal metabolomic studies among all livestock species (Table 3). With the growing interest in the microbiome and the clear role that gut (and rumen) microflora play in animal health, we were surprised by the complete absence of metabolomic papers on bovine fecal samples.

Given the importance of beef, sheep and goat meat, it is also surprising to see how little metabolomic data has been collected on meat samples. Indeed, only a total of 9 papers provided data on relatively small number (140) of meat metabolites. The most comprehensive meat metabolomics study was reported by Castejón et al. (2015). These authors profiled meat exudate using NMR to explore the effect of storage time on metabolite composition. They reported a total of 60 different metabolites. Overall, these data suggest that the livestock metabolomic literature is characterized by a significant under-representation of some important sample types, including milk, meat, fecal/rumen, semen samples and cerebrospinal fluid. These "gaps" in our knowledge and "gaps" in the published literature represent clear opportunities for livestock researchers to pursue.

With regards to the number of metabolites detected, quantified and/or reported among the different sample types, we found that the broadest level of coverage was for milk, plasma and serum (Table 4). Ruminal fluid, urine, feces and meat samples had slightly lower levels of coverage while the rest of the sample types reported in Figure 3 typically report <60 metabolites each. It is instructive to compare these livestock metabolite numbers to data reported for human metabolites identified in similar kinds of sample types. For instance, the most comprehensive human milk metabolomics paper reports just 129 identified metabolites (Andreas et al., 2015), which is >3X lower than what has been reported in the livestock milk. The total number of metabolites reported for plasma/serum in humans is 4229 (Psychogios et al., 2011), which is significantly more than what is reported for livestock plasma/serum (with 759). Likewise, the total number of human urine metabolites has been reported to be 445 (Bouatra et al., 2013), which is more than twice that found in the urine of livestock species. Given their genomic similarity, our expectation is that the number of metabolites measurable in livestock for each of the biofluids should be comparable to the

number of metabolites measured in humans. Currently, the Human Metabolome database recognized as the most comprehensive metabolomics database contains >40,000 metabolites derived from various human biosamples (Wishart et al., 2016). As a result, this suggests there is still a significant gap to be filled with regard to the depth and breadth of metabolome characterization in livestock.

Trends and Gaps in Analytical Instrumentation and Methodologies

Metabolomics uses a wide variety of analytical instruments that vary in terms of their sensitivity and breadth of coverage. Nuclear magnetic resonance (NMR) continues to be among the most commonly used analytical platforms in metabolomics (Wishart, 2009). It is often chosen for its reliability and utility in absolute quantitation however, NMR is relatively insensitive and is limited to measuring substances in micromolar to millimolar (μM-mM) concentrations (Figure 4). Mass spectrometry (MS) platforms (especially LC-ESI-MS) can detect metabolites at nanomolar (nM) to picomolar (pM) concentrations, allowing a much higher number of metabolites to be detected. However, MS instruments are prone to frequent breakdowns and, relative to NMR, it is often difficult to quantify chemical concentrations via MS techniques. Gas chromatography-MS (GC-MS) is less sensitive than liquid chromatography (LC)-MS, but is generally more robust and more reproducible. As a result, GC-MS can sometimes be used to identify and quantify the metabolome with higher precision and reproducibility than either NMR or LC-MS.

Each of the 149 livestock metabolomics papers was carefully analyzed to identify which analytical platforms (NMR, LC-MS, GC-MS) were used more frequently to conduct metabolomic analyses. In certain studies, more than one platform was used so, we simply counted the frequency that each technique or technology was used in each study. Interestingly, the most commonly used metabolomics platform for all animal categories is NMR spectroscopy, accounting for 28% of all

livestock metabolomics studies. Following closely behind NMR, in terms of frequency, is LC-MS with 25% of all studies using this analytical platform. It is noteworthy that the LC-MS category includes ultra performance liquid chromatography (UPLC)-MS, high-performance liquid chromatography (HPLC)-MS, and direct flow injection (DFI)-MS. Gas chromatography-MS is the third most prevalent (15%) analytical platform used in livestock metabolomics studies. The more limited use of GC-MS is typical of other metabolomic disciplines as well.

Other, less conventional or more targeted, methodologies account for the remaining 27% of the technologies used in livestock metabolomics studies. These methods include, but are not limited to, infrared spectroscopy (FTIR), silicic acid column chromatography, immunoassays, the Kjeldahl method (for organic nitrogen measurement), ELISAs, and miscellaneous, lab-specific methods. Relative to other fields of metabolomics, livestock metabolomics appears to use NMR spectroscopy somewhat more and LC-MS somewhat less. This may simply reflect the availability of instrumentation or the preferences of major research groups in livestock metabolomics. Certainly, sample abundance and supply is not a significant issue in livestock metabolomics so, the use of tools that require higher-volumes, but offer more quantitative results (such as NMR) is not unexpected. However, NMR is not the most sensitive technique and certainly if livestock metabolomics researchers wish to extend their coverage of the livestock metabolome, they will certainly need to make use of more LC-MS methods.

Another gap that was noted in livestock metabolomics research is the near complete absence of ICP (inductively coupled plasma)-MS studies to measure metal ion levels in tissues and biofluids. Indeed, only 2 studies used ICP-MS, with the most complete characterization being conducted by Saleem et al. (2013) who reported the identification and quantification of 20 metals in bovine ruminal fluid. The importance of metal ions as micronutrients for animal health and

animal productivity cannot be underestimated (McLaughlin et al., 1999; Singh et al., 2011). Therefore, it is surprising that so little metal ion data has been collected or analyzed in livestock metabolomic studies. It was also noted that the use of fluxomics (Winter and Krömer, 2013) or the measurement of metabolite flux using stable isotopes is completely absent in livestock metabolomics studies. Fluxomics is particularly useful in understanding metabolic sinks and sources. It is also useful for assessing nutrition and metabolic efficiency – topics, which are obviously important in livestock research. However, to conduct metabolic flux analysis, isotopically labeled (^{13}C or ^{2}H) feed needs to be used. Given the size of most livestock animals (relative to rats and mice) and the need for significant quantities of expensive, isotopically labeled feed, fluxomic studies are likely too difficult and costly to perform (Srivastava et al., 2013). Likewise, the use of imaging mass spectrometry or IMS (which is becoming very popular in human metabolomics studies) was completely absent in livestock studies. Imaging mass spectrometry is particularly useful for analyzing tissues and for understanding the metabolic changes that take place during tissue development or tissue transformation (Norris and Caprioli, 2013; Römpp et al., 2015).

A good metabolomics study should use more than one analytical platform, and ideally as many different (orthogonal) platforms as possible to broaden the metabolite coverage. In our analysis we found that 69% of the published studies used just 1 platform (either NMR, HPLC-UV, LC-MS, GC-MS or ICP-MS), 15% used 2 platforms and only 3% used 3 or more analytical platforms. The remaining 13% of studies used relatively non-conventional platforms or assays (immunoassays, FT-IR, etc.). The most comprehensive metabolomic analysis was a study that used 5 different platforms (NMR, HPLC-UV, LC-MS, GC-MS and ICP-MS) to characterize the bovine ruminal fluid metabolome (Saleem et al., 2013). Looking through the more recent studies, there is

a general trend towards using more than one platform and a growing trend towards using LC-MS techniques over NMR methods. However, the surprisingly high number of livestock metabolomic studies that still use only a single platform also represents a significant issue that the field must remedy. Certainly the trend in human metabolomic studies is to use at least 2 and often 3 or more different analytical platforms (Bouatra et al., 2013).

Another gap that was identified from this literature analysis was the general lack of integration of other omics techniques (proteomics, transcriptomics or SNP measurements) with reported livestock metabolomic studies. Indeed, only 5 papers (3 bovine and 2 swine metabolomics studies) used metabolomics in conjunction with genomics or proteomics. One paper of note was an investigation that used genomics and metabolomics to evaluate RFI (residual feed intake) from cross breeds of dairy and beef cattle (Widmann et al., 2015). This group of researchers used metabolomics and phenotypic data to support their genomics investigations and identified two genes (*TP53* and *TGFB1*) that were strongly associated with cellular functions driving feed efficiency. In another study by Lu and colleagues (2013), the effect of genetic polymorphisms on dairy milk characteristics was evaluated using a combination of metabolomics and proteomics. This paper identified alterations in triglyceride composition and reported changes in the milk metabolome and proteome of dairy cows with the K232A (lysine to alanine substitution) polymorphism in the well-studied *DGAT1* gene. Given the growing trend towards systems biology research and the more "holistic" interpretations of multi-omics data in other fields of life science, the near absence of multi-omics studies represents an important gap in livestock metabolomics (and omics) research.

Trends and Gaps in Metabolite Quantification

The majority of livestock metabolomics publications are non-quantitative or semi-quantitative (yielding relative quantification) while 28.18% of published studies provide fully (absolute) quantitative data. The metabolites tracked in this review were categorized in two main groups: 1) quantified and 2) non-quantified metabolites. Any metabolite that was associated with an absolutely quantified value (millimolar, micromolar, nanomolar, mg/mL, ug/mL, etc.) in a given sample type was placed in the quantified category. The non-quantified group consists of either metabolites with no quantified value or ones that have only relative quantification (i.e. reported as a fraction or a percentage). Over all livestock species and all sample types, we found a total of 404 quantified metabolites and 666 non-quantified. The majority of both quantified and non-quantified metabolites are lipids and lipid-like molecules. Temporal trends in metabolite quantification show that proportionally fewer livestock metabolomics papers are providing quantitative data. For instance, 69% of papers published prior to 1999 had quantitative data, while 34% of papers from 1999-2010 and just 21% from 2011-2015 generated quantitative metabolite data.

Overall, livestock metabolomics still has an impressive proportion (~28%) of publications that report absolute concentration values. In contrast, most other fields of metabolomics quantify metabolites far less frequently (Psychogios et al., 2011). Nevertheless, the steady decline in the proportion of livestock papers providing quantitative metabolomic data is not a good sign. The importance of absolute quantification in metabolomics cannot be over-emphasized. As a branch of analytical chemistry focusing on small molecule characterization, there is more than 100 years of history and a plethora of tools, standards and protocols designed specifically for absolute metabolite quantification (Wishart, 2009). Absolute quantification allows facile comparisons of readings between animals, research staff, platforms, laboratories and countries. Acquiring quantified values also allows one to determine normal and abnormal ranges for disease diagnosis,

prediction as well as other relevant production measures. Obtaining quantified data and recognizing normal physiological concentrations is also a requirement in biomarker discovery (Torii et al., 2016; Johnson et al., 2016). Indeed, absolute quantification and the existence of normal and abnormal ranges is the foundation to the entire field of clinical chemistry. In livestock metabolomics, having a "normal" quantified range specific for each animal species or breed is critical for defining referential "healthy" conditions. Likewise, being able to quantify specific changes in an animal's metabolome allows one to identify "abnormal" conditions such as overt disease, malnutrition, pregnancy difficulties, and most importantly subclinical conditions for which no obvious clinical indicators are visible (Sun et al., 2015; Ghazi et al., 2016).

Trends and Gaps in Metabolite Coverage

Based on our analysis of the literature and the definition of a metabolite given earlier, the majority of livestock metabolomics studies report ≤50 metabolites (79% of the total selected metabolomics publications) while the other 21% report >50 metabolites. The largest number of metabolites (or features) reported in a single paper was 647 (Sun et al., 2015) covering multiple biofluids for bovine samples while, the fewest reported was 8 (in a variety of papers from all different livestock species). As with metabolite quantification, there is a trend for more recent livestock metabolomics papers to report a greater number of metabolites. For instance, papers published prior to 1999 averaged 29 metabolites per study, those from 1999-2010 averaged 44 metabolites per study while, papers from 2011-2015 averaged 63. Among the later publications, the recent bovine study conducted by Sun et al. (2015) who investigated potential biomarkers of milk production and quality using GC-time-of-flight/MS analyses of rumen fluid, milk, serum and urine claimed to detect the highest number of metabolites (i.e., 647). However, careful reading of the manuscript shows that they only formally identified 123. The remaining "metabolites" were unidentified MS

peaks or features. In ovine metabolomic studies, Parveen and colleagues (2007) reported 168 out of 205 detected metabolites using GC-MS to investigate sheep plasma and feces. Clark et al. (2014) reported 97 metabolites out of the 571 detected features in caprine serum using a combination of both GC-MS and LC-MS. In equine metabolomics, the highest number of metabolites identified was from a study conducted by Escalona and colleagues (2015) with 102 metabolites identified via NMR analysis of plasma, urine and fecal water. A porcine metabolomics study by Metzler-Zebeli et al. (2015) reported 104 out of 132 detected serum metabolites using LC-MS.

Overall, our analysis shows a total of 1070 non-redundant or unique metabolites have been detected and/or quantified in the livestock metabolomics literature. Bovine studies covered the majority of detected metabolites (i.e., 768 different compounds) over multiple sample types. Porcine and ovine studies have the next highest number of detected metabolites with 412 and 285 different metabolites, respectively. Caprine and equine studies reported 167 and 109 different metabolites, respectively. The most frequently detected metabolites with >100 separate entries for different animals, biofluids or conditions include: alanine (124 times), valine (112 times), isoleucine (105 times), glycine (101 times), and lactate (101 times). In addition, 26 other metabolites were reported 50-100 times. Metabolites reported more than once and <50 times add to 560 while, 479 metabolites are reported only once.

It is important to provide some context to these numbers, especially with regard to metabolome studies reported for other animal or model species. The estimated size of the mammalian metabolome is >100,000 molecules (Wishart et al., 2007; Bouatra et al., 2013) and the total number of metabolites so far reported and/or theoretically expected to be in the human metabolome or HMDB is just over 42,000 (Wishart et al., 2016). While the number of expected or theoretical metabolites is large, the actual number of experimentally identified (and/or

quantified) metabolites is actually quite small. For instance, based on the HMDB, the number of experimentally identified metabolites in the human metabolome is 3821 (Wishart et al., 2016), in the *E. coli* metabolome it is 891 (Sajed et al., 2016) and in the yeast metabolome it is 625 (Jewison et al., 2012). Among the different livestock species, it is clear that the coverage of the bovine metabolome is quite extensive and is approaching or even exceeding that of other model organisms. However, there is an obvious gap in terms of the coverage of other livestock species with caprine and equine metabolomes being very poorly characterized. Much more work is needed on goat and horse metabolomes to bring them up to the level seen in the bovine metabolome.

Trends and Gaps in Animal Breeds

While we have largely focused on examining metabolomics data for different livestock species, we also noticed some interesting trends with regard to the choice of specific breeds in each livestock species. Similar to other fields of bovine research, the majority (45%) of bovine metabolomic studies use either pure- or cross-bred Holsteins. A smaller amount (11%) of other studies used cross breeds to investigate various aspects of the bovine metabolome. Other common bovine breeds used in metabolomic studies include Charolais (7%) and Jersey (3%). In ovine metabolomic studies, the main breed reported is Suffolk (19%) while other breeds (i.e., Sarda) are reported only once or twice. For caprine metabolomics studies, the preferred breeds have been Norwegian (22%) with other breeds such as Saanen and Alpine being reported only once. Likewise, among equine and porcine metabolomic studies, Standardbred horses (33%) and Landrace sows (22%) were most frequently used. Interestingly, no breed information was provided in 18%, 33%, 22%, 17%, and 9% of the bovine, ovine, caprine, equine, and porcine metabolomics manuscripts, respectively. It is surprising that this essential information is not provided in the

manuscripts. This suggests the reporting standards found in livestock metabolomics manuscripts still needs improvement.

Based on the above statistics, one of the more obvious gaps in current livestock metabolomics research is the limited variety of breeds being used in most metabolomic studies. The vast majority of the published research appears to be focused on just one or two main breeds i.e., Holstein in cattle, Suffolk in sheep, Standardbred in horses. Evidently, assessing breed differences and their potential impacts on the metabolome has not been a priority for most livestock researchers. However, it is important to remember that the existence of dozens of livestock breeds is a consequence of centuries of selection for very unique phenotypic qualities – some of which are likely determined by their metabolism or metabolome. Different breeds will be characterized by specific production or metabolic parameters and these may be fundamentally different between breeds. While the composition of mammalian (and livestock) metabolomes is likely to be highly similar, metabolite concentrations are expected to differ substantially between different breeds. Identifying the unique aspects affiliated with each breed's metabolome is therefore, an important component of livestock metabolomics that should be considered in future studies. This is particularly true for purebred and breeding stock herds that are limited to very few animals/herds worldwide. Breeding stock animals provide most of the genetic background found in most commercial herds, which means they have a significant influence on the metabolome associated with their progeny. We were also surprised by the very limited research on the neonatal livestock metabolome. Indeed, we found only 16 neonate metabolomic studies, with 1 study focused on calves, 4 on lambs, 1 on kids, 10 on piglets and no studies on colts or foals.

Trends and Gaps in Biomarker Discovery

One of the strengths of metabolomics lies in its utility for biomarker discovery (Xia et al., 2013). Because metabolites can be more easily, cheaply and routinely quantified than most other biological molecules, they are ideal for use in biomarker panels. Indeed, metabolite biomarkers continue to be developed and used in clinical applications at a much greater rate than genes or proteins (Wishart et al., 2016). In surveying the papers compiled for this review, we found a total of 11 livestock metabolomics papers that proposed candidate biomarkers. This included 5 papers in animal health, 1 in animal nutrition, 2 in animal production, 1 in animal reproduction and 2 for animal models of human health. These studies were limited to cattle, sheep and pigs with no metabolomic biomarker studies being reported for goats or horses. Of these papers, we observed that most reported fewer than 30 candidate biomarkers, with the lowest number being 2 (Klein et al., 2012). A few reports used higher number of metabolites, i.e., 64, as part of a statistical model to increase the accuracy of prediction (Osorio et al., 2013; Imhasly et al., 2014). The majority (55%) of metabolomic biomarker papers did not provide any quantitative data, but rather reported only relative metabolite trends (up or down relative to some indeterminate standard). This means that only 5 papers, all from the bovine group, effectively provided useful or verifiable biomarker data. Furthermore, only a single paper (Karisa et al., 2014) reported follow-up validation studies where the initially discovered biomarkers were subsequently validated on a separate cohort of samples.

Based on our data, most biomarker studies were conducted with relatively small sample sizes with the majority of studies being done on fewer than 100 animals. The largest biomarker study was one conducted on 321 animals (1587 samples), which investigated prognostic biomarkers of ketosis in dairy cows using NMR spectroscopy (Klein et al., 2012). Overall, the

quality of biomarker studies done for livestock metabolomics is not particularly good, especially given the standards expected of human biomarker studies (Xia et al., 2013).

Nevertheless, among the reported biomarker studies, we did find some very interesting and compelling results. One example is a biomarker study of RFI and other feed efficiency traits in beef steers (Karisa et al., 2014). In this study, NMR spectroscopy was used to identify and quantify plasma metabolites associated with RFI, initially in a discovery population and subsequently in the validation cohort. Karisa et al. (2014) reported 3 candidate biomarkers of RFI that significantly ($P<0.05$) account for >30% of the phenotypic variation for this trait. Other metabolites were proposed to be associated with average body weight, average feed intake, dry matter intake and average daily gain. In another interesting study, predictive biomarkers of transition diseases in dairy cows were investigated (Hailemariam et al., 2014). This study monitored only 12 dairy cows over four time points during the transition (pre- and post-calving) period. Blood samples were drawn to quantify the metabolome changes associated with various periparturient diseases post-calving. Using direct flow injection (DFI)-MS, Hailemariam and colleagues (2014) profiled 120 blood metabolites of which 3 were suggested as candidate biomarkers for transition diseases, with a sensitivity and specificity of ≥85%. Another study reported by Gray et al. (2015) looked into biomarkers associated with vaccine efficacy. Using UPLC-MS metabolomic measurement of plasma derived from Holstein male calves, Gray and colleagues (2015) found 12 metabolites that were altered post-vaccination. These biomarkers are being proposed as a newer, more efficient route to optimize vaccination and to make vaccine formulation and benchmarking much more efficient and targeted. This paper emphasizes on the importance of disease prevention and vaccination procedures in livestock, especially in using new technologies such as metabolomics to enhance evaluation of vaccine efficacy.

Identification of biomarkers will not only improve disease diagnosis but also allow the opportunity for disease prediction prior to manifestation of clinical signs. For example, if a metabolic disorder can be predicted well before (sub)clinical manifestation, farmers can make informative decisions with regards to their management, feeding, housing, etc., to change the cascade of biological events leading to that disease. Predictive attempts of such can make a significant financial and sustainability difference by maintaining production quantity and quality, saving on costs associated with treatment, veterinary visits, preventing animal culling and thus, maintaining longevity.

The Livestock Metabolome Database

In assembling the material for this review, we identified a total of 1070 metabolites that have been detected and/or quantified in livestock metabolomic studies of cattle, sheep, goats, horses and pigs. This information has been systematically categorized into LMDB with all of the metabolites being fully described including information about the degree or quality of quantification (i.e., quantified, non-quantified) and the source sample types for each livestock species. All of the metabolites with quantitative data had their concentrations converted into a standardized concentration unit (i.e., μM) to improve consistency. In addition to the chemical data and source information, an abbreviated description of the experimental context for each metabolite was extracted from the articles and included in the online database (www.lmdb.ca). This information includes data on the analytical platform(s), experimental conditions, field of research, and animal breed used in acquiring the metabolomic data. All metabolites are linked to a standard HMDB (http://www.hmdb.ca/) identification number, which provides a freely-accessible and detailed description of the metabolite. A PubMed and/or DOI identifier is also associated with each

metabolite entry, which provides a literature reference or a direct link to the article reporting that metabolite for readers who are interested in further details.

Only those metabolites that had reasonably complete descriptions (i.e., unique chemical names, sample types, source information, etc.) were included in the online database. A number of metabolites or "features" were identified during the review process but not included in the LMDB. These include those compounds that have either not been characterized at all (no chemical name, no data on sample types), or not fully characterized (unknown or undefined chemical structure). This collection of 415 "unknown" metabolites will be added to the LMDB once we can obtain sufficient structural and sample source information on them. Among the metabolites entered into the LMDB, 404 compounds were quantified and 666 were not. On a species level there were 768 bovine metabolites, 285 ovine metabolites, 167 caprine metabolites, 109 equine metabolites, and 412 porcine metabolites. Detailed descriptions of each compound are provided in the LMDB "metabocard" pages. Likewise, structural images, molecular formulas, names and synonyms, chemical classification/taxonomy information, physicochemical data (molecular weights, pI's, pKa's, boiling/melting points), referential spectral data (both experimental and theoretical NMR, MS/MS and EI-MS spectra), links to other online databases and full reference (authors, journals, volumes, etc.) information is also provided. The LMDB has been designed so that it can be easily browsed and it supports searches through standard text queries as well as via structure, mass, and spectral queries. Most of the information in the LMDB is hyperlinked to other resources within the LMDB, allowing for a more convenient and compact route to access the data. The LMDB is available at www.lmdb.ca. This database will be constantly updated with more metabolites and more detailed metabolite descriptions as more research in livestock metabolomics is published.

By assembling the LMDB and making this information freely available through both the web and this manuscript, we hoped to create a referential resource that other livestock researchers could readily use. Our past experience in assembling and maintaining the Human Metabolome Database (HMDB) clearly showed how useful a centralized, on-line resource could be in the field of human metabolomics (Wishart et al., 2013). Therefore, our expectation with the LMDB is that it will have a comparable impact on the field of livestock metabolomics. Indeed, we believe that establishing a comprehensive repository that stores and categorizes livestock metabolome information into a standardized format will be critical for future livestock research. It will also be important for identifying potential livestock disease biomarkers, improving animal selection (via metabolomic assays), enhancing animal nutrition and understanding novel biochemical mechanisms arising from various physiological perturbations. With more and more livestock metabolomics papers appearing each year and the continued growth in metabolite coverage, it will be challenging to maintain the LMDB. However, without even attempting to create the LMDB we suspect that livestock metabolomics would continue to lag behind the metabolomics activities seen in other areas (i.e. human, plant crops, microbes, food/beverage studies) and would face significant hurdles in the coming years trying to catch up.

Conclusion

Metabolomics is less than 15 years old, yet it has already delivered some remarkable achievements. This includes significant improvements in the ability to identify many environmental contaminants and toxins (Skelton et al., 2014), significant advances in food and nutrient characterization (Kim et al., 2016; Wishart, 2008), the identification of many novel biomarkers for disease risk including risk markers for diabetes (Wang-Sattler et al., 2012), heart disease (Shah et al., 2012) and cancer (Shajahan-Haq et al., 2015) as well as promising leads for a variety of drugs and therapies (Corona

et al., 2012). Metabolomics is also well-positioned to provide some important advances in both livestock research and the livestock industry, especially as it relates to livestock health, breeding and production. A number of examples were highlighted in this review including metabolome discovery for normal metabolite composition and concentrations (Saleem et al., 2013; Escalona et al., 2015), identification of biomarkers of transition diseases (Hailemariam et al., 2014) as well as production traits in dairy (Sun et al., 2015) and beef cattle (Karisa et al., 2014) with the goal of introducing prognostic strategies in animal health as well as increasing prediction accuracies. Our observations also showed that a wide variety of biofuids have received attention for metabolomics research such as metabolic profiling of milk, plasma, serum, and urine, minimizing animal welfare concerns.

However, in order for livestock metabolomics to deliver on the promise and the excitement seen in other areas of metabolomics research, it is important to carefully assess what has been accomplished, what is known and what still needs to be done. The intent of this review was to provide a critical overview of the trends and gaps in livestock metabolomics research. Specifically, we sought answers to 4 key questions: 1) What are the most common applications of metabolomics in animal science and where are they trending?, 2) What are the preferred metabolomics technologies livestock metabolomics and how are they evolving?, 3) What are the most obvious gaps or weaknesses in livestock metabolomics relative to other fields of metabolomics research? and 4) What are the known or measured metabolites for the 5 major livestock species (i.e., bovine, ovine, caprine, equine, and porcine) in different tissues and biofluids? In addressing the first 3 questions we focused on areas relating to: 1) Animal Choices; 2) Research Applications; 3) Sample Size; 4) Sample Type; 5) Instrumentation and Methodologies, 6) Quantification; 6) Metabolite Coverage; 7) Animal Breeds, and 8) Biomarker Identification. In many cases we were able to

identify some clear trends while at the same time identifying important shortcoming or areas where further improvements could be made. It was apparent that livestock metabolomics appears to be ahead with regard to metabolite quantification, the diversity of research applications and its efforts in biomarker identification. On the other hand, it was also clear that livestock metabolomics (especially with regard to sample size, instrumentation and metabolite coverage) was lagging somewhat further behind than human, microbial or plant crop metabolomics.

Based on our assessment of the shortcomings with current livestock metabolomics studies, it is clear that future metabolomics research should focus on expanding or extending metabolome discovery using healthy control animals, increase sample numbers, direct more effort towards metabolite quantification, perform more integrated multi-omics experiments, use a greater variety of analytical platforms or techniques (including ICP-MS, MSI and fluxomics), increase the breadth of metabolite coverage (by using more sensitive platforms, such as ESI-MS), investigate a greater and new varieties of biosamples such as semen, amniotic fluid, saliva and urine, extend the number and types of animal breeds used in metabolomic studies and be more conscientious in the design and implementation of biomarker studies.

Another important outcome of this study was the collection and consolidation of livestock metabolite information into a single, centralized resource (the LMDB). It became readily apparent in conducting this review that the livestock metabolomics literature is highly diffuse and that valuable information is being "lost" or is not readily available. By compiling the LMDB and making an on-line version of the database freely available, we hope it could serve as a hub for livestock researchers and the livestock industry to further advance the field of livestock metabolomics.

Literature Cited

1. Abarghuei, M. J., Y. Rouzbehan, A. Z. M. Salem, and M. J. Zamiri. 2014. Nitrogen balance, blood metabolites and milk fatty acid composition of dairy cows fed pomegranate-peel extract. Livest. Sci. 164:72-80. https://doi.org/10.1016/j.livsci.2014.03.021.
2. Alves, R. D. A. M., A. D. Dane, A. Harms, K. Strassburg, R. Maleki Seifar, L. B. Verdijk, S. Kersten, R. Berger, T. Hankemeier, and R. J. Vreeken. 2015. Global profiling of the muscle metabolome: method optimization, validation and application to determine exercise-induced metabolic effects. Metabolomics. 11:271-285. https://doi.org/10.1007/s11306-014-0701-7.
3. Andreas, N. J., M. J. Hyde, M. Gomez-Romero, M. A. Lopez-Gonzalvez, A. Villaseñor, A. Wijeyesekera, C. Barbas, N. Modi, E. Holmes, and I. Garcia-Perez. 2015. Multiplatform characterization of dynamic changes in breast milk during lactation. Electrophoresis. 36:2269–2285. https://doi.org/10.1002/elps.201500011.
4. Bouatra, S., F. Aziat, R. Mandal, A. C. Guo, M. R. Wilson, C. Knox, T. C. Bjorndahl, R. Krishnamurthy, F. Saleem, P. Liu, and Z. T. Dame. 2013. The human urine metabolome. PLoS One. 8:e73076. https://doi.org/10.1371/journal.pone.0073076.
5. Bussche, J. V., M. Marzorati, D. Laukens, and L. Vanhaecke. 2015. Validated high resolution mass spectrometry-based approach for metabolomic fingerprinting of the human gut phenotype. Anal. Chem. 87:10927–10934. https://doi.org/10.1021/acs.analchem.5b02688.
6. Castejón, D., J. M. García-Segura, R. Escudero, A. Herrera, and M. I. Cambero. 2015. Metabolomics of meat exudate: Its potential to evaluate beef meat conservation and aging. Anal. Chim. Acta. 901:1–11. https://doi.org/10.1016/j.aca.2015.08.032.
7. Cevallos-Cevallos, J. M., M. D. Danyluk, and J. I. Reyes-De-Corcuera. 2011. GC-MS based metabolomics for rapos simultaneous detection of Escherichia coli O157:H7, Salmonella

Typhimurium, Salmonella Muenchen, and Salmonella Hartford in ground beef and chicken. J. Food Sci. 76:M238-246. https://doi.org/10.1111/j.1750-3841.2011.02132.x.

8. Chapinal, N., M. E. Carson, S. J. LeBlanc, K. E. Leslie, S. Godden, M. Capel, J. E. P. Santos, M. W. Overton, and T. F. Duffield. 2012. The association of serum metabolites in the transition period with milk production and early-lactation reproductive performance. J. Dairy Sci. 95:1301-1309. https://doi.org/10.3168/jds.2011-4724.
9. Chen, X., C. Xie, L. Sun, J. Ding, and H. Cai. 2015. Longitudinal metabolomics profiling of Parkinson's disease-related α-synuclein A53T transgenic mice. PLoS One. 10:e0136612. https://doi.org/10.1371/journal.pone.0136612.
10. Clark, M., J. D. Murray, and E. A. Maga. 2014. Assessing unintended effects of a mammary-specific transgene at the whole animal level in host and non-target animals. Transgenic Res. 23:245–256. https://doi.org/10.1007/s11248-013-9768-6.
11. Corona, G., F. Rizzolio, A. Giordano, and G. Toffoli. 2012. Pharmaco-metabolomics: an emerging "omics" tool for the personalization of anticancer treatments and identification of new valuable therapeutic targets. J. Cell Physiol. 227:2827-2831. https://doi.org/10.1002/jcp.24003.
12. Duggan, G. E., D. S. Hittel, C. W. Sensen, A. M. Weljie, H. J. Vogel, and J. Shearer. 2011. Metabolomic response to exercise training in lean and diet-induced obese mice. J. Appl. Physiol. 110:1311-1318. https://doi.org/10.1152/japplphysiol.00701.2010.
13. Escalona, E. E., J. Leng, A. C. Dona, C. A. Merrifield, E. Holmes, C. J. Proudman, and J. R. Swann. 2015. Dominant components of the Thoroughbred metabolome characterized by 1H-nuclear magnetic resonance spectroscopy: A metabolite atlas of common biofluids. Equine Vet. J. 47:721-730. https://doi.org/10.1371/journal.pone.0246290.

14. Fiehn, O. 2002. Metabolomics – the link between genotypes and phenotypes. Plant Mol. Biol. 48:155-171. https://doi.org/10.1007/978-94-010-0448-0_11.

15. Fontanesi, L. 2016. Metabolomics and livestock genomics: Insights into a phenotyping frontier and its applications in animal breeding. Anim. Front. 6:73-79. https://doi.org/10.2527/af.2016-0011.

16. Ganna, A., T. Fall, S. Salihovic, W. Lee, C. D. Broeckling, J. Kumar, S. Hägg, M. Stenemo, P. K. Magnusson, J. E. Prenni, and L. Lind. 2016. Large-scale non-targeted metabolomic profiling in three human population-based studies. Metabolomics. 12: 4. https://doi.org/10.1007/s11306-015-0893-5.

17. Gilany, K., R. S. Moazeni-Pourasil, N. Jafarzadeh, and E. Savadi-Shiraz. 2014. Metabolomics fingerprinting of the human seminal plasma of asthenozoospermic patients. Mol. Repro. Dev. 81:84-86. https://doi.org/10.1002/mrd.22284.

18. Ghazi, N., M. Arjmand, Z. Akbari, A. O. Mellati, H. Saheb-Kashaf, and Z. Zamani. 2016. 1H NMR-based metabolomics approaches as non-invasive tools for diagnosis of endometriosis. Int. J. Reprod. Biomed. (Yazd). 14:1–8.

19. Gray, D. W., M. D. Welsh, S. Doherty, F. Mansoor, O. P. Chevallier, C. T. Elliott, and M. H. Mooney. 2015. Identification of systemic immune response markers through metabolomics profiling of plasma from calves given an intra-nasally delivered respiratory vaccine. Vet. Res. 46:7. https://doi.org/10.1186/s13567-014-0138-z.

20. Hailemariam, D., R. Mandal, F. Saleem, S. M. Dunn, D. S. Wishart, and B. N. Ametaj. 2014. Identification of predictive biomarkers of disease state in transition dairy cows. J. Dairy Sci. 97:2680–2693. https://doi.org/10.3168/jds.2013-6803.

21. Houle, D., D. R. Govindaraju, and S. Omholt. 2010. Phenomics: the next challenge. Nat. Rev. Genet. 11:855-866. https://doi.org/10.1038/nrg2897.

22. Imhasly, S., H. Naegeli, S. Baumann, M. von Bergen, A. Luch, H. Jungnickel, S. Potratz, and C. Gerspach. 2014. Metabolomic biomarkers correlating with hepatic lipidosis in dairy cows. BMC. Vet. Res. 10:122. https://doi.org/10.1186/1746-6148-10-122.

23. Jalali, A., A. Hatamie, T. Safarpour, A. Khajeamiri, T. Safa, and F. Buazar. 2016. Impact of pharmaceutical impurities in Ecstasy tablets: gas chromatography-mass spectrometry study. Iran. J. Pharm. Res. 15:221-229. https://search.informit.org/doi/10.3316/informit.883492148347757.

24. Jewison, T., V. Neveu, J. Lee, C. Knox, P. Liu, R. Mandal, R. Krishnamurthy, I. Sinelnikov, and M. Wilson. 2012. YMDB: The Yeast Metabolome Database. Nucleic Acids Res. 40:D815-20. https://doi.org/10.1093/nar/gkr916.

25. Johnson, C. H., J. Ivanisevic, and G. Siuzdak. 2016. Metabolomics: beyond biomarkers and towards mechanisms. Nat. Rev. Mol. Cell Biol. 17:451–459. https://doi.org/10.1038/nrm.2016.25.

26. Jones, D. P., Y. Park, and T. R. Ziegler. 2012. Nutritional metabolomics: progress in addressing complexity in diet and health. Annu. Rev. Nutr. 32:183-202. https://doi.org/10.1146/annurev-nutr-072610-145159.

27. Karisa, B. K., J. Thomson, Z. Wang, C. Li, Y. R. Montanholi, S. P. Miller, S. S. Moore, and G. S. Plastow. 2014. Plasma metabolites associated with residual feed intake and other productivity performance traits in beef cattle. Liv. Sci. 165:200–211. https://doi.org/10.1016/j.livsci.2014.03.002.

28. Karisa, B., S. Moore, and G. Plastow. 2013b. Complicated relationships: a review of biological interaction networks and pathways in animal science. Springer Sci. Rev. 1:73–83. DOI 10.1007/s40362-013-0005-8.

29. Karisa, B. K., J. Thomson, Z. Wang, H. L. Bruce, G. S. Plastow, and S. S. Moore. 2013a. Candidate genes and biological pathways associated with carcass quality traits in beef cattle. Can. J. Anim. Sci. 93:295-306. https://doi.org/10.4141/cjas2012-136.

30. Kim, S., J. Kim, E. J. Yun, and K. H. Kim. 2016. Food metabolomics: from farm to human. Curr. Opin. Biotechnol. 37:16-23. https://doi.org/10.1016/j.copbio.2015.09.004.

31. Klein, M. S., N. Buttchereit, S. P. Miemczyk, A. K. Immervoll, C. Louis, S. Wiedemann, W. Junge, G. Thaller, P. J. Oefner, and W. Gronwald. 2012. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. J. Proteome Res. 11:1373–1381. https://doi.org/10.1021/pr201017n.

32. Kordalewska, M., and M. J. Markuszewski. 2015. Metabolomics in cardiovascular diseases. J. Pharm. Biomed. Anal. 113:121-136. https://doi.org/10.1016/j.jpba.2015.04.021.

33. Kosmides, A. K., K. Kamisoglu, S. E. Calvano, S. A. Corbett, and I. P. Androulakis. 2013. Metabolomic fingerprinting: Challenges and opportunities. Crit. Rev. Biomed. Eng. 41:205–221. DOI: 10.1615/CritRevBiomedEng.2013007736.

34. Kuhn, C., R. Weikard, and P. Widmann. 2014. Metabolomics: a pathway for improved understanding of genetic modulation of mammalian growth and tissue deposition. Proc. 10th World Cong. Genet. App. Livest. Prod. 1-6. http://wcgalp.org/system/files/proceedings/2014/metabolomics-pathway-improved-understanding-genetic-modulation-mammalian-growth-and-tissue.pdf.

35. LeBlanc, S. J., K. E. Leslie, and T. F. Duffield. 2005. Metabolic predictors of displaced abomasum in dairy cattle. J. Dairy Sci. 88:159-170. https://doi.org/10.3168/jds.S0022-0302(05)72674-6.

36. Lu, J., E. Antunes Fernandes, A. E. Páez Cano, J. Vinitwatanakhun, S. Boeren, T. van Hooijdonk, A. van Knegsel, J. Vervoort, and K. A. Hettinga. 2013. Changes in milk proteome and metabolome associated with dry period length, energy balance, and lactation stage in postparturient dairy cows. J. Proteome Res. 12:3288-3296. https://doi.org/10.1021/pr4001306.

37. Mahdavi, V., M. M. Farimani, F. Fathi, and A. Ghassempour. 2015. A targeted metabolomics approach toward understanding metabolic variations in rice under pesticide stress. Anal. Biochem. 478:65-72. https://doi.org/10.1016/j.ab.2015.02.021.

38. Mahdavi, V., F. Ghanati, and A. Ghassempour. 2016. Integrated pathway-based and network-based analysis of GC-MS rice metabolomics data under diazinon stress to infer affected biological pathways. Anal. Biochem. 494:31-36. https://doi.org/10.1016/j.ab.2015.10.017.

39. Mathew, S., S. Krug, T. Skurk, A. Halama, A. Stank, A. Artati, C. Prehn, J. A. Malek, G. Kastenmüller, W. Römisch-Margl, and J. Adamski. 2014. Metabolomics of Ramadan fasting: an opportunity for the controlled study of physiological responses to food intake. J. Transl. Med. 12:161. https://doi.org/10.1186/1479-5876-12-161.

40. May, D. H., S. L. Navarro, I. Ruczinski, J. Hogan, Y. Ogata, Y. Schwarz, L. Levy, T. Holzman, M. W. McIntosh, and J. W. Lampe. 2013. Metabolomic profiling of urine: response to a randomized, controlled feeding study of select fruits and vegetables, and application to an observational study. Br. J. Nutr. 110:1760-1770. doi:10.1017/S000711451300127X.

41. McLaughlin, M. J., D. R. Parker, and J. M. Clarke. 1999. Metals and micronutrients - food safety issues. Field Crop. Res. 60:143-163. https://doi.org/10.1016/S0378-4290(98)00137-3.

42. Melzer, N., D. Wittenburg, S. Hartwig, S. Jakubowski, U. Kesting, L. Willmitzer, J. Lisec, N. Reinsch, and D. Repsilber. 2012. Investigating associations between milk metabolite profiles and milk traits of Holstein cows. J. Dairy Sci. 96:1521-1534. https://doi.org/10.3168/jds.2012-5743.

43. Melzer, N., D. Wittenburg, and D. Repsilber. 2013. Integrating milk metabolite profile information for the prediction of traditional milk traits based on SNP information for Holstein cows. PLOS One. 8:e70256. https://doi.org/10.1371/journal.pone.0070256.

44. Merrifield, C. A., M. Lewis, S. P. Claus, O. P. Beckonert, M. Dumas, S. Duncker, S. Kochhar, S. Rezzi, J. C. Lindon, M. Bailey, and E. Holmes. 2011. A metabolic system-wide characterization of the pig: a model for human physiology. Mol. Biosyst. 7:2577-2588. https://doi.org/10.1039/C1MB05023K.

45. Metzler-Zebeli, B. U., R. Ertl, D. Klein, and Q. Zebeli. 2015. Explorative study of metabolic adaptations to various dietary calcium intakes and cereal sources on serum metabolome and hepatic gene expression in juvenile pigs. Metabolomics. 11:545–558. https://doi.org/10.1007/s11306-014-0714-2.

46. Mickiewicz, B., B. J. Heard, J. K. Chau, M. Chung, D. A. Hart, N. G. Shrive, C. B. Frank, and H. J. Vogel. 2015. Metabolic profiling of synovial fluid in a unilateral Ovine model of anterior cruciate ligament reconstruction of the knee suggests biomarkers for early osteoarthritis. J. Orthop. Res. 33:71-77. https://doi.org/10.1002/jor.22743.

47. Minai-Tehrani, A., N. Jafarzadeh, and K. Gilany. 2015. Metabolomics: a state-of-the-art technology for better understanding of male infertility. Andrologia. 48:609-616. doi:10.1111/and.12496.

48. Mirsaeidi, M., M. M. Banoei, B. W. Winston, and D. E. Schraufnagel. 2015. Metabolomics: applications and promise in mycobacterial disease. Ann. Am. Thorac. Soc. 12:1278-1287. https://doi.org/10.1513/AnnalsATS.201505-279PS.

49. Moco, S., S. Collino, S. Rezzi, and F. J. Martin. 2013. Metabolomics perspectives in pediatric research. Pediatr. Res. 73:570-576. https://doi.org/10.1038/pr.2013.1.

50. Mohamad, N., R. I. Ismet, M. Rofiee, Z. Bannur, T. Hennessy, M. Selvaraj, A. Ahmad, F. Nor, T. A. Rahman, K. M. Isa, and A. Ismail. 2015. Metabolomics and partial least square discriminant analysis to predict history of myocardial infarction of self-claimed healthy subjects: validity and feasibility for clinical practice. J. Clin. Bioinform. 5:3. https://doi.org/10.1186/s13336-015-0018-4.

51. Monteiro, M. S., M. Carvalho, M. L. Bastos, and P. Guedes de Pinho. 2013. Metabolomics analysis for biomarker discovery: Advances and challenges. Curr. Med. Chem. 20:257-271. https://www.ingentaconnect.com/content/ben/cmc/2013/00000020/00000002/art00006.

52. Moore, E. R., J. Kirwan, M. K. Doherty, and P. D. Whitfield. 2007. Biomarker discovery in animal health and disease: the application of post-genomics technologies. Biomark. Insights. 2:185-196. https://doi.org/10.1177/117727190700200040.

53. Moore, S. S., F. D. Mujibi, and E. L. Sherman. 2009. Molecular basis for residual feed intake in beef cattle. J. Anim. Sci. 87:E41–E47. https://doi.org/10.2527/jas.2008-1418.

54. Morrow, A. L., A. J. Lagomarcino, K. R. Schibler, D. H. Taft, Z. Yu, B. Wang, M. Altaye, M. Wagner, D. Gevers, D. V. Ward, and M. A. Kennedy. 2013. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. Microbiome. 1:13. https://doi.org/10.1186/2049-2618-1-13.

55. Nielsen, K. L., M. L. Hartvigsen, M. S. Hedemann, H. N. Lærke, K. Hermansen, and K. E. B. Knudsen. 2014. Similar metabolic responses in pigs and humans to breads with different contents and compositions of dietary fibers: a metabolomics study. Am. J. Clin. Nutr. 99:941–949. https://doi.org/10.3945/ajcn.113.074724.

56. Norris, J. L., and R. M. Caprioli. 2013. Analysis of tissue specimens by matrix-assisted laser desorption/ionization imaging mass spectrometry in biological and clinical research. Chem. Rev. 113:2309–2342. https://doi.org/10.1021/cr3004295.

57. Oliver, S. G., M. K. Winson, D. B. Kell, and F. Baganz. 1998. Systematic functional analysis of the yeast genome. Trends Biotech. 16:373-378. https://doi.org/10.1016/S0167-7799(98)01214-1.

58. Osorio, M. T., G. Downey, A. P. Moloney, F. T. Röhrle, G. Luciano, O. Schmidt, and F. J. Monahan. 2013. Beef authentication using dietary markers: Chemometric selection and modelling of significant beef biomarkers using concatenated data from multiple analytical methods. Food Chem. 141:2795–2801. https://doi.org/10.1016/j.foodchem.2013.05.118.

59. Park, Y. W., M. Juarez, M. Ramosc, and G. F. W. Haenlein. 2007. Physico-chemical characteristics of goat and sheep milk. Small Rum. Res. 68:88–113. https://doi.org/10.1016/j.smallrumres.2006.09.013.

60. Parveen, I., J. M. Moorby, M. D. Fraser, G. G. Allison, and J. Kopka. 2007. Application of gas chromatography-mass spectrometry metabolite profiling techniques to the analysis of heathland plant diets of sheep. J. Agric. Food Chem. 55:1129-1138. https://doi.org/10.1021/jf062995w.

61. Pearson H. 2007. Meet the human metabolome. Nature. 446:8. https://doi.org/10.1038/446008a.

62. Peters, R. J. B., A. A. M. Stolker, J. G. J. Mol, A. Lommen, E. Lyris, Y. Angelis, A. Vonaparti, M. Stamou, C. Georgakopoulos, and M. W. F. Nielen. 2010. Screening in veterinary drug analysis and sports doping control based on full-scan, accurate-mass spectrometry. Tren. Anal. Chem. 29:1250-1268. https://doi.org/10.1016/j.trac.2010.07.012.

63. Psychogios, N., D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend, and D. S. Wishart. 2011. The human serum metabolome. PLoS ONE. 6:e16957. doi:10.1371/journal.pone.0016957.

64. Regal, P., C. Nebot, M. Díaz-Bao, R. Barreiro, A. Cepeda, and C. Fente. 2011. Disturbance in sex-steroid serum profiles of cattle in response to exogenous estradiol: a screening approach to detect forbidden treatments. Steriods. 76:365-375. https://doi.org/10.1016/j.steroids.2010.12.005.

65. Reinehr, T., B. Wolters, C. Knop, N. Lass, C. Hellmuth, U. Harder, W. Peissner, S. Wahl, H. Grallert, J. Adamski, T. Illig, C. Prehn, Z. Yu, R. Wang-Sattler, and B. Koletzko. 2014. Changes in the serum metabolite profile in obese children with weight loss. Eur. J. Nutr. 54:173-181. https://doi.org/10.1007/s00394-014-0698-8.

66. Robinson, T. P., G. R. W. Wint, G. Conchedda, T. P. Van Boeckel, V. Ercoli, E. Palamara, G. Cinardi, L. D'Aietti, S. I. Hay, and M. Gilbert. 2014. Mapping the global distribution of livestock. PLoS One. 9:e96084. https://doi.org/10.1371/journal.pone.0096084.

67. Römpp, A., J. Both, A. Brunelle, R. M. A. Heeren, O. Laprévote, B. Prideaux, A. Seyer, B. Spengler, M. Stoeckli, and D. F. Smith. 2015. Mass spectrometry imaging of biological tissue:

an approach for multicenter studies. Anal. Bioanal. Chem. 407:2329. https://doi.org/10.1007/s00216-014-8410-7.

68. Sajed, T., A. Marcu, M. Ramirez, A. Pon, A. Guo, C. Knox, M. Wilson, J. R. Grant, Y. Djoumbou, and D. S. Wishart. 2016. ECMDB 2.0: A richer resource for understanding the biochemistry of E. coli. Nucleic Acids Res. 44:D495-501. https://doi.org/10.1093/nar/gkv1060.
69. Saleem, F., B. N. Ametaj, S. Bouatra, R. Mandal, Q. Zebeli, S. M. Dunn, and D. S. Wishart. 2012. A metabolomics approach to uncover the effect of grain diets on rumen health in dairy cows. J. Dairy Sci. 95:6606-6623. https://doi.org/10.3168/jds.2012-5403.
70. Saleem, F., S. Bouatra, A. C. Guo, N. Psychogios, R. Mandal, S. M. Dunn, B. N. Ametaj, and D. S. Wishart. 2013. The bovine ruminal fluid metabolome. Metabolomics. 9:360-378. https://doi.org/10.1007/s11306-012-0458-9.
71. Shah, S. H., W. E. Kraus, and C. B. Newgard. 2012. Metabolomic profiling for the identification of novel biomarkers and mechanisms related to common cardiovascular diseases. Circulation. 169:1110-1120. https://doi.org/10.1161/CIRCULATIONAHA.111.060368.
72. Shajahan-Haq, A. N., M. S. Cheema, and R. Clarke. 2015. Application of metabolomics in drug resistant breast cancer research. Metabolites. 5:100-118. https://doi.org/10.3390/metabo5010100.
73. Simo, C., C. Ibanez, A. Valdes, A. Cifuentes, and V. Garcia-Canas. 2014. Metabolomics of genetically modified crops. Int. J. Mol. Sci. 15:18941-18966. https://doi.org/10.3390/ijms151018941.

74. Singh, R., N. Gautam, A. Mishra, and R. Gupta. 2011. Heavy metals and living systems: An overview. Indian J. Pharmacol. 43:246-53. doi: 10.4103/0253-7613.81505.

75. Skelton, D. M., D. R. Ekman, D. Martinović-Weigelt, G. T. Ankley, D. L. Villeneuve, Q. Teng, and T. W. Collette. 2014. Metabolomics for in situ environmental monitoring of surface waters impacted by contaminants from both point and nonpoint sources. Environ. Sci. Technol. 48:2395–2403. https://doi.org/10.1021/es404021f.

76. Srivastava, A., G. M. Kowalski, D. L. Callahan, P. J. Meikle, and D. J. Creek. 2016. Strategies for extending metabolomics studies with stable isotope labelling and fluxomics. Metabolites. 6:E32. https://doi.org/10.3390/metabo6040032.

77. Steinshamn, H., R. A. Inglingstadb, D. Ekebergb, J. Mølmanna, and M. Jørgensen. 2014. Effect of forage type and season on Norwegian dairy goat milk production and quality. Small Rum. Res. 122:18–30. https://doi.org/10.1016/j.smallrumres.2014.07.013.

78. Summer, L. W., Z. Lei, B. J. Nikolau, and K. Saito. 2015. Modern plant metabolomics: advanced natural product gene discoveries, improved technologies, and future prospects. Nat. Prod. Rep. 32:212-229. https://doi.org/10.1039/C4NP00072B.

79. Sun, H., D. Wang, B. Wang, J. Wang, H. Liu, L. L. Guan, and J. X. Liu. 2015. Metabolomics of four biofluids from dairy cows: potential biomarkers for milk production and quality. J. Proteome Res. 14:1287−1298. https://doi.org/10.1021/pr501305g.

80. Sundekilde, U. K., N. A. Poulsen, L. B. Larsen, and H. C. Bertram. 2013. Nuclear magnetic resonance metabonomics reveals strong association between milk metabolites and somatic cell count in bovine milk. J. Dairy Sci. 96:290-299. https://doi.org/10.3168/jds.2012-5819.

81. Thornton, P. K. 2010. Livestock production: recent trends, future prospects. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365:2853-2867. https://doi.org/10.1098/rstb.2010.0134.

82. Torii, Y., Y. Kawano, H. Sato, K. Sasaki, T. Fujimori, J. Kawada, O. Takikawa, C. K. Lim, G. J. Guillemin, Y. Ohashi, and Y. Ito. 2016. Quantitative metabolome profiling reveals the involvement of the kynurenine pathway in influenza-associated encephalopathy. Metabolomics. 12:84. https://doi.org/10.1007/s11306-016-1011-z.

83. Tweeddale, H., L. Notley-Mcrobb, and T. Ferenci. 1998. Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool ("metabolome") analysis. J. Bacteriol. 180:5109–5116. DOI: 10.1128/JB.180.19.5109-5116.1998.

84. Verma, M., M. J. Khoury, and J. P. A. Ioannidis. 2013. Opportunities and challenges for selected emerging technologies in cancer epidemiology: mitochondrial, epigenomic, metabolomic, and telomerase profiling. Cancer Epidemiol. Biomar. Prev. 22:189–200. DOI: 10.1158/1055-9965.EPI-12-1263.

85. Viswanathan, V. 2008. Rapid web application development: a ruby on rails tutorial. IEEE Software. 25:98-106. DOI: 10.1109/MS.2008.156.

86. Wang-Sattler, R., Z. Yu, C. Herder, A. C. Messias, A. Floegel, Y. He, K. Heim, M. Campillos, C. Holzapfel, B. Thorand, and H. Grallert. 2012. Novel biomarkers for pre-diabetes identified by metabolomics. Mol. Syst. Biol. 8:615. https://doi.org/10.1038/msb.2012.43.

87. Weikard, R., E. Altmaier, K. Suhre, K. M. Weinberger, H. M. Hammon, E. Albrecht, K. Setoguchi, A. Takasuga, and C. Kühn. 2010. Metabolomic profiles indicate distinct physiological pathways affected by two loci with major divergent effect on Bos taurus growth and lipid deposition. Physiol. Genomics. 42A:79-88. https://doi.org/10.1152/physiolgenomics.00120.2010.

88. Widmann, P., A. Reverter, R. Weikard, K. Suhre, H. M. Hammon, E. Albrecht, and C. Kuehn. 2015. Systems biology analysis merging phenotype, metabolomics and genomic data identifies

non-SMC condensing I complex, subunit G (NCAPG) and cellular maintenance processes as major contributors to genetic variability in bovine feed efficiency. PLoS One. 10:e0124574. https://doi.org/10.1371/journal.pone.0124574.

89. Winter, G., and J. O. Krömer. 2013. Fluxomics - connecting 'omics analysis and phenotypes. Environ. Microbiol. 15:1901-16. https://doi.org/10.1111/1462-2920.12064.
90. Wishart, D. S. 2009. Computational strategies for metabolite identification in metabolomics. Bioanalysis. 1:1579-1596. https://doi.org/10.4155/bio.09.138.
91. Wishart, D. S. 2008. Metabolomics: application to food science and nutrition research. Tren. Food. Sci. Tech. 19:482-493. https://doi.org/10.1016/j.tifs.2008.03.003.
92. Wishart, D. S., R. Mandal, A. Stanislaus, and M. Ramirez-Gaona. 2016. Cancer metabolomics and the human metabolome database. Metabolites. 2:6. https://doi.org/10.3390/metabo6010010
93. Wishart, D. S., D. Tzur, C. Knox, R. Eisner, A. C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, and C. Fung. 2007. HMDB: the Human Metabolome Database. Nucleic Acids Res. 35:D521–526. https://doi.org/10.1093/nar/gkl923.
94. Wishart, D. S., C. Knox, A. C. Guo, R. Eisner, N. Young, B. Gautam, D. D. Hau, N. Psychogios, E. Dong, S. Bouatra, and R. Mandal. 2009. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 37:D603–610. https://doi.org/10.1093/nar/gkn810.
95. Wishart, D. S., T. Jewison, A. C. Guo, M. Wilson, C. Knox, Y. Liu, Y. Djoumbou, R. Mandal, F. Aziat, E. Dong, and S. Bouatra. 2013. HMDB 3.0—The Human Metabolome Database in 2013. Nucleic Acids Res. 41:D801–807. https://doi.org/10.1093/nar/gks1065.

96. Xia, J., D. Broadhurst, M. Wilson, and D. Wishart. 2013. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics. 9:280-299. https://doi.org/10.1007/s11306-012-0482-9.

97. Zhang, A., H. Sun, P. Wang, Y. Han, and X. Wang. 2012. Modern analytical techniques in metabolomics analysis. Analyst. 137:293-300. https://doi.org/10.1039/C1AN15605E.

Tables

Table 1. Categorical comparison. Selected livestock metabolomics articles of 5 major livestock species were categorized based on the area of research, i.e., animal health, animal nutrition, animal production, animal reproduction, human health, animal physiology and animal products. It is noteworthy that articles in the area of human health mainly reflected animal models being used to study human related health issues.

	Bovine	**Ovine**	**Caprine**	**Equine**	**Porcine**
Animal Health	30	6	2	4	10
Animal Nutrition	10	6	5	0	14
Animal Production	22	3	2	2	11
Animal Reproduction	2	1	0	0	3
Human Health	6	4	2	0	14
Animal Products	16	1	0	0	2
Animal Physiology	13	2	0	1	0

Table 2. Sample size. Sample size reported in livestock metabolomics papers were divided into 5 categories with papers using ≤30 samples, 31-50, 51-100, or those that have not mentioned the number of samples used in the analysis.

	Bovine	**Ovine**	**Caprine**	**Equine**	**Porcine**
≤30	25	6	4	5	30
31-50	12	2	0	0	7
51-100	9	4	3	0	1
>100	16	2	0	0	2
Undetermined	13	3	0	0	3

Table 3. Sample types. Different varieties of samples have been used in livestock metabolomics analyses as identified by the number of published articles per sample per livestock specie.

	Bovine	Ovine	Caprine	Equine	Porcine
Adipose	1	0	0	0	0
Amniotic Fluid	0	2	0	0	0
Bile	0	0	0	0	2
Brain	0	1	0	0	1
Cerebral-Spinal Fluid	0	1	0	0	0
Cheese	0	1	0	0	0
Colostrum	1	0	0	0	0
Cream	1	0	0	0	0
Feces	0	1	0	1	0
Follicular Fluid/Media	3	0	0	0	2
Jejunal Tissue	0	0	0	0	2
Kidney	0	0	0	0	1
Kidney Perfusate	0	0	0	0	1
Liver	0	0	0	0	3
Lung	0	0	0	0	1
Meat	7	0	0	0	2
Milk	27	3	3	0	0
Muscle	1	0	0	0	1
Plasma	21	6	0	3	18
Proximal Colon	0	0	0	0	1
Rumen Fluid	7	1	1	0	0
Semen	1	0	0	0	0
Serum	14	3	1	1	15
Synovial Fluid	0	1	0	0	0
Urine	12	2	1	3	8
Vitreous Humor	0	0	1	0	0

Yogurt	0	1	0	0	0

Table 4. Metabolite coverage. The number of metabolites detected, quantified and/or reported among the commonly used sample types in the livestock metabolomics publications up to 2016 (counting publications that reported >8 metabolites).

	Milk	**Plasma**	**Serum**	**Ruminal Fluid**	**Urine**	**Feces**	**Meat**
Number of Metabolites	422	408	351	248	177	158	75

Figures

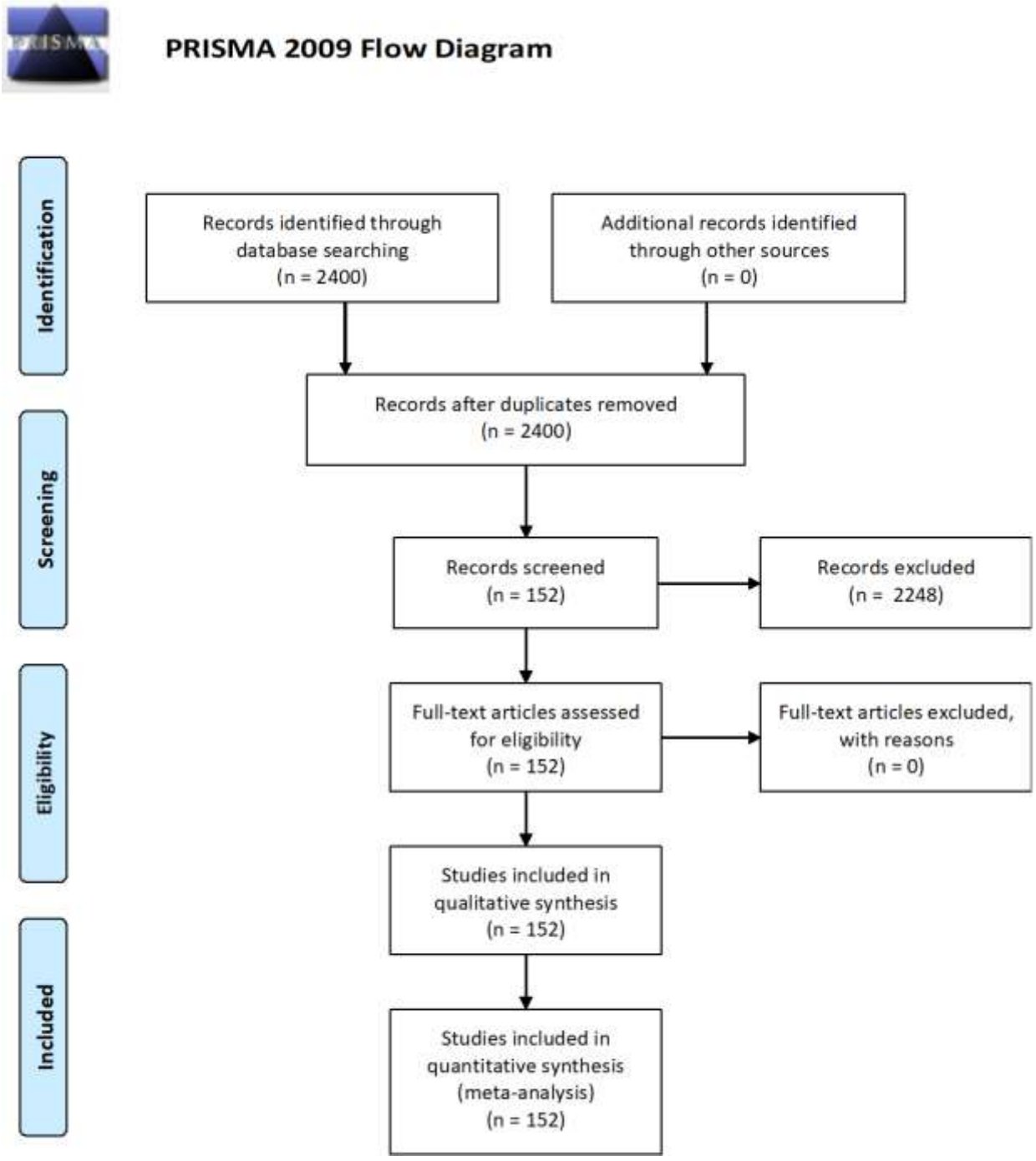


Figure 1. PRISMA diagram. The preferred reporting items for systematic reviews and meta-analysis (PRISMA) flow diagram identifies the total number of articles initially surveyed, the number of articles included and excluded for this systematic review.

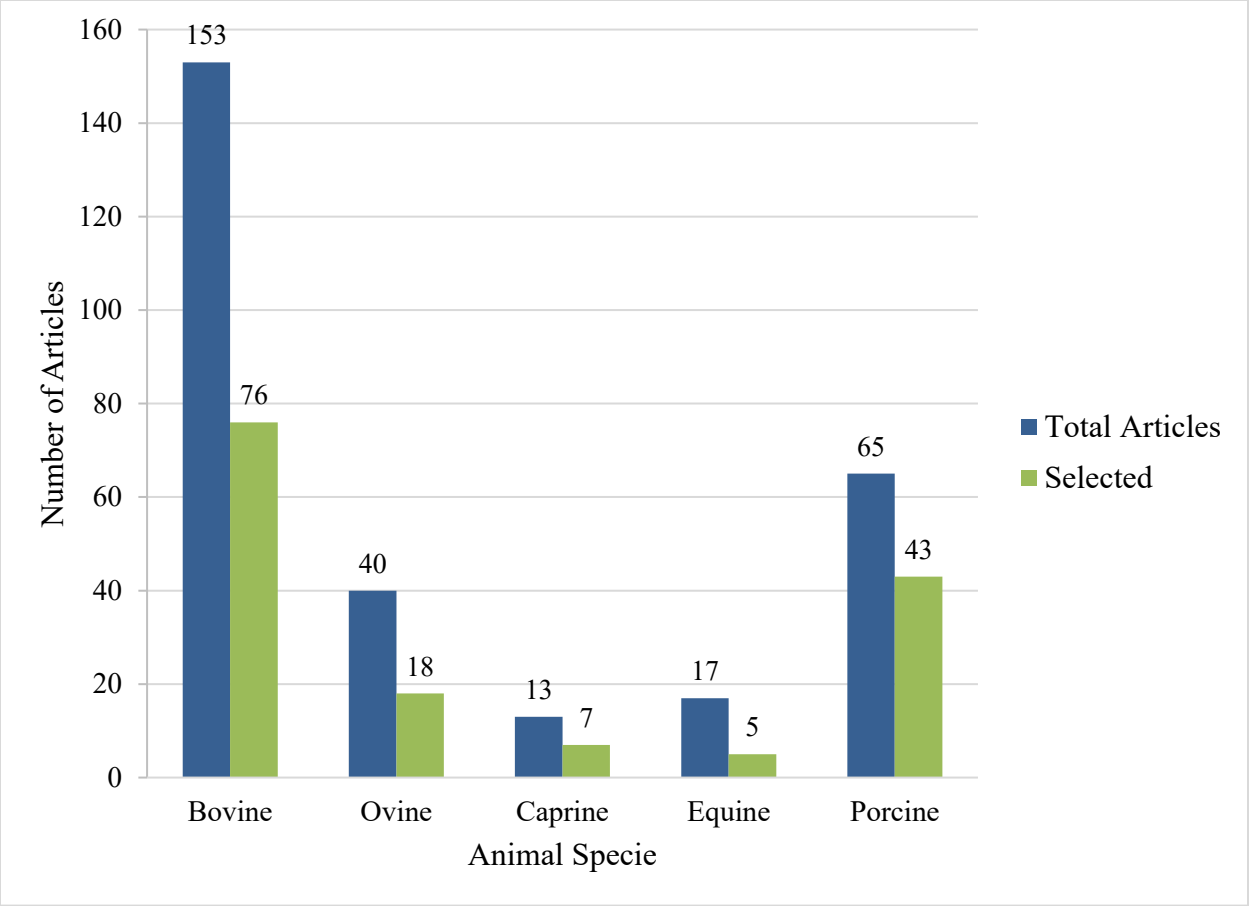


Figure 2. Literature mining. Total number of livestock metabolomics articles considering only articles that reported ≥8 metabolites resulted in selection of 149 manuscripts for this review.

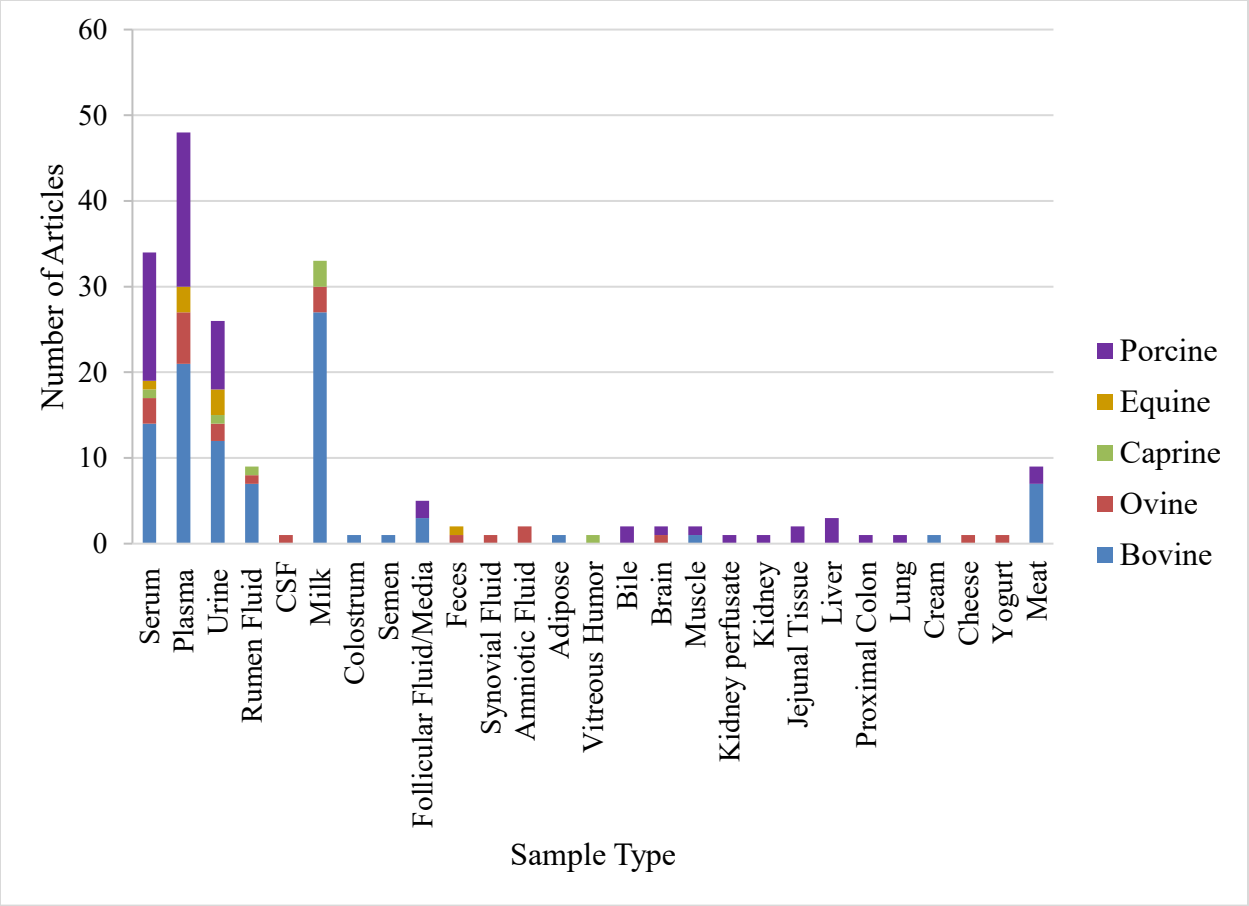


Figure 3. Sample types. Different varieties of samples and animal products have been analyzed in livestock metabolomics studies.

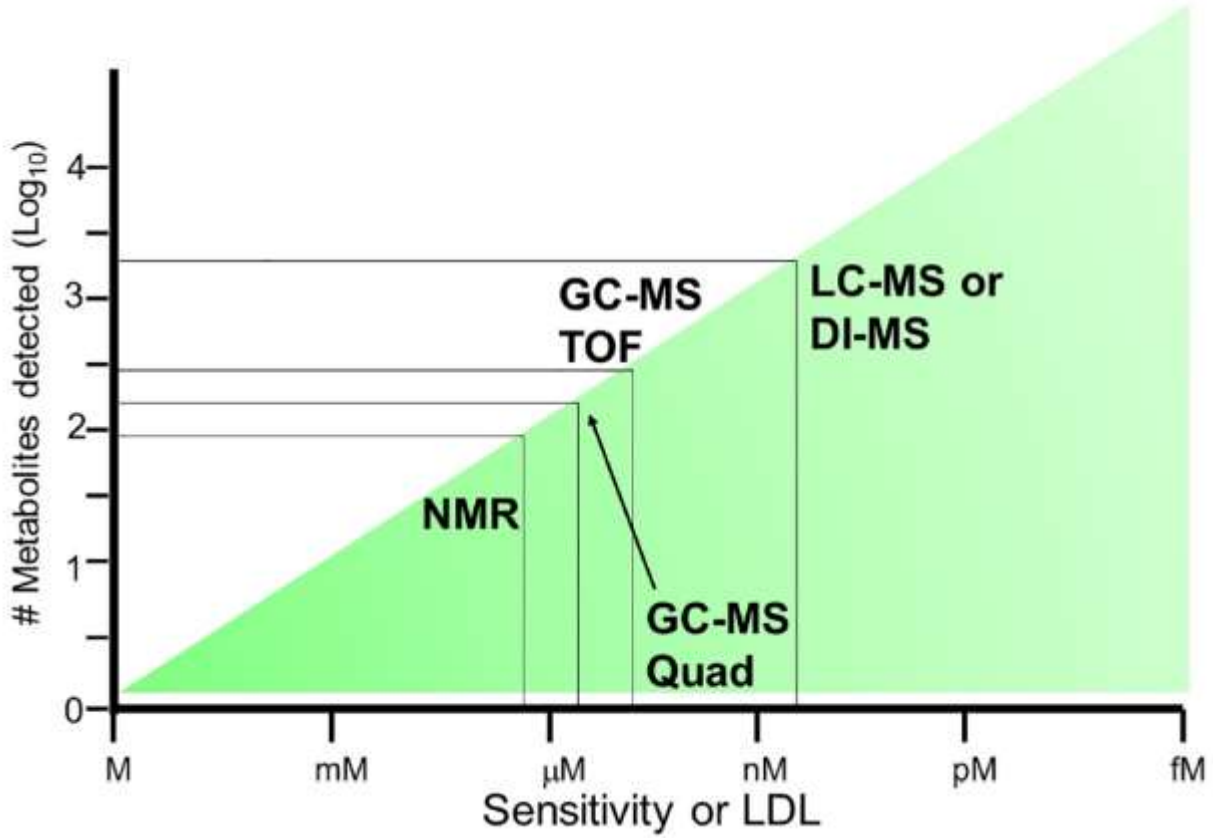


Figure 4. Relative sensitivity of metabolomics platforms. Nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography (LC)-MS are the commonly used metabolomics platforms with varying detection limits.

Chapter 3: Candidate Serum Metabolite Biomarkers of Residual Feed Intake and Carcass Merit in Sheep

Seyed Ali Goldansaz[*,#], Susan Markus[‡], Mark Berjanskii[#], Manoj Rout[#], An Chi Guo[#], Zhiquan Wang[*], Graham Plastow[*], David S. Wishart[#,§]

[*]Department of Agriculture, Food and Nutritional Sciences, University of Alberta, Edmonton, Canada, T6G 2P5

[#]Department of Biological Sciences, University of Alberta, Edmonton, Canada, T6G 2E9

[‡]Alberta Agriculture and Forestry, Stettler, Canada, T0C 2L0

[§]Department of Computing Science, University of Alberta, Edmonton, Canada, T6G 2E8

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Abstract

Mutton and lamb sales continue to grow globally at a rate of 5% per year. However, sheep farming struggles with low profit margins due to high feed costs and modest carcass yields. Selecting those sheep expected to convert feed efficiently and have high carcass merit, as early as possible in their life cycle, could significantly improve the profitability of sheep farming. Unfortunately, direct measurement of feed conversion efficiency (via residual feed intake (RFI)) and carcass merit is a labor-intensive and expensive procedure. Thus, indirect, marker-assisted evaluation of these traits has been explored as a means of reducing the cost of its direct measurement. One promising and potentially inexpensive route to discover biomarkers of RFI and/or carcass merit is metabolomics. Using quantitative metabolomics, we profiled the blood serum metabolome (i.e., the sum of all measurable metabolites) associated with sheep RFI and carcass merit, and identified candidate biomarkers of these traits. The study included 165 crossbred ram-lambs that underwent direct measurement of feed consumption to determine their RFI classification (i.e., low versus high) using the GrowSafe System over a period 40 days. Carcass merit was evaluated after slaughter using standardized methods. Prior to being sent to slaughter one blood sample was drawn from each animal, and serum prepared and frozen at -80 ^{o}C to limit metabolite degradation. A subset of the serum samples was selected based on divergent RFI and carcass quality for further metabolomic analyses. The analyses were conducted using three analytical methods (nuclear magnetic resonance spectroscopy, liquid chromatography mass spectrometry and inductively coupled mass spectrometry), which permitted the identification and quantification of 161 unique metabolites. Biomarker analyses identified three significant (P-value < 0.05) candidate biomarkers of sheep RFI (AUC=0.80), seven candidate biomarkers of carcass yield grade (AUC=0.77) and one candidate biomarker of carcass muscle to bone ratio (AUC=0.74). The identified biomarkers

appear to have roles in regulating energy metabolism and protein synthesis. These results suggest that serum metabolites could be used to categorize and predict sheep for their RFI and carcass merit. Further validation using a larger (3X) and more diverse cohort of sheep is required to confirm these findings.

Key words: Metabolomics, residual feed intake, carcass merit, sheep, biomarker, blood

Introduction

Animal feed contributes up to 85% of the cost of production in livestock farming (Norton 2005; Spring, 2013; Holmgren and Feuz 2015). One approach to mitigate feed costs and increase farm profitability is to select for livestock with higher feed efficiency (Jackson et al., 2014; Muir et al., 2018). Feed-efficient livestock are expected to grow at a rate similar to the rest of the herd while at the same time consuming less feed and producing less manure and methane (Basarab et al., 2003). Residual feed intake (RFI) is an effective method for evaluating feed efficiency, particularly in beef and dairy cattle. The RFI score of each animal is the residual amount of feed the animal consumes compared to the predicted value obtained from similar animals and literature standards (Koch et al., 1963). These calculations are based on animal maintenance and production requirements that are corrected for body weight, fat and animal performance. Therefore, animals with lower RFI will consume less feed than expected and produce less waste while not sacrificing productivity, body weight or size. Because of its utility in identifying high performing animals, along with its moderate heritability (0.11-0.46), RFI is a relevant feed-efficiency trait considered for genetic selection (Muir et al., 2018; Marie-Etancelin et al., 2019).

The concept of RFI has also been used in evaluating feed efficiency of other livestock, including sheep and lambs (Paula et al., 2013). One recent study showed that low-RFI lambs had 12% less dry matter intake (DMI) compared to high-RFI lambs while having similar growth performance as high-RFI lambs (Rajaei Sharifabadi et al., 2012). Other sheep-based RFI studies have shown that DMI can vary up to 30% between the most efficient and least efficient sheep (Muro-Reyes et al., 2011; Redden et al, 2014). Despite its value, direct measurement of RFI has been limited in sheep. Based on standards established for cattle, RFI measurement requires costly equipment and intensive data collection (Rincon-Delgado et al., 2011) for a period of 40 to 90 days

(Wang et al., 2006; Cockrum et al., 2013; Meyer et al., 2015; Manafiazar et al., 2017). Ideally, a set of biomarkers predictive of RFI performance could greatly reduce the cost of direct RFI measurement while making the detection process cheaper and more feasible.

There have been a number of attempts to indirectly measure RFI in sheep using biological markers (Rajaei Sharifabadi et al., 2012) in readily accessible biofluids such as blood (Table 1). Some of the proposed approaches include hormone measurement (Zhang et al., 2017; Knott et al., 2008) as well as hematological and other biochemical measurements (Paula et al., 2013; Rincon-Delgado et al., 2011). These results suggest that multiple biochemical measures of blood serum, if combined together, might yield a useful, indirect measure of RFI. Serum and plasma measurements are widely used in livestock biomarker analyses (Goldansaz et al., 2017). Indeed, serum metabolites (characterized via metabolomic methods) have already been shown to yield useful biomarkers of RFI in cattle (Karisa et al., 2014).

In addition to feed efficiency, carcass yield is another contributing factor to the profitability of livestock farming – especially for sheep. The common practice to evaluate sheep carcass merit on a live animal is via ultrasound measurement of back fat at different locations along the spine (Grill et al., 2015; Morales-Martinez et al., 2020). Most other evaluation methods are limited to post-mortem assessment of the carcass. Unfortunately, this approach to measurement means losing the genetic potential of that animal. Therefore, developing methods to measure carcass merit on live animals would be beneficial. To date, marker-assisted measurement of sheep carcass merit using metabolomics has yet to be explored. However, a few studies have investigated this approach in other livestock species (Connolly et al., 2019).

Here we describe the application of high throughput metabolomics to comprehensively characterize the serum metabolome of sheep and to use this information to identify candidate

serum biomarkers of sheep RFI and carcass merit. In particular, we used a combination of quantitative mass spectrometry (MS) and nuclear magnetic resonance (NMR)-based metabolomics methods to characterize the serum metabolome of 165 ram-lambs. These animals had their feed intake monitored in order to calculate their RFI and measure their carcass quality (muscle-to-bone ratio [MBR] and yield grade [YG]) to determine carcass merit. This work allowed us to identify several promising serum metabolite markers of sheep RFI and carcass merit.

Materials and Methods

Animal Use and Experimental trials

All animal procedures were approved by the respective College Animal Care Committees (2015-RES1 for Lakeland College and Policy No. A20 for Olds College) and the University of Alberta Animal Care Committee (2016.006Wang). Animal trials were conducted at two locations in Alberta at Lakeland College, in the town of Vermilion, and at Olds College, in the town of Olds, in 2015. A total of 165 intact ram-lambs (83 Suffolk x Dorset crossbreds from Lakeland College and 82 Rideau Arcott from Olds College) were used in this study. Lambs at Lakeland College were born, raised and fed at the location and began the feeding trials at an average age of 104 days. Lambs at Olds College were sourced from a private flock and transported to the College, after a minimum period of 6 weeks from date of weaning, and began the trial with an average age of 96±8 days. At each location, the ram-lambs were divided into 2 feeding pens and group-housed with approximately 40 lambs per pen with a similar distribution of animals between pens. Lambs were housed in outdoor pens approximately 20 x 15 meters with protection from wind, shelter from sun, and wood shavings for bedding.

Feed Intake and Feed Efficiency Measurements

Individual daily feed consumption for the lambs was collected using the GrowSafe automated feeding system (GrowSafe Systems Ltd., Airdrie, Canada) following standard feed intake procedures for sheep as outlined in Cammack et al. (2005). This information was used to determine individual RFI values. The GrowSafe system was adapted to be used for sheep by elevating the bottom of the feeding bunks, and the lambs were provided with a platform to raise them 48 cm from the ground level. Each lamb was fitted with an electronic identification device (EID) in one of its ears. Each time a lamb inserted his head into the bunk, the GrowSafe system scanned his EID to record the amount of feed consumed (as measured by feed weight loss from the feeder) and the time spent for each feeding event. Lambs at both farm locations had equal access to four GrowSafe nodes in each pen. Animal feed consisted of either wheat and barley-based total mixed ration (TMR) in a pellet form (PEL) or home mixed whole barley with protein supplement (BAR) ration. In either case, the nutrient content of the rations was exactly the same. All ram-lambs were given *ad libitum* access to their trial diets (Table 4) and water.

Lambs were adapted to their trial diets incrementally during a 14-day period prior to commencement of the experiment. During the adaptation period, the lambs received 75% as fed basis of their creep ration (barley and oat-based feed) with 25% as fed basis of their trial ration (TMR in PEL or home mixed BAR ration). Every three days, 25% would be added to the trial ration and 25% would be reduced from the creep ration. By day 10 of the adaptation period, 100% of the feed consisted of the trial ration.

Residual Feed Intake Calculation

The duration of the experiment and feed intake measurements were 77 to 101 days at the Lakeland and Olds College sites, respectively. However, a 40-day data collection period was used to make

RFI calculations (Wang et al., 2006; Manafiazar et al., 2017). Lambs were weighed on days one and two at the beginning of the trial and the weights were then averaged to obtain the initial start-of-test weights. In addition, in the morning and prior to new feed being added to the bunks, weekly measurements of live animal weights were taken throughout the trial. Initially, the observed feed intake of each animal was converted to DMI based on the moisture percentage of the feed. Then the energy content of the DMI was converted into MJ/kg of DMI for each animal. Furthermore, the RFI for each animal was estimated by regressing the standardized energy content (SMJ/kg) to the average daily gain (ADG), as well as the ADG adjusted for backfat content, the metabolic body weight and the ultrasound BF. The residual of the above regression line was deemed as the estimated RFI of each animal.

Carcass Measurements

Ultrasound BF and loin area measurements measured via an A6V portable ultrasound system equipped with a L761V linear transducer set at 507 MHz (SonoScape Medical Corp., China). Measurements were taken from live animals at the start of the trial on day 1 and again at the end of the trial after the lambs were transported to the slaughter location. Measurements of BF were required for RFI calculations to account for any differences in initial BF values. The BF measurements were taken as fat depth at three locations: 1) above maximum muscle depth, 2) 10 mm further away from spine, and 3) 20 mm further away from spine.

When lambs reached a suitable market weight (typically 52 kg) they were grouped and transported to a federally inspected commercial plant (SunGold Specialty Meats, Innisfail, Alberta) for processing following standard slaughtering procedures. Carcass measurements, including YG and MBR, were determined for each lamb upon slaughter at the slaughterhouse. The YG measurement includes the total tissue depth (both fat and lean tissue) measured 11 cm from

the centerline over the 12th and 13th ribs. In addition, leg muscle circumference (taken at the cod level from the right leg) and bone circumference (taken 2 cm below the hock joint on the right leg) were measured on chilled carcasses 24 hours after slaughter to calculate a leg MBR.

Blood Collection and Serum Processing

Blood samples were drawn from the jugular vein at the end of each trial before the lambs were sent to the slaughterhouse and approximately three hours prior to feeding. Blood (n=165) was collected using 21-gauge needles (PrecisionGlide®, USA) and vacutainers coated with no anti-coagulant (BD Vacutainer, USA) for a maximum volume of 10 ml. Blood samples were kept on ice upon collection. Samples were spun with a centrifuge (Beckman Coulter, USA) for 30 minutes at 16,000 x g at 4 °C. The serum was then transferred to Eppendorf tubes (Axygen, USA) and snap frozen on dry ice. Frozen serum samples were stored at -80 °C until used for metabolomic analyses.

Metabolomics Experiments

Nuclear Magnetic Resonance (NMR) Spectroscopy. Serum samples contain a large proportion of macromolecules (i.e., proteins and lipoproteins), which affects the identification of metabolites with low molecular weight via NMR. Therefore, samples were deproteinized by ultra-filtration as described by Psychogios et al. (2011) and further processed with buffer, phasing and chemical shift reference standards as described by Foroutan et al. (2019). The NMR sample (total volume of 250 µL including serum and buffer solution) was then transferred to a 3 mm SampleJet NMR tube for spectral analysis. All ^{1}H-NMR spectra were collected on a 700 MHz Avance III (Bruker, USA) spectrometer equipped with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) cryoprobe. All spectra were acquired at 25 °C using the first transient of the NOESY pre-saturation pulse sequence (noesy1dpr), chosen for its high degree of quantitative accuracy (Saude et al., 2006). All FID's (free induction decays) were zero-filled to 250 K data points. The singlet produced by the

sodium trimethylsilylpropanesulfonate (DSS) methyl groups was used as an internal standard for chemical shift referencing (set to 0.00 ppm). For quantification, all ^{1}H-NMR spectra were processed and analyzed using a modified version of the Bayesil automated analysis software package with a custom metabolite library (Ravanbakhsh et al., 2015). The modified version of Bayesil allows for qualitative and quantitative analysis of an NMR spectrum by automatically fitting spectral signatures from an internal database to the spectrum. Based on the library, most visible peaks were assigned and annotated with a compound name. It has been previously shown that this fitting procedure provides absolute concentration accuracy of 90% or better (Ravanbakhsh et al., 2015). This method allows the identification and quantification of more than 50 metabolites including amino acids, biogenic amines, carboxylic acids, organo-nitrogens and keto acids (please refer to Table 5 for further details).

Direct Injection Liquid Chromatography Mass Spectrometry/Mass Spectrometry (DI/LC-MS/MS). A targeted, fully quantitative metabolite profiling approach was employed that combined DI-MS/MS with reverse-phase liquid chromatography to determine the concentrations of a wide range of metabolites. These analyses were performed using an in-house quantitative metabolomics kit (called The Metabolomics Innovation Center [TMIC] Prime). This kit, when used with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA) coupled with an AB SCIEX QTRAP® 4000 mass spectrometer (Sciex Canada, Concord, Canada), can identify and quantify up to 119 compounds (including amino acids, biogenic amines, glucose, organic acids, acylcarnitines, phosphatidylcholines (PCs), lysophosphatidylcholines (LysoPCs), sphingomyelins (SMs), and hydroxysphingomyelins (SM(OHs)). The absolute quantification of water-soluble compounds including amino acids, organic acids, and biogenic amines was ensured by using two separate UHPLC injections with C18 column separations. On the other hand, glucose and the

various lipid classes (acylcarnitines, PCs, LysoPCs, SMs, etc.) are measured by two column-free, DI-MS methods. While initially designed and calibrated for human metabolomic studies, the measurable ranges of metabolite concentrations available through the TMIC Prime kit match very closely with the known or expected metabolite concentrations in sheep biofluids (as determined via orthogonal NMR experiments and high levels of agreement with published literature data).

The detection of each metabolite in the TMIC Prime kit relies on multiple reaction monitoring (MRM). The kit incorporates both isotope-labeled internal standards and other quality control (QC) standards into its 96-well filter plate to ensure accurate compound quantification. The 96 deep-well plate contained a filter plate attached with sealing tape, and reagents and solvents used to prepare the plate assay. The first 14 wells were used for one blank, seven standards and three quality control samples. These initial wells are used for building calibration curves and QCs, while the other 82 wells are used for sample analysis. For all biofluids analyzed with this assay, both the original sample (without dilution) and diluted samples (10×) were analyzed to ensure correct calibration and quantification. For all metabolites except organic acid, samples were thawed on ice and were vortexed and centrifuged at 13,000x g. Ten µL of each sample was loaded onto the center of the filter on the upper 96-well plate and dried in a stream of nitrogen. Subsequently, phenyl-isothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator. Extraction of the metabolites was then achieved by adding 300 µL of extraction solvent. The extracts were obtained by centrifugation into the lower 96-deep well plate, followed by a dilution step with MS running solvent.

For organic acid analysis, 150 µL of ice-cold methanol and 10 µL of isotope-labeled internal standard mixture was added to 50 µL of serum sample for overnight protein precipitation. Then it was centrifuged at 13000x g for 20 min. Fifty µL of supernatant was loaded into the center

of wells of a 96-deep well plate, followed by the addition of 3-nitrophenylhydrazine reagent. After incubation for 2h, BHT stabilizer and water were added before LC-MS injection.

All LC-MS/MS and DI-MS/MS assays were performed on a Qtrap® 4000 tandem mass spectrometry instrument (Sciex Canada, Concord, Canada) equipped with an Agilent 1260 series HPLC system (Agilent Technologies, Palo Alto, CA). The Analyst software 1.6.2 (Concord, Canada) was used to control the entire assay's workflow.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS). All trace elemental analysis was performed on a Perkin-Elmer NexION 350x ICP-MS (Perkin-Elmer, Woodbridge, Canada), operating in a kinetic energy discrimination (KED) mode. Argon (ICP/MS grade, 99.999 %) was used as a nebulizer (0.9 mL min^{-1}), an auxiliary (1 mL min^{-1}) and a plasma gas (15 mL min^{-1}). Helium (He) was used as non-reactive collision gas (Cell gas A: 4.3) to eliminate/minimize chemical interference. Prior to ICP-MS analysis, a total of 200 µL of each serum sample was collected in a metal free tube and was centrifuged at 14,000 x g for 2 minutes in order to obtain a homogeneous dispersion. The sample was then diluted to 2 mL (10x dilution) using 5% hydrogen peroxide and 1% nitric acid solution. Internal standard (indium, 10 ppb) was also added to the solution. Blank subtraction was applied after internal standard correction. Typically, a 3 point-calibration curve was used to quantify all compounds. The accuracy of the ICP-MS analytical protocol was periodically evaluated via the analysis of certified reference materials (serum toxicology controls; UTAK Laboratories Inc.).

Statistical Analyses

Metabolomics datasets from all three platforms were pre-processed and normalized using standard methods available via MetaboAnalyst 4.0 (Chong et al., 2019). MetaboAnalyst is a widely used, open-access web server for processing and analyzing metabolomic data. As recommended by

MetaboAnalyst, metabolites with >20% missing values were removed from the dataset prior to statistical analyses. Univariate and multivariate statistical analyses, including fold change, Student's t-test, volcano plot analysis, and partial least squares-discriminant analysis (PLS-DA) were conducted with statistical significance set to a *P*-value < 0.05 and a Benjamini-Hochberg false discovery rate set to $q \leq 0.05$. The volcano plot displays data based on their *P*-value (determined by Student's t-test) versus their fold change. Volcano plots are widely used to rapidly detect and visually display highly significant differences in metabolite, gene or protein expression. Data visualization was also conducted via PLS-DA to observe the separation between the animal groups based on their corresponding serum metabolomic data. The significance of the PLS-DA separation was verified using permutation testing (n=1000). Datasets were then evaluated for candidate biomarkers using receiver operating characteristic (ROC) analysis conducted by logistic regression and measuring the area under the curve (AUC) values. Individual or multiple metabolites with an AUC ≥ 0.70 and a significant permutation test (n=1000; *P*-value < 0.05) were considered as candidate biomarkers for each trait.

Results

Measurements of RFI and Carcass Merit. All 165 sheep had their RFI and carcass merit quantitatively determined. The RFI values ranged from -0.11 to +0.16 kg DM, while YG measurements ranged from 5 to 23 mm and MBR ranged from 2.57 to 3.69 mm. Based on the distribution and precision of the RFI measurements, we determined that an RFI cut-off of ±0.02 kg DM was appropriate to distinguish high-RFI animals from low-RFI animals. Therefore, we selected 69 animals as being low-RFI (RFI≤-0.02) and 33 animals as being high-RFI (RFI≥+0.02). The remaining 63 animals, with RFI values ranging from -0.02 to +0.02, were excluded from both RFI groups, as their RFI measurements were insufficiently distinct (given the RFI measurement

error of ~15%). Based on the distribution and precision of the YG measurements, we excluded animals with a YG score of 11-15 mm and identified 37 animals as having low YG (categorized as YG1 with measurement ≤11 mm) and 41 animals as having high YG (categorized as YG2 with measurement ≥15 mm). The other 87 animals were excluded, as their YG measurements were insufficiently distinct (given the YG measurement error of ~5%) to suggest they were statistically different from one another. Similarly, we excluded animals with an MBR ratio of between 2.80 and 3.00 which led to 27 animals being identified as having low MBR (categorized as MBR1 with a ratio ≤2.80) and 28 animals as having high MBR (categorized as MBR2 with a ratio ≥3.00). By categorizing animals into two groups (high/low-RFI, YG1/YG2 and MBR1/MBR2), biomarker discovery for these traits could be simplified into a standard categorical analysis.

The Serum Metabolome of Sheep. The first objective of this study was to comprehensively and quantitatively characterize the serum metabolome of sheep. As noted in our previous work on the Livestock Metabolome Database (LMDB; Goldansaz et al., 2017), sheep metabolomic studies are relatively scarce. To date, only 52 metabolites had previously been quantified in sheep serum. By performing a comprehensive, quantitative metabolomic analyses of sheep serum over three analytical platforms (NMR, ICP-MS, DI/LC-MS/MS) we were able to identify 161 serum metabolites with unique chemical structures. These metabolites could be classified into 16 broad chemical classes using the ClassyFire ontology (Djoumbou Feunang et al., 2016), and included 42 carboxylic acids and derivatives, 39 fatty acyl derivatives (acylcarnitines), 24 glycerophospholipids, 18 metals, 10 sphingolipids, 9 organo-oxygen compounds, 7 organo-nitrogen compounds, 5 keto acids and derivatives, and 8 “other” groups consisting of chemical classes having less than 5 metabolites. The NMR, ICP-MS and DI/LC-MS/MS platforms measured 60 metabolites, 18 metals and 83 metabolites, respectively. The most frequently measured

metabolites reported by NMR were carboxylic acids and derivatives (33 metabolites), organonitrogen compounds (12 metabolites) and keto acids and derivatives (5 metabolites). The main metabolite classes measured by DI/LC-MS/MS comprised of fatty acyl derivatives (36 metabolites), glycerophospholipids (24 metabolites), sphingolipids (10 metabolites), as well as carboxylic acids and derivatives (9 metabolites). Twenty-seven metabolites (identified by * in Table 5) were new to the LMDB while 100 were new to the sheep serum/plasma metabolome (identified by $^{+}$ in Table 5). In this regard, our study represents the most complete and comprehensive metabolomic study yet reported for sheep serum.

Metabolomic platforms exhibit different levels of sensitivity and coverage. Therefore, metabolite identification and quantification will vary between different analytical platforms. Sample analysis with NMR is able to robustly identify and quantify compounds from the millimolar (mM) to micromolar (μM) range whereas, MS-based methods are more sensitive and can identify and quantify metabolites at lower concentrations, i.e., nanomolar (nM), concentrations (Pinu et al., 2019). Based on our data, the range of metabolite concentrations detected in sheep serum varied from 0.3 μM (dimethylglycine) to 7923 μM (L-Lactic acid) for NMR, from 0.002 μM (spermidine) to 354 μM (citrulline) for LC-MS/MS, and from 0.002 μM (cesium) to 223667.3 μM (sodium) for ICP-MS.

Significant Metabolites Associated with Sheep RFI. The second major objective for this study was to identify those serum metabolites that could distinguish lambs based on their RFI category (high vs. low-RFI). Feature selection was performed using a combination of univariate and multivariate statistics to identify significant (P-value < 0.05, Q-value < 0.05) metabolites that could discriminate the two RFI groups. The features obtained from the uni/multivariate analyses were

then used to inform the ROC curve analysis which led to a high-performing logistic regression model to categorize high/low-RFI animals from serum metabolite measurements.

Univariate analyses. A total of 24 significant metabolites (across all three platforms) were identified by the Student's t-test that distinguished low-RFI from high-RFI sheep (Table 2). These metabolites include four from NMR, three from ICP-MS, and 16 from DI/LC-MS/MS.

Multivariate Analyses. Multivariate statistical analyses produced a variable importance plot (VIP) with 15 metabolites (Figure 1) that contributed significantly to the PLS-DA categorization of the RFI groups. These 15 metabolites consist of nine acylcarnitines [C6, C0, C5, L-acetylcarnitine, C5-OH], two biogenic amines (acetyl-ornithine, spermidine), one amino acid (aminoadipic acid), two fatty acids (butyrate, 2-hydroxyisovalerate), two ketones (ketoleucine, acetone), one alcohol (isopropyl alcohol), one benzenoid (hippuric acid) and one metal (Cs). Seven of these metabolites were detected by DI/LC-MS/MS (C6, C0, C5, C5-OH, acetyl-ornithine, spermidine, aminoadipic acid) and seven were detected by NMR (isopropyl alcohol, L-acetylcarnitine, butyrate, 2-hydroxyisovalerate, ketoleucine, acetone, hippuric acid) while only one was detected by ICP-MS (Cs). All, except four metabolites (L-acetylcarnitine, butyrate, 2-hydroxyisovalerate, acetyl-ornithine), identified via this multivariate method overlap with the significant metabolites identified by univariate methods.

Biomarker detection for sheep RFI. From the significant metabolites identified via univariate and multivariate analysis, we selected a panel of three metabolites (isopropyl alcohol, aminoadipic acid and acetone), based on their VIP values and individual *Q*-values, to serve as candidate biomarkers of sheep RFI. A logistic regression equation for these three candidate biomarkers was used to generate a model with a final AUC of 0.80 (Figure 2) and permutation testing (n=1000) confirmed

its significance (*P*-value = 0.01). The logistic regression model developed for this panel of metabolites is given as follows:

logit(P) = log(P / (1-P)) = 1.174 + 0.645 isopropyl alcohol - 0.465 aminoadipate - 0.037 acetone

where P is the probability of y=1/x with a cut-off of 0.66. Because the concentrations of the metabolites used in this study were sum normalized, log transformed and scaled via mean centering, the value for isopropyl alcohol in the above equation corresponds to (Log_2([isopropyl alcohol]/2442.34) – 9.80)/2.29 (where [isopropyl alcohol] is the measured concentration of this compound by NMR). Likewise, the value for aminoadipic acid corresponds to (Log_2([aminoadipate]/508.18) – 7.12)/1.19 (where [aminoadipate] is the measured concentration of this compound by DI/LC-MS/MS). Similarly, the value for acetone corresponds to (Log_2([acetone]/835.73) – 7.03)/0.99 (where [acetone] is the measured concentration of this compound by NMR).

Significant metabolites of sheep carcass merit. We also sought to identify those serum metabolites that could distinguish high from low carcass merit lambs. Feature selection was performed as previously described to discriminate the YG1 and YG2 groups as well as the MBR1 and MBR2 groups. The features obtained from the analyses were then used to inform the ROC curve analysis and to develop a robust, high-performing logistic regression model to categorize carcass merit traits.

Univariate analyses of MBR. Parametric and non-parametric t-tests did not identify any of the metabolites to be significantly different between the two groups while a volcano plot identified three significant metabolites (lysoPC a C26:1, lysoPC a C28:0, lysoPC a C17:0), all of which were phospholipids detected by DI/LC-MS/MS (Table 6).

Multivariate analyses of MBR. Multivariate statistical analyses using PLS-DA identified the top 15 metabolites that contribute most to the PLS-DA categorization of the MBR groups via VIP analysis (Figure 3). Of these 15 metabolites, two consist of alcohols and polyols (ethanol and isopropyl alcohol), two fatty acids (2-hydroxyisovalerate and butyrate), two biogenic amines (acetyl-ornithine and spermine), three phospholipids (lysoPC a C26:1, lysoPC a C28:0, lysoPC a C17:0), two organic acids (citric acid and acetic acid), two acylcarnitines [C12 (dodecanoylcarnitine) and C3 (propionylcarnitine)], one keto acid (3-methyl-2-oxovaleric acid) and one amino acid (L-aspartic acid). Eight of these metabolites were detected by NMR (ethanol, 2-hydroxyisovalerate, butyrate, isopropyl alcohol, 3-methyl-2-oxovaleric acid, L-aspartate, citric acid, acetic acid) and seven were detected by DI/LC-MS/MS (acetyl-ornithine, lysoPC a C26:1, lysoPC a C28:0, lysoPC a C17:0, C12, C3, spermine). Only three metabolites identified via multivariate analysis overlap with the metabolites detected by univariate analysis (lysoPC a C26:1, lysoPC a C28:0, lysoPC a C17:0).

Biomarker detection for sheep MBR. Among the list of identified metabolites, a combination of three phospholipids overlapping between the volcano plot and VIP (lysoPC a C26:1, lysoPC a C28:0, lysoPC a C17:0) for ROC curve analysis yielded the highest ROC AUC value of 0.68 which had only a tendency towards significance (*P*-value = 0.06). This is while the individual AUC value of lysoPC a C26:1 was 0.74 (Figure 4). Therefore, we selected this single metabolite as the candidate biomarker of sheep MBR.

Univariate analyses of YG. Parametric and non-parametric t-tests did not identify any of the metabolites to be significantly different between the two YG groups while a volcano plot identified two significant metabolites (hippuric acid and citric acid), both of which were detected by NMR (Table 3).

Multivariate analyses of YG. Multivariate statistical analyses identified 15 metabolites that contribute most to the PLS-DA categorization of the YG groups via VIP analysis (Figure 5). Of these metabolites, 10 were detected by NMR (butyrate, propylene glycol, hippuric acid, 2-hydroxyisovalerate, citric acid, dimethylglycine, L-threonine, dimethylamine, L-ornithine, L-acetylcarnitine) and five were detected by DI/LC-MS/MS [trimethylamine N-oxide, C7-DC, spermidine, total dimethylarginine, C5 (valerylcarnitine)]. Only two metabolites identified via multivariate statistics (with a VIP score of more than 2) overlap with those metabolites detected by univariate analysis (hippuric acid and citric acid).

Biomarker detection for sheep YG. From the metabolites identified via univariate and multivariate analysis, we selected a panel of seven metabolites to serve as candidate biomarkers of sheep carcass merit based on their YG measurements. These metabolites include total dimethylarginine, citric acid, hypoxanthine, hippuric acid, asymmetric dimethylarginine, L-phenylalanine, and SM C16:1. A logistic regression model using these seven metabolites yielded a final AUC of 0.77 (Figure 6) and permutation testing (n=1000) confirmed its significance (*P*-value = 0.01). The logistic regression model developed is given as follows:

logit(P) = log(P / (1-P)) = 0.148 + 1.789 SM C16:1 + 1.754 L-phenylalanine – 0.345 asymmetric dimethylarginine + 0.551 hippuric acid + 1.501 hypoxanthine + 1.181 citric acid – 1.953 total dimethylarginine

where P is the probability of y=1/x. The concentrations of the metabolites used in this study had been sum normalized, log transformed and scaled via mean centering. Therefore, the value for candidate biomarkers in the above equation correspond to the following equations: (Log2([SM C16:1]/1.48) – 6.37)/0.51 for SM C16:1 (where [SM C16:1] is the measured concentration of this compound by DI/LC-MS/MS); (Log_2([phenylalanine]/3116.92) – 6.35)/0.46 for L- phenylalanine

(where [phenylalanine] is the measured concentration of this compound by NMR); (Log_2([asymmetric dimethylarginine]/346.63) – 6.42)/0.64 for asymmetric dimethylarginine (where [asymmetric dimethylarginine] is the measured concentration of this compound by DI/LC-MS/MS); (Log_2([hippuric acid]/431.62) – 6.68)/1.28 for hippuric acid (where [hippuric acid] is the measured concentration of this compound by NMR); (Log_2([hypoxanthine]/1411.25) – 6.35)/0.47 for hypoxanthine (where [hypoxanthine] is the measured concentration of this compound by NMR); (Log_2([citric acid]/10600.48) – 6.43)/0.73 for citric acid (where [citric acid] is the measured concentration of this compound by NMR); and (Log_2([total dimethylarginine]/594.74) – 6.42)/0.66 for total dimethylarginine (where [total dimethylarginine] is the measured concentration of this compound by DI/LC-MS/MS). This model was assessed for its significance using a permutation test (n=1000) and was found to be significant (*P*-value = 0.01).

Discussion

The Serum Metabolome of Sheep. In recent years, the application of metabolomics to livestock research has gained momentum. However, the application of metabolomics to sheep research is still lagging (Goldansaz et al., 2017). In an effort to expand application of metabolomics to sheep research, we used three different high throughput metabolomics platforms (NMR, DI/LC-MS/MS, ICP-MS) to comprehensively and quantitatively analyze the sheep serum metabolome. In total, we identified and quantified 161 serum metabolites. These data along with the known literature values of the sheep metabolome have been deposited into the freely accessible LMDB (www.lmdb.ca; Goldansaz et al., 2017). Prior to this work, the sheep metabolome (as contained in LMDB), listed only 288 sheep-associated metabolites of which just 18% were quantified. From the total sheep metabolome revealed in LMDB, only 200 were identified in serum or plasma. Reference values obtained from healthy sheep in LMDB make up 29% of the reported metabolites while the

remainder were gathered from treated sheep. With the addition of these 161 compounds, the serum/plasma metabolome in sheep has grown from 200 to 300 compounds, the total sheep metabolome has increased from 288 to 375 compounds and the percentage of quantified metabolites reported in sheep has nearly tripled from 18% to 49%. Data from this experimental work also adds to the reference values obtained from healthy sheep in LMDB. Moreover, our data present 100 unique metabolites that had not been previously reported in the sheep serum/plasma metabolome.

Literature-Reported Biomarkers of Sheep RFI. There have been limited studies in the literature that investigate markers associated with sheep RFI. One study measured lamb plasma concentrations of five different hormones, two of which (thyroxine and adrenocorticotropic hormone) were identified to have a positive correlation with RFI (Zhang et al., 2017). Another report by Paula et al. (2013) also evaluated serum concentrations of 10 metabolites and enzymes and seven hematological parameters in plasma of ram-lambs. They reported RFI to be associated with protein metabolism as measured by serum albumin and creatinine. Creatinine is also positively correlated with muscle mass and negatively correlated with back fat in sheep (Caldeira et al., 2007; Paula et al., 2013). In another study conducted by Rincon-Delgado and colleagues (2011), evaluation of blood capacity for gas transportation and exchange, as well as immunological characteristics of ewes and rams revealed a positive correlation between RFI and red and white blood cells. This study also reported a significant positive correlation between RFI and serum glucose of ewes and rams, and a tendency for a positive correlation between RFI and triglycerides (only in ewes). Among these studies, none used metabolomics to evaluate the blood profile. More importantly, previously published RFI studies only described correlations while none conducted

quantitative ROC analysis to evaluate the biomarker potential of each significant blood component.

In addition to blood, the rumen microbiome has also been associated with RFI (Patil et al., 2018). A recent study (Ellison et al., 2019) reported six microbial species to have significant correspondence with RFI measurement in sheep. These authors point out that diet dictates the rumen microbiome to a large extent. As a consequence, the results of a rumen analysis may change depending on the ration provided to the animals. Moreover, the Ellison et al. (2019) study used a small cohort of animals (12 ewes) and it did not evaluate the candidate microbial species using standard ROC analysis. It is noteworthy that collecting rumen fluid samples is an expensive and invasive procedure and would not likely be practical or applicable for predicting RFI in large commercial flocks.

Categorizing Sheep RFI via Metabolomics. In this project we used standard methods to determine the RFI in 165 sheep at two different farms and then used serum metabolic profiling to identify metabolites that could be used to distinguish high-RFI from low-RFI animals. To the best of our knowledge, this is the first attempt to evaluate serum biomarkers of sheep RFI using metabolomics. A small number of studies have investigated a small number of blood components associated with sheep RFI however, none of them conducted formal or rigorous biomarker analyses to verify if the compounds could serve as proxies of RFI. On the other hand, a number of studies have used metabolomics to explore biomarkers for RFI in other livestock species such as beef (Karisa et al., 2014; Clemmons et al., 2017; Novais et al., 2019; Jorge-Smeding et al., 2019) and dairy cattle (Wang and Kadarmideen, 2019).

Karisa and colleagues (2014) identified and validated three significant metabolites (creatine, carnitine and hippurate) associated with beef RFI which explained more than 30% of the

phenotypic variation in this trait. Their prediction model, which included these three metabolites, yielded a prediction accuracy of 95%. In another untargeted attempt (Clemmons et al., 2017), four serum metabolites (pantothenate, homocysteine, glutamine, carnitine) were found to be associated with different classes of RFI in steers. Using an MS-based platform, Novais and colleagues (2019) suggested a single unidentified metabolic feature could be associated with the RFI of bulls, and the vitamin A metabolism pathway was critical to RFI differentiation. Another non-targeted evaluation of heifer serum samples (Jorge-Smeding et al., 2019) suggested that the urea cycle and some of its associated metabolites (ornithine, carbamoyl-P, citrulline, aspartate, lysine, valine) were correlated with RFI. Similarly, in dairy cattle, Wang and Kadarmideen (2019) reported three metabolic pathways to be strongly associated with RFI. In addition, individual plasma metabolites were associated with dairy cattle RFI however, these metabolites varied between different breeds of dairy cattle.

Candidate Serum Biomarkers of Sheep RFI. Three metabolites were identified in our project as candidate biomarkers to classify sheep into high and low-RFI groups: acetone, isopropyl alcohol and aminoadipic acid. Acetone was elevated in the serum of low-RFI lambs and reduced in the serum of high-RFI lambs (Figure 1). In addition, low-RFI animals which are more feed efficient have a lower DMI, make less frequent visits to the feed bunk, and spend less time eating (Rajaei Sharifabadi et al., 2012; Redden et al, 2014). Moreover, high levels of ketone bodies, including acetone, are associated with lower insulin levels in ewes (Henze et al., 1998; Senchuk, 2019). When there is an insufficient supply of glucose (from metabolism of feed or due to low insulin levels) to support normal energy demands, the body catabolizes its internal energy sources to produce ketone bodies, such as acetone, to compensate for the energy requirement (Hanuš et al.,

2011; Jones et al., 2018). Considering these facts, we speculate that acetone may have an essential role in energy compensation due to lower feed intake of low-RFI animals.

Similar to acetone, concentrations of isopropyl alcohol were higher in the low-RFI lambs (Figure 1). Isopropyl alcohol is a precursor of acetone (Davis et al., 1984) and its intravenous injection in healthy sheep leads to increasing levels of acetone in the body (Araújo et al., 2013). Ketone bodies, including acetone, possess a glucose-sparing role in ruminants (Heitmann et al., 1987). Furthermore, ketone bodies are often used as a source of energy in the small intestine and peripheral tissues of ruminants (Penner et al., 2011) and are involved in regulation of feed intake (Laeger et al., 2010). Therefore, we speculate that in low-RFI lambs, due to lower DMI and the need for alternative energy substrates such as acetone, higher levels of blood isopropyl alcohol would be used to convert to acetone. This may lead to the high concentration of acetone in the serum of our low-RFI lambs.

The third candidate biomarker of sheep RFI identified in this project was aminoadipic acid which is a product of lysine catabolism (Guidetti and Schwarcz, 2003). Lysine is an essential amino acid which plays a key role in stimulating energy metabolism and protein synthesis. It has been previously associated with RFI in heifers (Jorge-Smeding et al., 2019). In our project, lower levels of aminoadipic acid in low-RFI lambs also corresponded to higher levels of lysine in these lambs. It has been previously shown that *in vitro* administration of aminoadipic acid in cell culture (i.e., increasing levels of aminoadipic acid) decreases protein synthesis (Nishimura et al., 2000). Therefore, low levels of aminoadipic acid (and correspondingly high levels of lysine) would be expected to lead to increased protein synthesis and thereby increase muscle tissue or muscle mass. Low-RFI animals are, in some cases, characterized by greater muscle mass (Herd and Arthur, 2009), a higher proportion of lean meat and lower levels of adipose tissue while high-RFI animals

have the opposite phenotype (Paula et al., 2013; Zhang et al., 2017). While it is interesting to speculate on the possible biological roles of these candidate biomarkers, confirming their roles is beyond the scope of this study and will require further verification in a separate experiment.

Literature-Reported Biomarkers of Sheep Carcass Merit. As far as we are aware, there has been no report on the application of metabolomics for pre-mortem, marker-assisted evaluation of sheep carcass quality. However, a small number of studies have reported a handful of metabolites associated with carcass traits in sheep. Creatinine and creatine have been reported to correspond with carcass lean and fat content (Caldeira et al., 2007; Paula et al., 2013). Sheep having a body condition score of 3 are considered to have an optimal ratio of muscle to fat content. These sheep tend to have lower levels of blood creatinine due to a low rate of protein turnover (Caldeira et al., 2007). Paula and associates (2013) reported serum creatinine having a negative correlation with back fat and a positive correlation with muscle mass. Others have also confirmed an increased amount of blood urea and a decreased concentration of creatinine correlate with higher fat deposition and lower lean growth in sheep and steers (Richardson et al., 2004; Herd et al., 2004). In other livestock species, metabolomics has been more frequently used to evaluate carcass quality. In steers, for example, NMR was used to identify a panel of blood metabolites (3-hydroxybutyrate, propionate, acetate, creatine, histidine, valine, isoleucine, glucose, leucine, anserine, arabinose, aspartate, and arginine) that were associated with carcass marbling, rump fat thickness, carcass weight and growth rate (Connolly et al., 2019). The correlation of these metabolites with different carcass features were used to investigate the underpinning biology of carcass fat and muscle development and to recommend early identification of high value carcasses. To date, the metabolites reported in the literature and associated with carcass merit are too few and do not portray a clear trend. Some of this may be due to the differences in age and maturity of the

experimental animals. For example, growing livestock have a higher rate of protein synthesis and turnover while mature animals, have greater fat deposition (Herd et al., 2004) hence, their metabolite concentrations will vary.

Candidate Serum Biomarkers of Sheep Carcass Merit. Seven metabolites were identified as candidate biomarkers of YG and one metabolite as the candidate biomarker of MBR. The candidate biomarker of MBR, lysoPC a C26:1 is a glycerophospholipid, and has only been associated with increased mobilization of blood fatty acids in dairy cows (Ehret et al., 2015; Klein et al., 2012). There is not enough evidence in the literature to draw a direct relationship between this metabolite and carcass merit in sheep.

As with MBR, we did not identify any previously reported metabolite markers for sheep YG. However, six of the seven candidate biomarkers (except for SM C16:1) for sheep YG identified in this study have been correlated with physiology of muscle development and reduction of carcass fat. For example, phenylalanine, an essential amino acid, hippuric acid and hypoxanthine are three candidate biomarkers that have previously been linked to muscle development, protein synthesis and meat quality in sheep and beef (Liang et al., 2019). Measurement of phenylalanine in meat is quite often used as an index of muscle development (Wester et al., 2000). Hippurate is also recognized as a candidate urine biomarker for beef meat authentication (Osorio et al., 2012). Likewise, hypoxanthine is associated with beef aging and meat quality evaluation (Yano et al., 1995; Escudero et al., 2011). The other group of candidate biomarkers include the methylated-arginine metabolites such as ADMA and total dimethylarginine. These metabolites are known to enhance vasodilatation and endothelial function to improve nutrient delivery to organs via regulating nitric oxide production (Kadkhodaei Elyaderani et al., 2013; Tsikas et al., 2000). Interestingly, ewes consuming a lower amount of feed

have increasing concentrations of circulating ADMA (Berlinguer et al., 2020). In addition to muscle development, the other two candidate biomarkers of sheep YG, hippuric acid and citric acid, are reported to associate with carcass fat content. Hippurate is reported to have a positive linear relationship with visceral fat content (Pallister et al., 2017) while citric acid leads to significant reduction in abdominal fat when introduced in the diet of broilers (Ul-Haq et al., 2014). Considering the physiological role of these metabolites, at least six of the seven compounds we identified appear to have biological relevance to carcass merit and may qualify as confirmed biomarkers upon validation.

Financial Benefits of Sheep RFI Biomarkers. Profit margins in the Canadian sheep industry are very tight. However, producers do have room to improve this margin by reducing the costs of production. When over 40% of the cost of production in a sheep flock is feed-related, improvements in feed efficiency can bring increased profitability to the industry (Norton 2005; Spring, 2013; Holmgren and Feuz 2015). Here we present a brief, high-level financial evaluation of how selecting for sheep with improved feed efficiency (i.e., low-RFI) could improve sheep farming economics.

Early marker-assisted selection for feed-efficient lambs has shown a 12-30% reduction in dry matter consumption while reaching the same market weight (Paula et al., 2013). If farmers target sheep based on animal feed efficiency for their replacement breeding stock or when designing mating groups, we anticipate a 5-10% increase in feed efficiency can be attained in the progeny. We believe that screening for feed efficiency can be much more easily, quickly and far more cheaply attained through rapid screening of the serum metabolites we have identified here (once validated) compared to conventional methods. By doing so, the genetic gain of the progeny towards RFI would save on feed costs, which in Alberta, Canada, over a 5 year period would

average $2.44/feedlot lamb per year or about $351,000.00 per year in total savings (assuming Alberta ewe flock is 100,000 head with a lambing rate of 1.8 lambs/ewe per year, a weaning rate of 80%; 0.29 kg/day/lamb feed savings for 56 days at $0.15/kg). In addition, feed savings from the selection of efficient breeding stock for replacement would also be realized. Over a five-year period, using a ewe replacement rate of 20%, breeding ewes with improved feed efficiency would equate to saving $10.68/ewe per year just on animal feed. In the Albertan sheep industry, this would result in savings of over $1.3 million per year in lamb and ewe feed costs.

As noted earlier, the standard measurement of RFI is expensive (estimated to be $200-300 per animal over a duration of 70-90 days for cattle; Basarab et al., 2013). However, if indirect, marker-assisted RFI measurements could be conducted via metabolomic analysis, the costs for selection or selective breeding could be greatly reduced. A defined panel of 3-4 serum metabolites can typically be detected and quantified by tandem mass spectrometers and NMR in as little as 1-2 minutes and 10-13 minutes per sample, respectively, (Wishart, 2016; Pinu et al., 2019) and cost as low as $5 per sample (or $5 per animal). Centralized animal testing facilities are starting to emerge which would make these kinds of measurements (at even lower costs) quite feasible. Furthermore, if the panel of metabolites identified here could be converted to a pen-side test or a portable handheld device (Koczula and Gallotta, 2016), then measurement costs could be further reduced.

In addition to the direct financial gains, the indirect benefit of marker-assisted selection for low-RFI sheep leads to a lower environmental footprint and a more sustainable farm production. Selecting for multiple generations of feed-efficient ewes (for the maternal flock) and rams (for the breeding stock) will have a positive additive effect on lowering methane production in the long-run (Paganoni et al., 2017). Feed efficient sheep have 12-19% lower methane emissions, while in

ewes this could reach up to 29%, without negatively impacting other production traits (Muro-Reyes et al., 2011). Moreover, selection for low-RFI animals (i.e., higher efficiency) will further improve environmental costs by decreasing grazing area, stocking rate, waste production (>15% decrease in manure N, P, and K), and lead to >25% reduction in methane production (Basarab et al., 2003; Cockrum et al., 2013).

Future prospects. The focus of this discovery-based study was to evaluate the effectiveness of using metabolomics to identify candidate biomarkers of sheep RFI and carcass merit. We successfully identified three significant candidate biomarkers of sheep RFI (AUC=0.80), seven candidate biomarkers of carcass yield grade (AUC=0.77) and one candidate biomarker of carcass muscle to bone ratio (AUC=0.74). While these initial results are promising, further validation using a larger cohort of sheep (approximately 3X larger, based on power analysis) with more diverse genetic backgrounds and from different management settings will be required to confirm the robustness and the potential of these biomarkers. By increasing the size and diversity of the cohort, it may also be possible to extend the work to not only categorize (high vs. low) animals but to predict numerical values of RFI and carcass traits using the biomarkers we have identified.

Disclosures

The authors confirm no conflicts of interest that may affect their ability to objectively present research or data.

Literature Cited

1. Araújo, C. A. S. C., F. A. M. L. Rodrigues, A. H. H. Minervino, B. F. Trivelatto, L. F. D. Reis, C. S. Mori, and E. L. Ortolani. 2013. Clinical evaluation of nervous ketosis induced by isopropanol in sheep. Braz. J. Jet. Jes. Anim. Sci. 50:493-496. https://doi.org/10.11606/issn.1678-4456.v50i6p493-496.
2. Basarab, J. A., M. A. Price, J. L. Aalhus, E. K. Okine, W. M. Snelling, and K. L. Lyle. 2003. Residual feed intake and body composition in young growing cattle. Can. J. Anim. Sci. 83:189-204. https://doi.org/10.4141/A02-065.
3. Basarab, J. A., K. A. Beauchemin, V. S. Baron, K. H. Ominski, L. L. Guan, S. P. Miller, and J. J. Crowley. 2013. Residual feed intake: An indirect approach for reducing GHG emissions. Greenhouse Gases & Animal Agriculture Conference. Dublin. Ireland.
4. Berlinguer, F., C. Porcu, G. Molle, A. Cabiddu, M. Dattena, M. Gallus, V. Pasciu, S. Succu, F. D. Sotgiu, P. Paliogiannis, S. Sotgia, A. A. Mangoni, A. Gonzalez-Bulnes, C. Carru, and A. Zinellu. 2020. Circulating concentrations of key regulators of nitric oxide production in undernourished sheep carrying single and multiple fetuses. Animals. 10:65. https://doi.org/10.3390/ani10010065.
5. Caldeira, R. M., A. T. Belo, C. C. Santos, M. I. Vazques, and A. V. Portugal. 2007. The effect of body condition score on blood metabolites and hormonal profiles in ewes. Sm. Rumin. Res. 68:233-241. https://doi.org/10.1016/j.smallrumres.2005.08.027.
6. Cammack, K. M., K. A. Leymaster, T. G. Jenkins, M. K. Nielsen. 2005. Estimates of genetic parameters for feed intake, feeding behavior, and daily gain in composite ram lambs. J. Anim. Sci. 83:777–785. https://doi.org/10.2527/2005.834777x.

7. Chong, J., D. S. Wishart, and J. Xia. 2019. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. Cur. Prot. Bioinf. 68:e86. doi: 10.1002/cpbi.86.
8. Clemmons, B. A., R. I. Mihelic, R. C. Beckford, J. B. Powers, E. A. Melchior, Z. D. McFarlane, E. R. Cope, M. M. Embree, J. T. Mulliniks, S. R. Campagna, B. H. Voy, and P. R. Myer. 2017. Serum metabolites associated with feed efficiency in black angus steers. Metabolomics. 13:147. https://doi.org/10.1007/s11306-017-1282-z.
9. Cockrum, R. R., R. H. Stobart, S. L. Lake, and K. M. Cammack. 2013. Phenotypic variation in residual feed intake and performance traits in rams. Sm. Rumin. Res. 113:313-322. https://doi.org/10.1016/j.smallrumres.2013.05.001.
10. Connolly, S., A. Dona, L. Wilkinson-White, D. Hamblin, M. D'Occhio, and L. A. González. 2019. Relationship of the blood metabolome to subsequent carcass traits at slaughter in feedlot Wagyu crossbred steers. Sci. Rep. 9:15139. doi:10.1038/s41598-019-51655-2.
11. Davis, P. L., L. A. Dal Cortivo, and J. Maturo. 1984. Endogenous isopropanol: forensic and biochemical implications. J. Anal. Toxicol. 8:209-212. doi:10.1093/jat/8.5.209.
12. Djoumbou Feunang, Y., R. Eisner, C. Knox, L. Chepelev, J. Hastings, G. Owen, E. Fahy, C. Steinbeck, S. Subramanian, E. Bolton, R. Greiner, and D. S. Wishart. 2016. ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. J. Cheminform. 8:61. doi:10.1186/s13321-016-0174-y.
13. Ehret, A., D. Hochstuhl, N. Krattenmacher, J. Tetens, M. S. Klein, W. Gronwald, and G. Thaller. 2015. Short communication: Use of genomic and metabolic information as well as milk performance records for prediction of subclinical ketosis risk via artificial neural networks. J. Dairy Sci. 98:322-329. https://doi.org/10.3168/jds.2014-8602.

14. Ellison, M. J., G. C. Conant, W. R. Lamberson, K. J. Austin, E. van Kirk, H. C. Cunningham, D. C. Rule, and K. M. Cammack. 2019. Predicting residual feed intake status using rumen microbial profiles in ewe lambs. J. Anim. Sci. 97:2878–2888. https://doi.org/10.1093/jas/skz170

15. Escudero, E., L. Mora, M. C. Aristoy, and F. Toldrá. 2011. Possible biological markers of the time of processing of dry-cured ham. Meat. Sci. 89:536-539. https://doi.org/10.1016/j.meatsci.2011.05.002.

16. Foroutan, A., S. A. Goldansaz, M. Lipfert, and D. S. Wishart. 2019. Protocols for NMR analysis in livestock metabolomics. In: Bhattacharya S. (eds) Metabolomics. Methods in Molecular Biology, vol 1996. Humana, New York, NY. https://doi.org/10.1007/978-1-4939-9488-5_23.

17. Goldansaz, S. A., A. C. Guo, T. Sajed, M. A. Steele, G. S. Plastow, and D. S. Wishart. 2017. Livestock metabolomics and the livestock metabolome: A systematic review. PLoS ONE. 12:e0177675. https://doi.org/10.1371/journal.pone.0177675.

18. Grill, L., F. Ringdorfer, R. Baumung, and B. Fuerst-Waltl. 2015. Evaluation of ultrasound scanning to predict carcass composition of Austrian meat sheep. Sm. Rumin. Res. 123:260-268. https://doi.org/10.1016/j.smallrumres.2014.12.005.

19. Guidetti, P., and R. Schwarcz. 2003. Determination of α-aminoadipic acid in brain, peripheral tissues, and body fluids using GC/MS with negative chemical ionization. Brain. Res. Mol. Brain. Res. 118:132-139. https://doi.org/10.1016/j.molbrainres.2003.08.004.

20. Hanuš, O., P. Roubal, M. Vyletělová, T. Yong, M. Bjelka, and A. Dufek. 2011. The relations of some milk indicators of energy metabolism in cow, goat and sheep milk. Sci. Agri. Bohem. 42:102-112.

21. Heitmann, R. N., D. J. Dawes, and S. C. Sensenig. 1987. Hepatic Ketogenesis and Peripheral Ketone Body Utilization in the Ruminant. J. Nutr. 117:1174–1180. https://doi-org.login.ezproxy.library.ualberta.ca/10.1093/jn/117.6.1174.

22. Henze, P., K. Bickhardt, H. Fuhrmann, and H. P. Sallmann. 1998. Spontaneous pregnancy toxaemia (ketosis) in sheep and the role of insulin. Zentralbl Veterinarmed A. 45:255-66. doi:10.1111/j.1439-0442.1998.tb00825.x.

23. Herd, R. M., V. H. Oddy, and E. C, Richardson. 2004. Biological basis for variation in residual feed intake in beef cattle. 1. Review of potential mechanisms. Aust. J. Exp. Agr. 44:423-430. doi:10.1071/EA02221.

24. Herd, R. M., and P. F. Arthur. 2009. Physiological basis of residual feed intake. J. Anim. Sci. 87(Suppl.):E64–E71.

25. Holmgren, L., and D. Feuz. 2015. 2015 costs and returns for a 200 cow, cow-calf operation. All Curr. Pub. 711. http://digitalcommons.usu.edu/extension_curall/711.

26. Jackson, T., J. Heard, and B. Malcolm. 2014. System changes to a lamb farm in south-west Victoria: some pre-experimental modelling. AFBM. J. 11:1–18.

27. Jorge-Smeding, E., G. Renand, D. Centeno, M. Pétéra, S. Durand, S. Polakof, and G. Cantalapiedra-Hijar. 2019. Metabolomics reveals changes in urea cycle associated to residual feed intake in growing heifers. Ene. Pro. Metab. Nutr. 138:231-232. https://doi.org/10.3920/978-90-8686-891-9_50.

28. Jones, A. K., R. E. Gately, T. D. Kellogg, S. A. Zinn, K. E. Govoni, S. A. Reed. 2018. Evaluation of the Nova Vet Meter for sheep-side monitoring of β-hydroxybutyric acid (BHBA) and description of ewe BHBA during late gestation in three flocks from the Northeastern U.S. Res. Vet. Sci. 118:491-497. https://doi.org/10.1016/j.rvsc.2018.05.002.

29. Kadkhodaei Elyaderani, M., A. Malek, M. Ali, M. Rostami, M. Aberomand, and A. Kheirollah. 2013. Inhibitory effect of asymmetric dimethylarginine and NG-Monomethyl-L-arginine methyl ester on nitric oxide synthase activity. J. Gorgan Uni. Med. Sci. 15:84-92.

30. Karisa, B. K., J. Thomson, Z. Wang, C. Li, Y. R. Montanholi, S. P. Miller, S. S. Moore, and G. S. Plastow. 2014. Plasma metabolites associated with residual feed intake and other productivity performance traits in beef cattle. Liv. Sci. 165:200–211. https://doi.org/10.1016/j.livsci.2014.03.002.

31. Klein, M. S., N. Buttchereit, S. P. Miemczyk, A. K. Immervoll, C. Louis, S. Wiedemann, W. Junge, G. Thaller, P. J. Oefner, and W. Gronwald. 2012. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. J. Proteome Res. 11:1373–1381. https://doi.org/10.1021/pr201017n.

32. Knott, S. A., L. J. Cummins, F. R. Dunshea, and B. J. Leury. 2008. The use of different models for the estimation of residual feed intake (RFI) as a measure of feed efficiency in meat sheep. Anim. Feed Sci. Technol. 143:242–255. https://doi.org/10.1016/j.anifeedsci.2007.05.013.

33. Knott, S. A., L. J. Cummins, F. R. Dunshea, and B. J. Leury. 2010. Feed efficiency and body composition are related to cortisol response to adrenocorticotropin hormone and insulin-induced hypoglycemia in rams. Domest. Anim. Endocrinol. 39:137-46. doi: 10.1016/j.domaniend.2010.03.003.

34. Koch, R. M., L. A. Swiger, D. Chambers, and K. E. Gregory. 1963. Efficiency of feed use in beef cattle. J. Anim. Sci. 22:486–494. https://doi.org/10.2527/jas1963.222486x.

35. Koczula, K. M., and A. Gallotta. 2016. Lateral flow assays. Ess. Biochem. 60:111–120. doi: 10.1042/EBC20150012.

36. Laeger, T., C. C. Metges, and B. Kuhla. 2010. Role of β-hydroxybutyric acid in the central regulation of energy balance. Appetite. 54:450-455. https://doi.org/10.1016/j.appet.2010.04.005.

37. Liang, X., X. Bi, M. Kamruzzaman, and H. Sano. 2019. Effect of Chinese herbal medicine on kinetics of plasma phenylalanine, tyrosine and whole body protein synthesis in sheep. Anim. Sci. J. 90:533–538. https://doi.org/10.1111/ asj.13180.

38. Manafiazar, G., J. A. Basarab, L. McKeown, J. Stewart-Smith, V. Baron, M. D. MacNeil, and G. Plastow. 2017. Optimizing feed intake recording and feed efficiency estimation to increase the rate of genetic gain for feed efficiency in beef cattle. Can. J. Anim. Sci. 97:456-465. https://doi.org/10.1139/cjas-2016-0118.

39. Marie-Etancelin, C., D. Francois, J. L. Weisbecker, D. Marcon, C. Moreno-Romieux, F. Bouvier, and F. Tortereau. 2019. Detailed genetic analysis of feeding behaviour in Romane lambs and links with residual feed intake. J. Anim. Breed. Genet. 136:174-182. doi:10.1111/jbg.12392.

40. Meyer, A. M., R. A.Vraspir, M. J. Ellison, and K. M. Cammack. 2015. The relationship of residual feed intake and visceral organ size in growing lambs fed a concentrate- or forage-based diet. Livest. Sci. 176:85-90. https://doi.org/10.1016/j.livsci.2015.03.019.

41. Moore, S. S., F. D. Mujibi, and E. L. Sherman. 2009. Molecular basis for residual feed intake in beef cattle. J. Anim. Sci. 87:E41–E47. https://doi.org/10.2527/jas.2008-1418.

42. Morales-Martinez, M. A., C. Arce-Recinos, M. M. Mendoza-Taco, C. Luna-Palomera, M. A. Ramirez-Bautista, Á. T. Piñeiro-Vazquez, R. Vicente-Perez, L. O. Tedeschi, and A. J. Chay-Canul. 2020. Developing equations for predicting internal body fat in Pelibuey sheep using

ultrasound measurements. Sm. Rumin. Res. 183:106031. https://doi.org/10.1016/j.smallrumres.2019.106031.

43. Muir, S. K., N. Linden, M. Knight, R. Behrendt, and G. Kearney. 2018. Sheep residual feed intake and feeding behaviour: are ‘nibblers’ or ‘binge eaters’ more efficient. Anim. Pro. Sci. 58:1459–1464. doi:10.1071/AN17770.
44. Muro-Reyes, A., H. Gutierrez-Banuelos, L. H. Diaz-Garcia, F. J. Gutierrez-Pina, L. M. Escareno-Sanchez, R. Banuelos-Valenzuela, C. A. Medina-Flores, and A. Corral Luna. 2011. Potential environmental benefits of residual feed intake as strategy to mitigate methane emissions in sheep. J. Anim. Vet. Adv. 10:1551-1556. doi:10.3923/javaa.2011.1551.1556.
45. Nishimura, R. N., D. Santos, S. T. Fu, and B. E. Dwyer. 2000. Induction of cell death by L-alpha-aminoadipic acid exposure in cultured rat astrocytes: relationship to protein synthesis. Neurotoxicology. 21:313–320.
46. Norton, M. 2005. Factors affecting beef and cattle producer prices movements. Mont. Lab. Rev. 128:32.
47. Novais, F. J., P. R. L. Pires, P. A. Alexandre, R. A. Dromms, A. H. Iglesias, J. B. S. Ferraz, M. P. Styczynski, and H. Fukumasu. 2019. Identification of a metabolomic signature associated with feed efficiency in beef cattle. BMC Genomics. 20:8. doi:10.1186/s12864-018-5406-2.
48. Osorio, M., A. Moloney, L. Brennan, and F. Monahan. 2012. Authentication of beef production systems using a metabolomic-based approach. Animal. 6:167-172. doi:10.1017/S1751731111001418.
49. Pallister, T., M. Jackson, T. C. Martin, C. A. Glastonbury, A. Jennings, M. Beaumont, R. P. Mohney, K. S. Small, A. MacGregor, C. J. Steves, A. Cassidy, T. D. Spector, C. Menni, and

A. M. Valdes. 2017. Untangling the relationship between diet and visceral fat mass through blood metabolomics and gut microbiome profiling. Int. J. Obes. 41:1106–1113. https://doi.org/10.1038/ijo.2017.70.

50. Patil, R. D., M. J. Ellison, S. M. Wolff, C. Shearer, A. M. Wright, R. R. Cockrum, K. J. Austin, W. R. Lamberson, K. M. Cammack, and G. C. Conant. 2018. Poor feed efficiency in sheep is associated with several structural abnormalities in the community metabolic network of their ruminal microbes. J. Anim. Sci. 96:2113–2124. https://doi.org/10.1093/jas/sky096.
51. Paganoni, B., G. Rose, C. Macleay, and C. Jones. 2017. More feed efficient sheep produce less methane and carbon dioxide when eating high-quality pellets. J. Anim. Sci. 95:3839–3850. doi:10.2527/jas2017.1499.
52. Paula, E. F. E. de, D. F. de Souza, A. L. G. Monteiro, M. H. de A. Santana, S. Gilaverte, P. Rossi Junior, and R. Locatelli Dittrich. 2013. Residual feed intake and hematological and metabolic blood profiles of Ile de France lambs. Rev. Bras. Zoot. 42:806-812. https://dx.doi.org/10.1590/S1516-35982013001100007.
53. Penner, G. B., M. A. Steele, J. R. Aschenbach, and B. W. McBride. 2011. RUMINANT NUTRITION SYMPOSIUM: Molecular adaptation of ruminal epithelia to highly fermentable diets. J. Anim. Sci. 89:1108–1119. https://doi.org/10.2527/jas.2010-3378.
54. Pinu, F. R., S. A., Goldansaz, and J. Jaine. 2019. Translational metabolomics: current challenges and future opportunities. Metabolites. 9(6):108. doi:10.3390/metabo9060108.
55. Psychogios, N., D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman,

T. Goodfriend, and D. S. Wishart. 2011. The human serum metabolome. PLoS ONE. 6:e16957. doi:10.1371/journal.pone.0016957.

56. Rajaei Sharifabadi, H., M. J. Zamiri, E. Rowghani, W. G. Bottje. 2012. Relationship between the activity of mitochondrial respiratory chain complexes and feed efficiency in fat-tailed Ghezel lambs. J. Anim. Sci. 90:1807–1815, https://doi.org/10.2527/jas.2011-4791.
57. Ravanbakhsh, S., P. Liu, T. C. Bjorndahl, R. Mandal, J. R. Grant, M. Wilson, R. Eisner, I. Sinelnikov, X. Hu, C. Luchinat, R. Greiner, and D. S. Wishart. 2015. Accurate, fully-automated NMR spectral profiling for metabolomics. PLoS ONE. 10(5): e0124219. doi: 10.1371/journal.pone.0124219.
58. Redden, R. R., L. M. M. Surber, A. V. Grove, and R. W. Kott. 2014. Effects of residual feed intake classification and method of alfalfa processing on ewe intake and growth. J. Anim. Sci. 92:830–835. https://doi.org/10.2527/jas.2013-6768.
59. Richardson, E. C., and R. M. Herd. 2004. Biological basis for variation in residual feed intake in beef cattle. 2. Synthesis of results following divergent selection. Aus. J. Exp. Agr. 44:431-440. https://doi.org/10.1071/EA02221.
60. Rincon-Delgado, R. M., H. Gutierrez, E. D. Perez-Vszq, A. Muro-Reyes, L. H. Diaz-Garci, R. Banuelos-V, F. J. Gutierrez, C. A. Medina-Flo, L. M. Escareno-S, J. I. Aguilera-S, M. A. Lopez-Carl, and C. F. Arechiga-F. 2011. Relationship of residual feed intake on specific hematological and biochemical parameters in Rambouillet sheep. Agri. J. 6:87-91. doi:10.3923/aj.2011.87.91.
61. Saude, E. J., C. M. Slupksy, and B. D. Sykes. 2006. Optimization of NMR analysis of biological fluids for quantitative accuracy. Metabolomics. 2:113-123. https://doi.org/10.1007/s11306-006-0023-5.

62. Senchuk, I. 2019. Evaluation of metabolic status of ewes infected with ketosis. Agri. Sci. Euro-North-East. 20:265-272. doi:10.30766/2072-9081.2019.20.3.265-272.

63. Spring, P. 2013. The challenge of cost effective poultry and animal nutrition: optimizing existing and applying novel concepts. Lohm. Info. 48:38-47.

64. Tsikas, D., R. H. Böger, J. Sandmann, S. M. Bode-Böger, and J. C. Frölich. 2000. Endogenous nitric oxide synthase inhibitors are responsible for the l-arginine paradox. FEBS Lett. 478:1–3. https://doi.org/10.1016/S0014-5793(00)01686-0.

65. Ul-Haq, A., M. Tabassum Ch., F. Ahmad, J. Shafi, M. Ashraf, M. Javed, and S. Ur-Rehman. 2014. Effect of dietary acidification with citric acid on carcass characteristics, haemogram and serum metabolite values of broiler chicken. Pakistan J. Life Soc. Sci. 12:36-41.

66. Wang, X., and H. N. Kadarmideen. 2019. Metabolomics analyses in high-low feed efficient dairy cows reveal novel biochemical mechanisms and predictive biomarkers. Metabolites. 9:151. https://doi.org/10.3390/metabo9070151.

67. Wang, Z., J. D. Nkrumah, C. Li, J. A. Basarab, L. A. Goonewardene, E. K. Okine, D. H. Crews, and S. S. Moore. 2006. Test duration for growth, feed intake, and feed efficiency in beef cattle using the GrowSafe System. J. Anim. Sci. 84:2289-2298. https://doi.org/10.2527/jas.2005-715.

68. Wester, T. J., G. E. Lobley, L. M. Birnie, and M. A. Lomax. 2000. Insulin stimulates phenylalanine uptake across the hind limb in fed lambs. J. Nutr. 130:608–611. https://doi.org/10.1093/jn/130.3.608.

69. Wishart, D. S. 2016. Emerging applications of metabolomics in drug discovery and precision medicine. Nat. Rev. Drug Discov. 15:473. doi: 10.1038/nrd.2016.32.

70. Yano, Y., N. Kataho, M. Watanabe, T. Nakamura, and Y. Asano. 1995. Evaluation of beef aging by determination of hypoxanthine and xanthine contents: application of a xanthine sensor. Food Chem. 52:439-445. https://doi.org/10.1016/0308-8146(95)93297-5.

71. Zhang, X., W. Wang, F. Mo, Y. La, C. Li, and F. Li. 2017. Association of residual feed intake with growth and slaughtering performance, blood metabolism, and body composition in growing lambs. Sci. Rep. 7:12681. doi:10.1038/s41598-017-13042-7.

Tables

Table 1. Literature-reported blood components associated with sheep RFI. A limited number of research have investigated significant blood components associated with sheep RFI. None of these studies implemented metabolomics and no rigorous biomarker analyses was conducted to verify if these compounds could serve as proxies of RFI.

Biomarker	Biomarker Classification	Sample Type	Correlation	Reference
Thyroxine, adrenocorticotropic hormone, cortisol	Hormone	Serum	Positive	Zhang et al., 2017; Richardson and Herd, 2004; Knott et al., 2010
Albumin, creatinine, total plasma protein, glucose	Metabolites	Serum	Positive	Paula et al., 2013; Rincon-Delgado et al., 2011
Red blood cells, white blood cells	Hematological and biochemical parameters	Serum	Positive	Rincon-Delgado et al., 2011
Mean corpuscular volume, mean corpuscular hemoglobin, eosinophils, monocyte	Hematological and biochemical parameters	Serum	Negative	Paula et al., 2013; Rincon-Delgado et al., 2011

Table 2. **Univariate analyses of metabolites associated with sheep RFI.** These metabolites differentiated between high-RFI and low-RFI sheep.

	Metabolite	Fold Change	P-value	Metabolite Category	Platform
1	Alpha-aminoadipic acid	1.88	<0.001	Biogenic amines	DI/LC-MS/MS
2	Ketoleucine	1.72	<0.05	Ketones	NMR
3	Acetone	1.78	<0.005	Ketones	NMR
4	Isopropyl alcohol	20.83	<0.001	Alcohol or polyol	NMR
5	Cesium (Cs)	<1.5	<0.001	Metal	ICP-MS
6	C5 (Valerylcarnitine)	<1.5	<0.001	Acylcarnitines	DI/LC-MS/MS
7	C5-OH (C3-DC-M) (Hydroxyvalerylcarnitine)	<1.5	<0.001	Acylcarnitines	DI/LC-MS/MS
8	PC aa C40:2	<1.5	<0.001	Phospholipids	DI/LC-MS/MS
9	C6 (C4:1-DC) (Hexanoylcarnitine)	<1.5	<0.001	Acylcarnitines	DI/LC-MS/MS
10	C0 (Carnitine)	<1.5	<0.001	Acylcarnitines	DI/LC-MS/MS
11	Asymmetric dimethylarginine (ADMA)	<1.5	<0.001	Biogenic amines	DI/LC-MS/MS
12	Spermidine	<1.5	<0.005	Biogenic amines	DI/LC-MS/MS
13	PC ae C36:0	<1.5	<0.005	Phospholipids	DI/LC-MS/MS
14	PC aa C40:1	<1.5	<0.005	Phospholipids	DI/LC-MS/MS
15	C14:1-OH (Hydroxytetradecenoyl carnitine)	<1.5	<0.005	Acylcarnitines	DI/LC-MS/MS
16	Glycerol	<1.5	<0.005	Sugar alcohols	NMR
17	C16 (Hexadecanoylcarnitine)	<1.5	<0.005	Acylcarnitines	DI/LC-MS/MS
18	LysoPC a C16:1	<1.5	<0.005	Phospholipid	DI/LC-MS/MS
19	PC aa C32:2	<1.5	<0.005	Phospholipid	DI/LC-MS/MS
20	Copper (Cu)	<1.5	<0.005	Metal	ICP-MS
21	Potassium (K)	<1.5	<0.005	Metal	ICP-MS
22	LysoPC a C18:1	<1.5	<0.005	Phospholipid	DI/LC-MS/MS
23	PC ae C40:6	<1.5	<0.005	Phospholipid	DI/LC-MS/MS
24	Hippuric acid	<1.5	<0.005	Benzenoid	NMR
25	Taurine	<1.5	<0.005	Biogenic amine	DI/LC-MS/MS

Table 3. **Univariate analyses of metabolites associated with sheep carcass merit (YG).** These metabolites differentiated between the two groups of YG1 and YG2.

	Metabolite	Fold Change	P-value	Metabolite Class	Platform
1	Citric acid	0.80	<0.01	Carboxylic acid	NMR
2	Hippuric acid	0.74	<0.05	Benzenoid	NMR

Table 4. Ingredient and chemical composition of the ration.

	Whole Barley (BAR)		Pelleted TMR (PEL)	
Ingredient, % As Fed basis				
Whole barley	79		-	
Grower/Finisher Protein Supplement[a]	20		-	
Grower-Finisher Pellet[b]	-		100	
Limestone	1		-	
Chemical Composition	SEM	P Value	SEM	
DM, % (as fed)	89.1	0.15	88.1	0.19
ADF %	7.04	0.15	11.4	0.19
CP, % of DM	14.7	0.15	18.4	0.19
TDN, % of DM	81.7	0.26	78.5	0.33

[a] Contained 3% Calcium; 1% Phosphorus; 0.3% Magnesium; 1% Sodium; 1.5% Potassium; 125 mg kg^{-1} Fluorine; 4 mg kg^{-1} Cobalt; 4 mg kg^{-1} Iodine; 600 mg kg^{-1} Manganese; 440 mg kg^{-1} Zinc; 450 mg kg^{-1} Iron; 85 000 IU kg^{-1} Vit A; 14 500 IU kg^{-1} Vit D; 155 IU kg^{-1} Vit E; 1.5 mg kg^{-1} Selenium.

[b] Contained 0.9% Calcium; 0.4% Phosphorus; 0.25% Sodium; 8800 IU kg^{-1} Vit A; 2000 IU kg^{-1} Vit D_3; 10 IU kg^{-1} Vit E; 0.25 mg kg^{-1} Selenium.

Table 5. Sheep metabolome associated with RFI and carcass merit. Metabolites include those identified and quantified by NMR, ICP-MS and DI/LC-MS/MS from serum of healthy sheep assessed for RFI and carcass merit. Metabolites identified by * or $^{+}$ are reported for the first in the LMDB (www.lmdb.ca) and the sheep metabolome, respectively. Metabolite IDs identified by ^ refer to an isomer of that lipid. Note that total dimethylarginine does not have a LMDB ID since it consists of the sum of two metabolites (symmetrical and asymmetric dimethylarginine).

Platform	Metabolite	LMDB ID	ClassyFire Chemical Classification
NMR	2-Hydroxybutyric acid^{+}	LMDB00003	Hydroxy acids and derivatives
	2-Hydroxyisovalerate*$^{+}$	LMDB01096	Fatty Acyl derivatives
	3-Hydroxybutyric acid	LMDB00144	Hydroxy acids and derivatives
	3-Hydroxyisovaleric acid^{+}	LMDB00238	Fatty Acyl derivatives
	3-Methyl-2-oxovaleric acid*$^{+}$	LMDB01097	Keto acids and derivatives
	Acetic acid	LMDB00014	Carboxylic acids and derivatives
	Acetoacetate	LMDB00026	Keto acids and derivatives
	Acetone	LMDB00352	Organooxygen compounds
	L-Arginine	LMDB00171	Carboxylic acids and derivatives
	L-Asparagine	LMDB00075	Carboxylic acids and derivatives
	Betaine	LMDB00015	Carboxylic acids and derivatives
	Choline^{+}	LMDB00041	Organonitrogen compounds
	Citric acid	LMDB00040	Carboxylic acids and derivatives
	Creatine	LMDB00029	Carboxylic acids and derivatives
	Creatinine	LMDB00180	Carboxylic acids and derivatives
	Dimethylamine	LMDB00037	Organonitrogen compounds
	Dimethyl sulfone^{+}	LMDB00459	Sulfonyl compounds
	Dimethylglycine^{+}	LMDB00039	Carboxylic acids and derivatives
	D-Mannose^{+}	LMDB00076	Organooxygen compounds
	Ethanol^{+}	LMDB00044	Organooxygen compounds
	Formate	LMDB00060	Carboxylic acid and derivatives
	Fumaric acid	LMDB00057	Carboxylic acid and derivative
	Glucose	LMDB00048	Organooxygen compounds
	Glycerol	LMDB00055	Organooxygen compounds
	Glycine	LMDB00049	Carboxylic acid and derivatives
	Hippuric acid	LMDB00227	Benzene and substituted benzene derivatives
	Hypoxanthine^{+}	LMDB00067	Imidazopyrimidines
	Isobutyric acid^{+}	LMDB00357	Carboxylic acid and derivatives
	Isoleucine	LMDB00077	Carboxylic acid and derivatives
	Isopropyl alcohol^{+}	LMDB00266	Organooxygen compound

	Ketoleucine^{+}	LMDB00220	Keto acid and derivatives
	L-Acetylcarnitine	LMDB00091	Fatty Acyl derivatives
	L-Alanine	LMDB00070	Carboxylic acids and derivatives
	L-Alpha-aminobutyric acid	LMDB00157	Carboxylic acids and derivatives
	L-Aspartic acid	LMDB00085	Carboxylic acids and derivatives
	L-Carnitine	LMDB00027	Organonitrogen compounds
	L-Glutamic acid	LMDB00063	Carboxylic acids and derivatives
	L-Glutamine	LMDB00202	Carboxylic acids and derivatives
	L-Histidine	LMDB00080	Carboxylic acids and derivatives
	L-Lactic acid	LMDB00084	Hydroxy acids and derivatives
	L-Leucine	LMDB00215	Carboxylic acids and derivatives
	L-Ornithine	LMDB00099	Carboxylic acids and derivatives
	L-Phenylalanine	LMDB00069	Carboxylic acids and derivatives
	L-Proline	LMDB00071	Carboxylic acids and derivatives
	L-Serine	LMDB00083	Carboxylic acids and derivatives
	L-Threonine	LMDB00074	Carboxylic acids and derivatives
	L-Lysine	LMDB00081	Carboxylic acids and derivatives
	Malonic acid	LMDB00217	Carboxylic acids and derivatives
	Methanol	LMDB00358	Organooxygen compounds
	Methionine	LMDB00221	Carboxylic acids and derivatives
	1-Methylhistidine^{+}	LMDB00001	Carboxylic acids and derivatives
	Oxoglutaric acid	LMDB00094	Keto acids and derivatives
	Propylene glycol	LMDB00360	Organooxygen compounds
	Pyruvic acid	LMDB00112	Keto acids and derivatives
	Sarcosine^{+}	LMDB00124	Carboxylic acids and derivatives
	Succinic acid	LMDB00118	Carboxylic acids and derivatives
	Trimethylamine N-oxide	LMDB00278	Organonitrogen compounds
	Tyrosine	LMDB00068	Carboxylic acids and derivative
	Urea	LMDB00131	Organic carbonic acids and derivatives
	Valine	LMDB00271	Carboxylic acids and derivatives
DI/LC-MS/MS	SM (OH) C14:1	LMDB00624	Sphingolipids
	SM C16:0	LMDB00524	Sphingolipids
	SM C16:1^{+}	LMDB00656	Sphingolipids
	SM (OH) C16:1	LMDB00780	Sphingolipids
	SM C18:0	LMDB00569	Sphingolipids
	SM C18:1*$^{+}$	LMDB01208	Sphingolipids
	SM C20:2^{+}	LMDB00626	Sphingolipids
	SM (OH) C22:1	LMDB00627	Sphingolipids
	SM (OH) C22:2	LMDB00628	Sphingolipids
	SM (OH) C24:1	LMDB00630	Sphingolipids
	Acetylornithine^{+}	LMDB00430	Carboxylic acids and derivatives
	Alpha-aminoadipic acid	LMDB00168	Carboxylic acids and derivatives
	Asymmetric dimethylarginine (ADMA)	LMDB00344	Carboxylic acids and derivatives

	C0 (Carnitine)	LMDB00027	Organonitrogen compounds
	C10 (Decanoylcarnitine)* $^{+}$	LMDB01099	Fatty Acyl derivatives
	C10:1 (Decenoylcarnitine)$^{+}$	LMDB00993	Fatty Acyl derivatives
	C10:2 (decadienylcarnitine)* $^{+}$	LMDB01102	Fatty Acyl derivatives
	C12 (dodecanoylcarnitine)* $^{+}$	LMDB01101	Fatty Acyl derivatives
	C12:1 (Dodecenoylcarnitine)$^{+}$	LMDB01008	Fatty Acyl derivatives
	C12-DC (dodecanedioylcarnitine)* $^{+}$	LMDB01104	Carboxylic acids and derivatives
	C14 (tetradecanoylcarnitine)$^{+}$	LMDB00462	Fatty Acyl derivatives
	C14:1 (tetradecenoylcarnitine)$^{+}$	LMDB01011	Fatty Acyl derivatives
	C14:1-OH (Hydroxytetradecenoyl carnitine)* $^{+}$	LMDB01105	Fatty Acyl derivatives
	C14:2 (Tetradecadienylcarnitine)* $^{+}$	LMDB01227	Fatty Acyl derivatives
	C14:2-OH (hydroxytetradecadienylcarnitine)	LMDB01010	Fatty Acyl derivatives
	C16 (Hexadecanoylcarnitine)	LMDB00102	Fatty Acyl derivatives
	C16:1 (Hexadecenoylcarnitine)* $^{+}$	LMDB01224	Fatty Acyl derivatives
	C16:1-OH (Hydroxyhexadecenoylcarnitine)$^{+}$	LMDB01012	Hydroxy acid and derivative
	C16:2 (Hexadecadienylcarnitine)$^{+}$	LMDB00757	Fatty Acyl derivatives
	C16:2-OH (hydroxyhexadecadienylcarnitine)* $^{+}$	LMDB01225	Fatty Acyl derivatives
	C16-OH (hydroxyhexadecanoylcarnitine)$^{+}$	LMDB00941	Fatty Acyl derivatives
	C18 (Octadecanoylcarnitine)	LMDB00260	Fatty Acyl derivatives
	C18:1 (Octadecenoylcarnitine)* $^{+}$	LMDB01107	Fatty Acyl derivatives
	C18:1-OH (Hydroxyoctadecenoylcarnitine)* $^{+}$	LMDB01106	Fatty Acyl derivatives
	C18:2 (Octadecadienylcarnitine)$^{+}$	LMDB00475	Fatty Acyl derivatives
	C2 (Acetylcarnitine)	LMDB00091	Fatty Acyl derivatives
	C3 (Propionylcarnitine)$^{+}$	LMDB00253	Fatty Acyl derivatives
	C3:1 (Propenoylcarnitine)$^{+}$	LMDB00762	Fatty Acyl derivatives
	C3-OH (hydroxyPropionylcarnitine)$^{+}$	LMDB00578	Fatty Acyl derivatives
	C4 (butyrylcarnitine)	LMDB00374	Fatty Acyl derivatives
	C4:1 (Butenylcarnitine)$^{+}$	LMDB00579	Fatty Acyl derivatives
	C4-OH (C3-DC) (Hydroxybutyrylcarnitine)$^{+}$	LMDB00580	Fatty Acyl derivatives
	C5 (Valerylcarnitine)$^{+}$	LMDB00581	Fatty Acyl derivatives
	C5:1 (Tiglylcarnitine)$^{+}$	LMDB00397	Fatty Acyl derivatives
	C5:1-DC (Glutaconylcarnitine)$^{+}$	LMDB00582	Fatty Acyl derivatives
	C5-DC (C6-OH) (Glutarylcarnitine)$^{+}$	LMDB00766	Fatty Acyl derivatives
	C5-M-DC (methylglutarylcarnitine)$^{+}$	LMDB00927	Fatty Acyl derivatives
	C5-OH (C3-DC-M) (hydroxyvalerylcarnitine)$^{+}$	LMDB01080	Fatty Acyl derivatives
	C6 (C4:1-DC) (Hexanoylcarnitine)$^{+}$	LMDB00769	Fatty Acyl derivatives
	C6:1 (Hexenoylcarnitine)$^{+}$	LMDB00940	Fatty Acyl derivatives

	C7-DC (pimelylcarnitine)$^{+}$	LMDB00584	Carboxylic acids and derivatives
	C8 (Octanoylcarnitine)*$^{+}$	LMDB01100	Fatty Acyl derivatives
	C9 (Nonaylcarnitine)*$^{+}$	LMDB01103	Fatty Acyl derivatives
	Carnosine^{+}	LMDB00010	Peptides
	Citrulline	LMDB00274	Carboxylic acid and derivatives
	Kynurenine	LMDB00214	Organooxygen compounds
	lysoPC a C14:0^{+}	LMDB00525	Glycerophospholipids
	lysoPC a C16:0^{+}	LMDB00526	Glycerophospholipids
	lysoPC a C16:1^{+}	LMDB00527	Glycerophospholipids
	lysoPC a C17:0^{+}	LMDB00571	Glycerophospholipids
	lysoPC a C18:0^{+}	LMDB00528	Glycerophospholipids
	lysoPC a C18:1^{+}	LMDB00409	Glycerophospholipids
	lysoPC a C18:2^{+}	LMDB00530	Glycerophospholipids
	lysoPC a C20:3^{+}	LMDB00533	Glycerophospholipids
	lysoPC a C20:4^{+}	LMDB00534	Glycerophospholipids
	lysoPC a C24:0*$^{+}$	LMDB01207	Glycerophospholipids
	lysoPC a C26:0^{+}	LMDB00653	Glycerophospholipids
	lysoPC a C26:1*$^{+}$	LMDB01226	Glycerophospholipids
	lysoPC a C28:0^{+}	LMDB00654	Glycerophospholipids
	lysoPC a C28:1^{+}	LMDB00657	Glycerophospholipids
	Methionine sulfoxide^{+}	LMDB00373	Carboxylic acids and derivatives
	PC aa C32:2*$^{+}$	LMDB01211^	Glycerophospholipids
	PC aa C36:0*$^{+}$	LMDB01212 ^	Glycerophospholipids
	PC ae C36:0*$^{+}$	LMDB01210 ^	Glycerophospholipids
	PC aa C36:6*$^{+}$	LMDB01110 ^	Glycerophospholipids
	PC aa C38:0*$^{+}$	LMDB01111 ^	Glycerophospholipids
	PC aa C38:6*$^{+}$	LMDB01122 ^	Glycerophospholipids
	PC aa C40:1*$^{+}$	LMDB01119 ^	Glycerophospholipids
	PC aa C40:2*$^{+}$	LMDB01125 ^	Glycerophospholipids
	PC aa C40:6*$^{+}$	LMDB01140 ^	Glycerophospholipids
	PC ae C40:6^{+}	LMDB00599	Glycerophospholipids
	Serotonin^{+}	LMDB00120	Indole and derivatives
	Spermidine	LMDB00311	Organonitrogen compounds
	Spermine^{+}	LMDB00310	Organonitrogen compounds
	Taurine^{+}	LMDB00115	Organic sulfonic acids and derivatives
	Total dimethylarginine	N/A	Carboxylic acids and derivatives
	trans-Hydroxyproline (t4-OH-Pro)	LMDB00230	Carboxylic acids and derivatives
	Tryptophan	LMDB00279	Indoles and derivatives
ICP-MS	Barium (Ba)$^{+}$	LMDB00450	Homogeneous alkaline earth metal compounds
	Calcium (Ca)$^{+}$	LMDB00159	Homogeneous alkaline earth metal compounds
	Cobalt (Co)*$^{+}$	LMDB01098	Homogeneous transition metal compounds

	Cesium $(Cs)^+$	LMDB00634	Homogeneous alkali metal compounds
	Copper $(Cu)^+$	LMDB00204	Homogeneous transition metal compounds
	Iron $(Fe)^+$	LMDB00651	Homogeneous transition metal compounds
	Potassium $(K)^+$	LMDB00185	Homogeneous alkali metal compounds
	Lithium $(Li)^+$	LMDB00466	Homogeneous alkali metal compounds
	Magnesium $(Mg)^+$	LMDB00178	Homogeneous alkaline earth metal compounds
	Molybdenum $(Mo)^+$	LMDB00316	Homogeneous transition metal compounds
	Sodium $(Na)^+$	LMDB00186	Homogeneous alkali metal compounds
	Phosphorus $(P)^+$	LMDB00317	Homogeneous other non-metal compounds
	Rubidium $(Rb)^+$	LMDB00320	Homogeneous alkali metal compounds
	Selenium $(Se)^+$	LMDB00323	Homogeneous other non-metal compounds
	Strontium $(Sr)^+$	LMDB00439	Homogeneous alkaline earth metal compounds
	Titanium $(Ti)^+$	LMDB00368	Homogeneous transition metal compounds
	Vanadium $(V)^+$	LMDB00403	Homogeneous transition metal compounds
	Zinc $(Zn)^+$	LMDB00652	Homogeneous transition metal compounds

Table 6. Univariate analyses of metabolites associated with sheep carcass merit (MBR). These metabolites differentiated between the two groups of MBR1 and MBR2.

	Metabolite	Fold Change	*P*-value	Metabolite Class	Platform
1	LysoPC a C26:1	1.24	0.004	Phospholipids	DI/LC-MS/MS
2	LysoPC a C17:0	1.32	0.02	Phospholipids	DI/LC-MS/MS
3	LysoPC a C28:0	1.26	0.03	Phospholipids	DI/LC-MS/MS

Figures

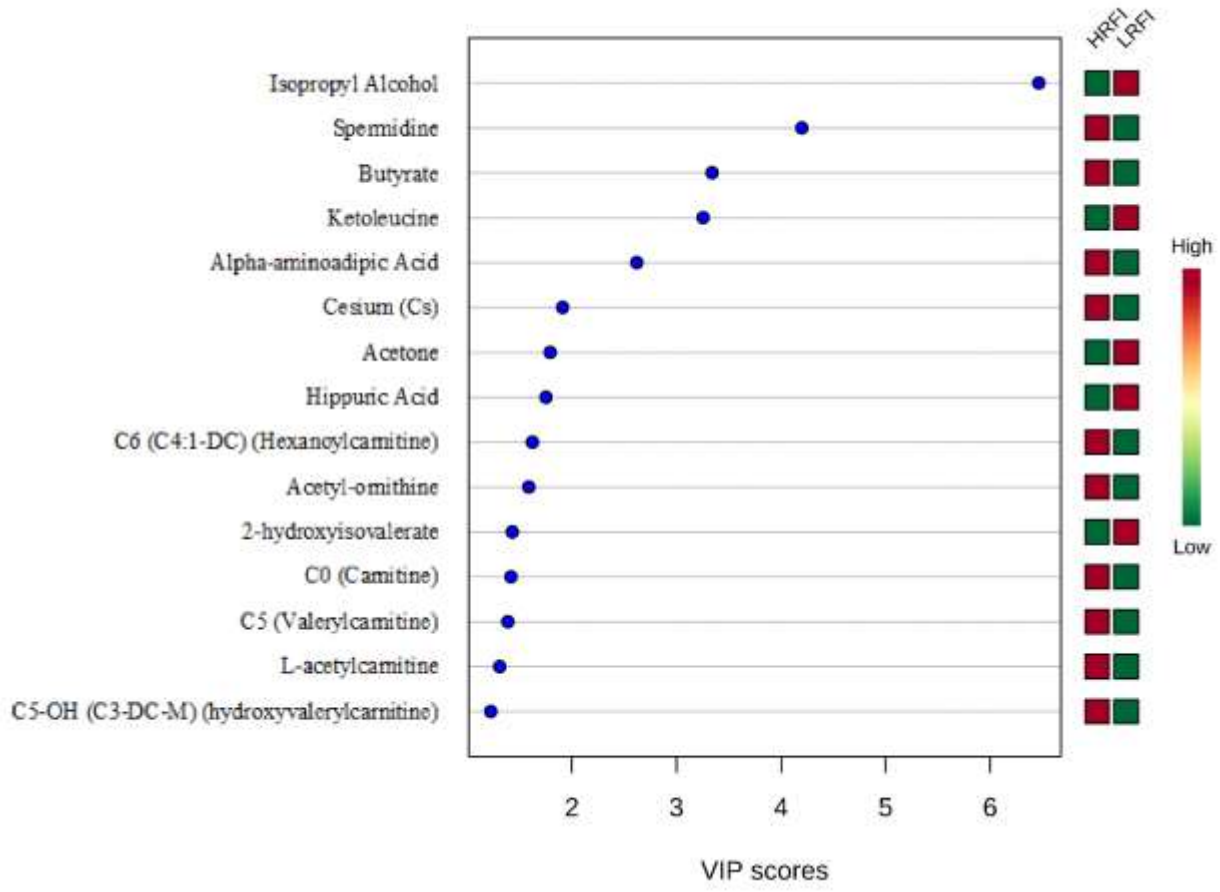

Figure 1. The VIP metabolites of sheep RFI. PLS-DA multivariate analysis identified the top 15 serum metabolites by VIP scores to have the highest influence in grouping of the low (LRFI) and high (HRFI) RFI groups.

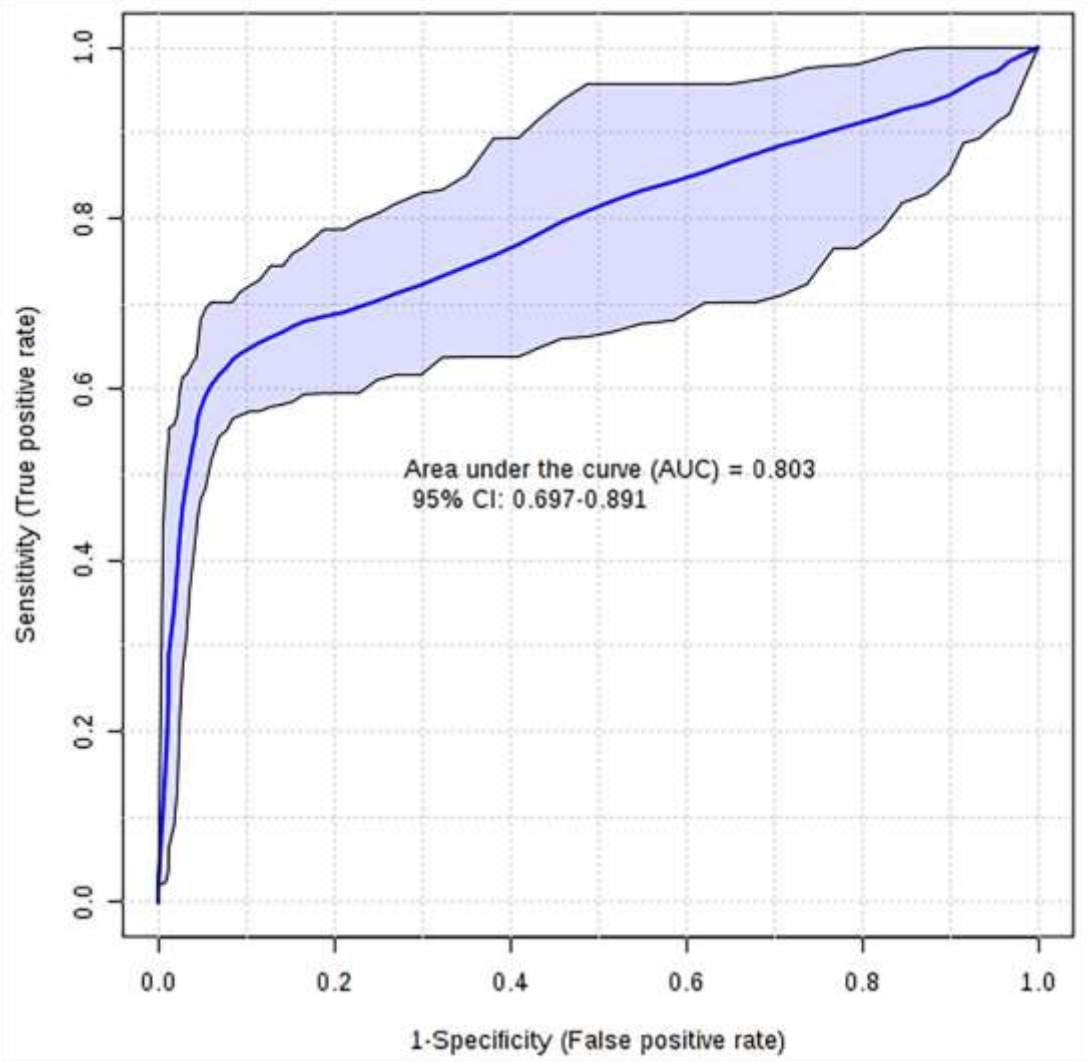

Figure 2. Logistic regression ROC curve for sheep RFI. Biomarker analysis identifying a panel of three candidate biomarkers (isopropyl alcohol, alpha-aminoadipic acid, acetone) from sheep serum samples yields an AUC of 0.80 (*P*-value < 0.05).

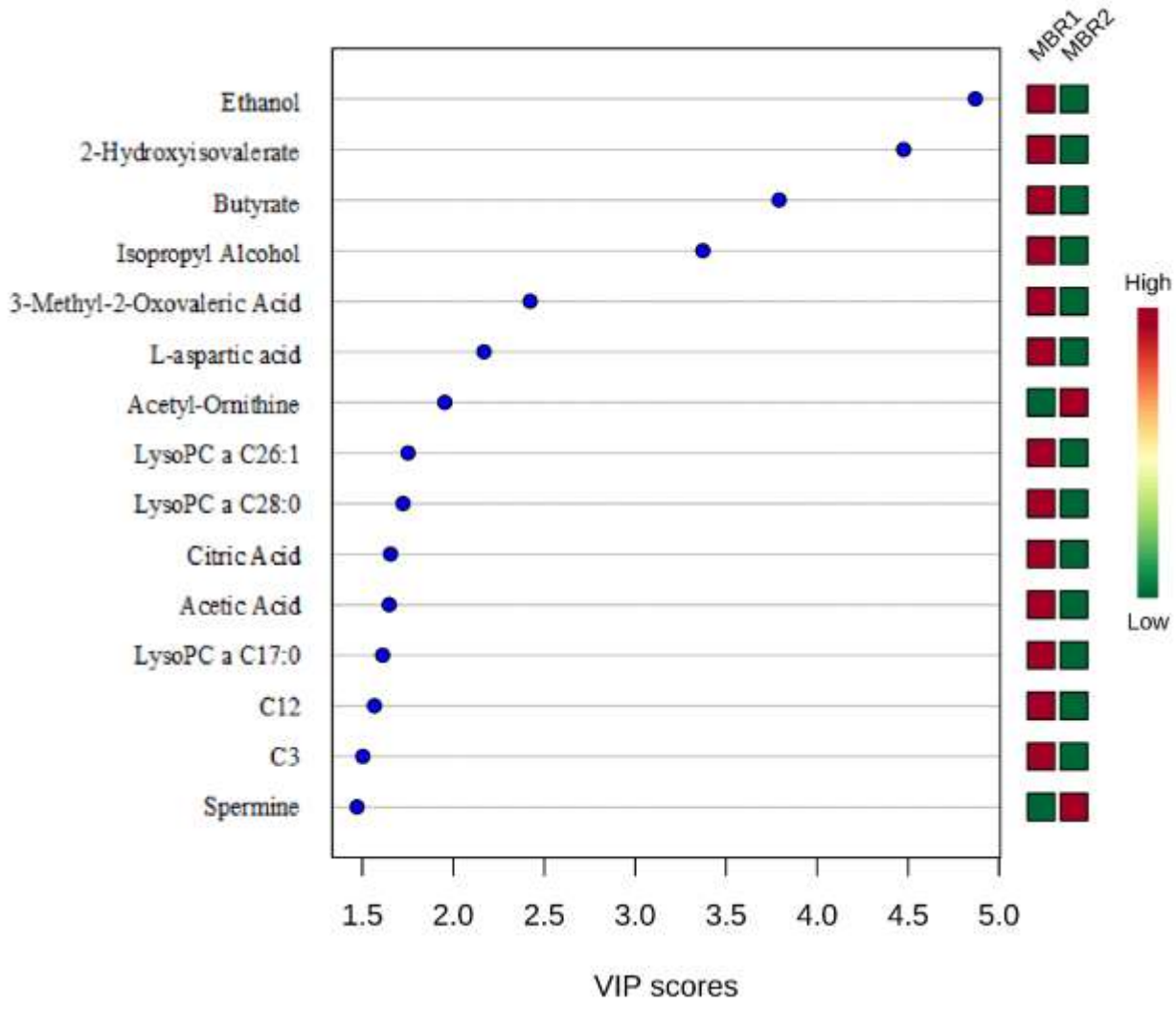

Figure 3. The VIP metabolites of sheep carcass merit (MBR). PLS-DA multivariate analysis identified the top 15 serum metabolites by VIP scores to have the highest influence in differentiating the MBR carcass groups.

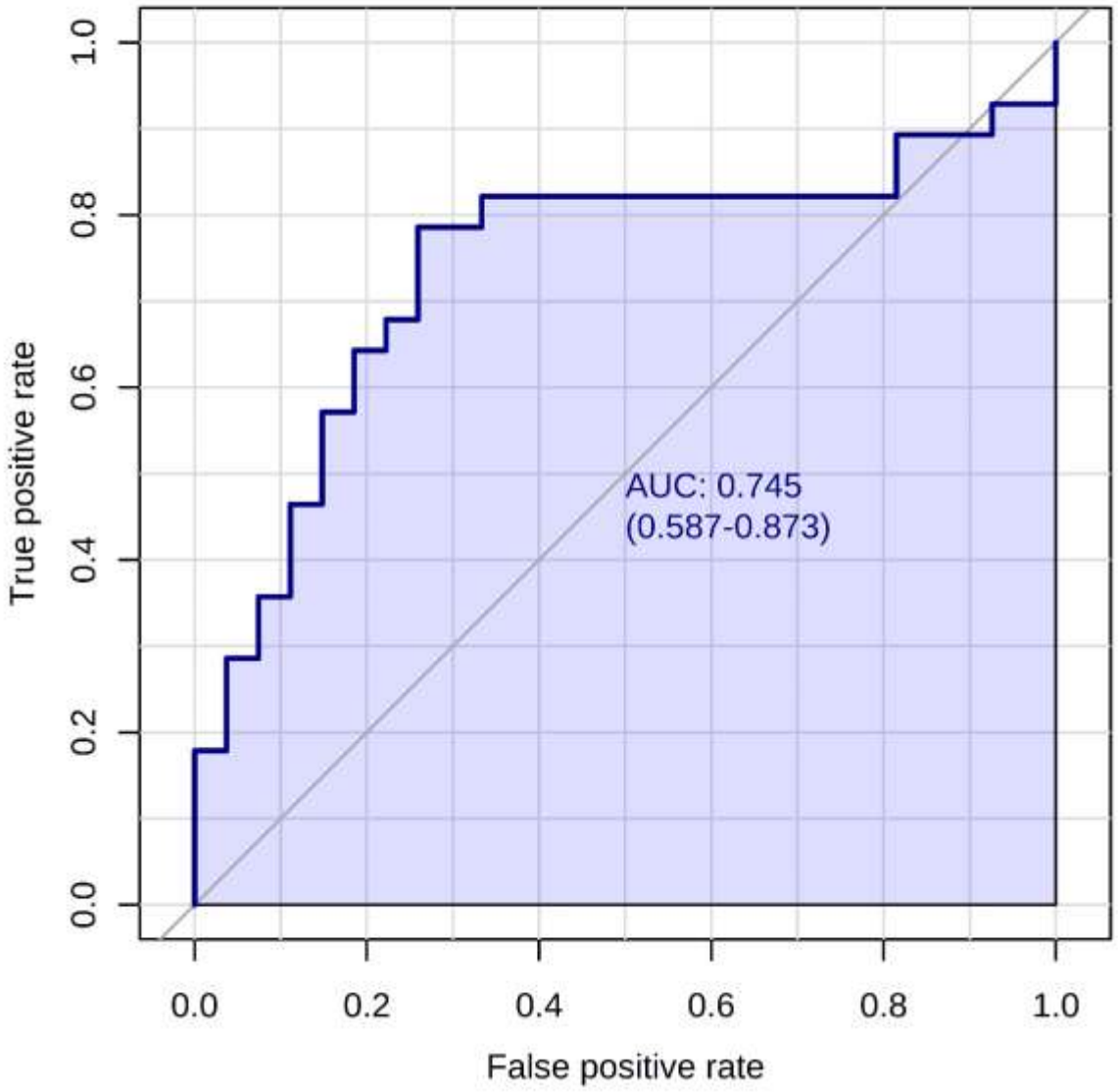

Figure 4. Biomarker analysis of sheep carcass merit (MBR). ROC curve analysis of a candidate biomarker (lysoPC a C26:1) from sheep serum samples yields an AUC of 0.74.

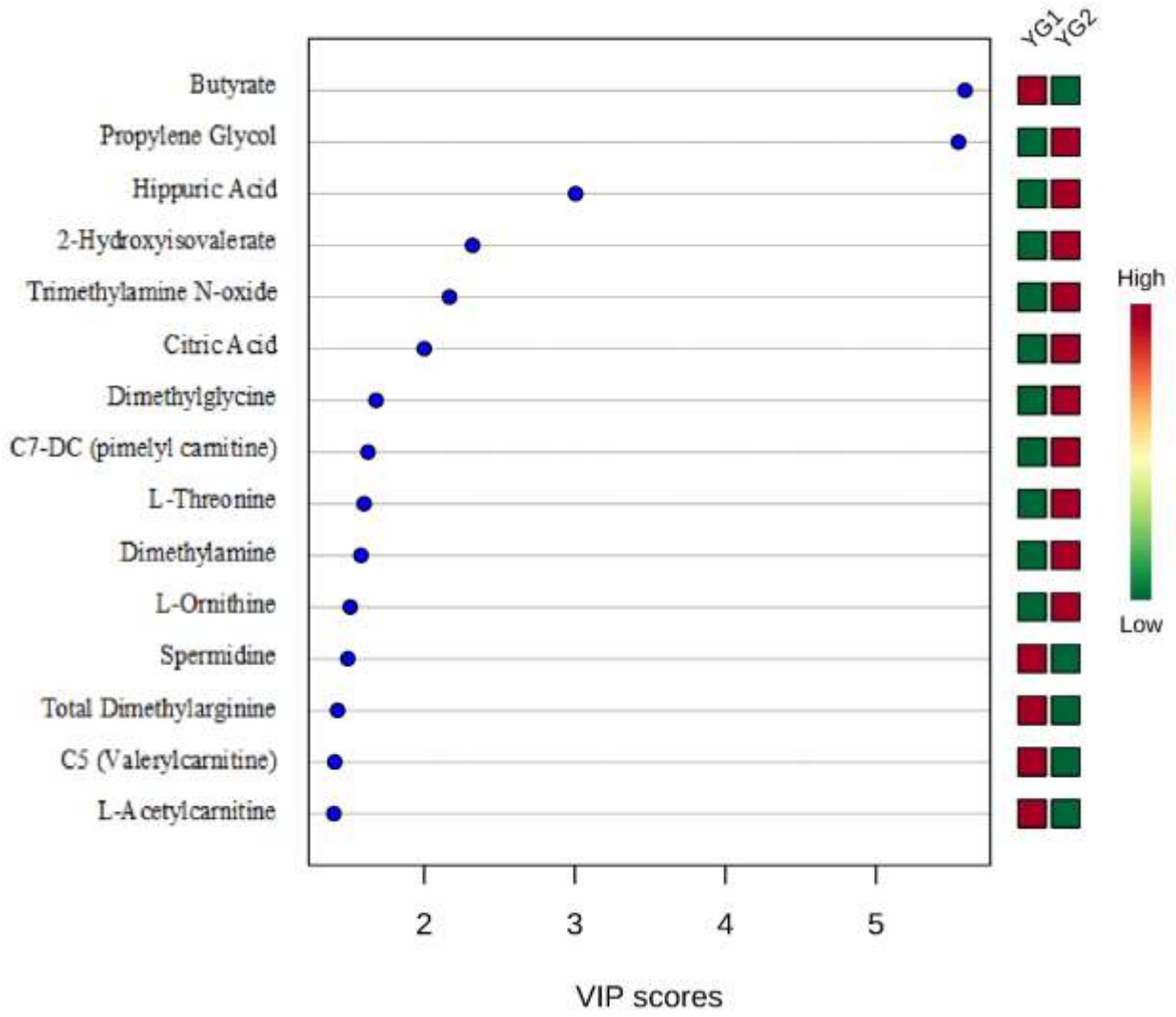

Figure 5. The VIP metabolites of sheep carcass merit (YG). PLS-DA multivariate analysis identified the top 15 serum metabolites by VIP scores to have the highest influence in differentiating the YG carcass groups.

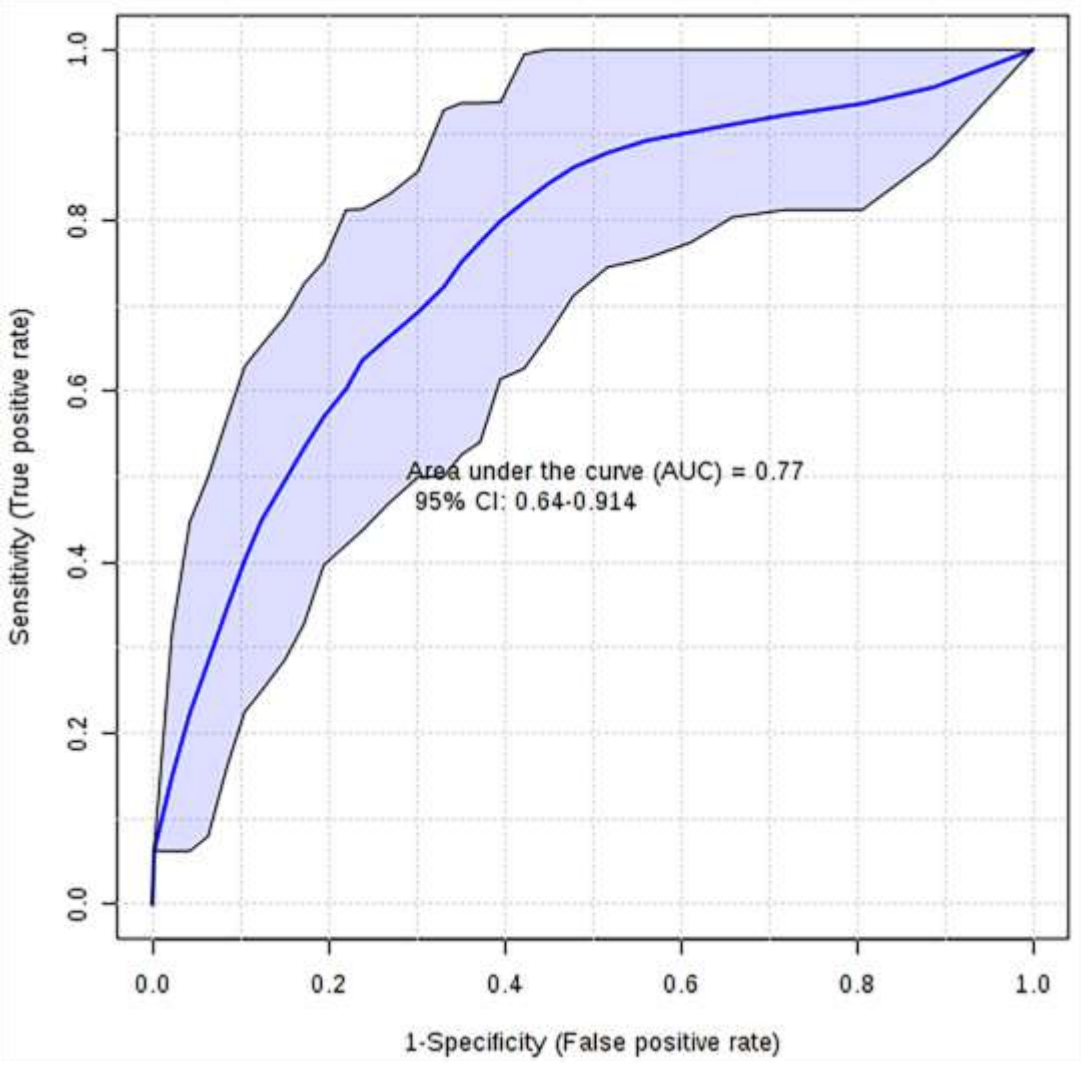

Figure 6. Logistic regression ROC curve for sheep carcass merit (YG). Biomarker analysis identified a panel of seven candidate biomarkers (total dimethylarginine, citric acid, hypoxanthine, hippuric acid, asymmetric dimethylarginine, L-phenylalanine, SM C16:1) from sheep serum samples yields an AUC of 0.77 (*P*-value < 0.05).

Chapter 4: Predictive Blood Biomarkers of Sheep Pregnancy and Litter Size

Seyed Ali Goldansaz*[,#], Susan Markus[‡], Graham Plastow*, David S. Wishart[#,§,£,$]

*Department of Agriculture, Food and Nutritional Sciences, University of Alberta, Edmonton, Canada, T6G 2P5

[#]Department of Biological Sciences, University of Alberta, Edmonton, Canada, T6G 2E9

[‡]Lakeland College, 5707 College Drive, Vermillion, AB, Canada, T9X 1K5

[§]Department of Computing Science, University of Alberta, Edmonton, Canada, T6G 2E8

[£]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada, T6G 2R3

[$]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada, T6G 2H7

*This chapter has been reformatted from the originally submitted paper format to make it consistent with the formatting used throughout the thesis.

Abstract

Early detection of sheep pregnancy and the prediction of how many lambs a pregnant ewe delivers affects sheep farmers in a number of ways, most notably with regard to feed management, lambing rate, and sheep/lamb health. The standard practice for direct detection of sheep pregnancy and litter size (PLS) is ultrasonography. However, this approach has a number of limitations. Indirect measurement of PLS using blood biomarkers could offer a simpler, faster and earlier route to PLS detection. Therefore, we undertook a large-scale study to identify and validate predictive biomarkers of sheep PLS using metabolomics of sheep serum. We conducted a longitudinal experiment that analyzed 131 blood samples over five timepoints (from seven days pre-conception to 70 days post-conception) from six commercial flocks in Alberta and Ontario, Canada. Using LC-MS/MS and NMR, we identified and quantified 107 metabolites in each sample. We also identified three panels of serum metabolite biomarkers that can predict ewe PLS as early as 50 days after breeding. These biomarkers were then validated in separate flocks consisting of 243 animals yielding areas-under-the-receiver-operating-characteristic-curve (AU-ROC) of 0.81-0.93. The development of a simple, low-cost blood test to measure PLS at an early stage of pregnancy could help optimize reproductive management on sheep farms.

Key words: Sheep, pregnancy, litter size, metabolomics, livestock metabolomics, blood biomarker.

Introduction

Sheep are relatively prolific small ruminants and an important source of animal protein contributing to human diets worldwide. Sheep gestation is relatively short (about 150 days) and litter sizes consisting of two or more offspring are preferred. As a result, sheep farm profitability is highly correlated to reproductive efficiency. Formally, reproductive efficiency for sheep farmers is expressed as the number of lambs born annually per ewe exposed to a ram at breeding. Breed type and prolificacy, nutrition, environment, age at first mating, conception rate, embryo and fetus viability, and flock age structure are some of the determining factors contributing to reproductive efficiency. However, outcomes of ewe fertility management can vary considerably among flocks. Identifying pregnant ewes and determining the number of fetuses they carry are key components of breeding management in sheep production (Haibel, 1990). Pregnancy testing during the critical early period of the mating season allows for re-breeding or the culling of non-pregnant ewes, resulting in increasing flock pregnancy rates (Zaher et al., 2020). If producers miss this opportunity, they can adjust their management practices by separating the open ewes from the pregnant mob to feed each group based on their physiological needs. Another benefit to early determination of pregnancy and litter size (PLS) is the acquisition of valuable data for selection and breeding purposes.

In addition to detecting pregnancy, predicting or determining litter size is instrumental to successful reproductive management. Maternal nutrition during gestation directly impacts ewe prolificacy (Rosales-Nieto et al., 2021) as well as lamb survivability and performance. These lamb performance traits include growth (Ghafouri-Kesbi and Eskandarinasab, 2008; Du et al., 2010), reproductive capacity (Bielli et al., 2002) and hormonal development (Bloomfield et al., 2004). Thus, early detection of ewe PLS elevates income for producers by increasing the number of

pregnant ewes and the number of healthy lambs born. Costs of production are reduced by preventing over-feeding of open ewes, and optimizing rations based on nutritional needs of the pregnant animals in an attempt to reduce the number of overweight singles, small sized multiples and the incidence of pregnancy toxemia.

Ultrasonography is the gold standard and the most commonly performed method for PLS detection in sheep (Jones et al., 2016). This method requires producers to either invest in an ultrasound machine and develop the appropriate skills for scanning or producers must contract the services from a veterinarian. Ultrasound pregnancy detection is commonly practiced between 45-90 days into gestation (Ishwar, 1995). However, detecting the number of fetuses is not straightforward and depends on the time of scanning as well as operator experience (Jones and Reed, 2017). The breeding season is also a busy time for ultrasound professionals, limiting the number of farms they can serve. The cost of ultrasonography, currently CAD$5-8/ewe in Alberta, also varies depending on flock size and geographical location of the farm. This makes ultrasonography more expensive for medium-to-small size flocks and those that are not conveniently accessible. In some jurisdictions, including the province of Alberta in Canada, delivering ultrasound services is restricted to veterinarian professionals, which limits its widespread use.

Molecular biomarkers, such as proteins or metabolites found in blood, urine or milk, are a promising alternative for the indirect measurement or prediction of different traits in many livestock species (Fontanesi, 2016; Goldansaz et al., 2017). Biomarkers are most suited for traits that have higher economic value. Likewise, biomarkers are particularly useful if the trait measurement needs to be performed within a short timeframe, or if the direct measurement of the trait involves lengthy trials, is labour-intensive, leads to loss of the animal or is expensive. While

plasma progesterone (P4) levels can be used to detect sheep pregnancy as early as 18 days, P4 does not accurately detect open, non-pregnant ewes (Susmel and Piasentier, 1992; Karen et al., 2003) and there is no commercial kit that provides the service to farmers in any part of the world (including Alberta, Canada). Recent literature indicates promising results when applying metabolomics to detect pregnancy in other livestock species (Fontanesi, 2016; de Nicola et al., 2020; Gómez et al., 2020). However, there are no publications using high throughput metabolomics platforms to characterize non-hormonal metabolite biomarkers that can be used for sheep PLS detection in readily accessible biofluids at early stages of gestation. Therefore, a metabolomic study on early-stage sheep PLS detection is warranted.

Livestock metabolomics is an emerging field that has led to the discovery of useful biomarkers in many livestock species (Goldansaz et al., 2017). However, only one study has used metabolomics to investigate non-hormonal metabolic changes during ewe pregnancy (Sun et al., 2017). Most other metabolomic studies have measured hormones or individual metabolites associated with ewe pregnancy (See et al., 2007; Huang et al., 2012; Washburn et al., 2015; Kandiel et al., 2016; Cihan et al., 2016). Previously, we have shown that metabolomics can be used to identify candidate blood biomarkers for detecting several economically important traits in sheep, such as residual feed intake and carcass merit (Goldansaz et al., 2020). Based on that success, we decided to investigate if blood biomarkers of sheep PLS could be identified and validated.

Given the metabolic changes that occur due to pregnancy, we hypothesized that ewe pregnancy and the number of lambs delivered per pregnant ewe can be predicted at early stages of pregnancy using blood biomarkers. Therefore, the objectives of this study were to: (1) profile the blood metabolome associated with ewe PLS, and (2) identify and validate blood biomarkers of

ewe PLS prior to 60 days of gestation. These findings could provide an alternative route for ewe pregnancy detection and enhance the reproductive management of sheep flocks. Indirect measurement of sheep PLS through blood biomarkers is also expected to increase the profitability of sheep production by reducing the proportion of open ewes during the breeding season. It will also improve the health and welfare of pregnant ewes through better nutritional management based on their pregnancy requirements.

Material and Methods

All animal procedures were approved by the University of Alberta Animal Care Committee (AUP00002510).

Experimental Design

The experiments were designed in two phases: 1) a discovery phase to identify candidate serum biomarkers of ewe pregnancy and litter size at the earliest timepoint in gestation, and 2) a validation phase to validate the candidate biomarkers using a sample size three times larger than that used in the discovery phase.

Discovery Phase Sampling

In the discovery phase, ewes were selected from two farms (Olds College and a private farm) in Alberta, Canada, consisting of Suffolk x Dorset crosses (n=91) and Rideau Arcott (n=152) ewes, respectively. Blood was drawn from all animals over five timepoints throughout this phase, including seven days prior to exposing the ewes to rams (day -7), day 0 (day of ram turnout for breeding), days 35, 50 and 70 of gestation. These animals were synchronized for estrus and the number of lambs delivered was recorded.

Based on the pregnancy outcome of all the animals included in this phase, two broad groups were formed for statistical analyses: controls (CNT; n=32) composed of non-pregnant, open ewes,

and pregnant ewes (PRG) that delivered one or more lambs (n=99). The CNT animals were comprised of ewes that were bred and did not deliver any lambs (n=9) as well as the negative controls (n=23) which were not exposed to rams. We divided the PRG animals to form three subgroups including ewes that delivered a single lamb (SNG; n=30), ewes that delivered a twin (TWN; n=36) and those that delivered a triplet or more (TRP; n=33). The remaining ewes (n=113) were not included in the analyses due to poor sample collection, missing data, and/or the producer's decision to cull the animal.

Animal Feed

During the discovery phase, the Olds College ewes were group-housed outdoors and fed a ration of grass mix alfalfa hay with whole barley grain and a mineral supplement. Ewes at the private farm were group housed indoors in a climate-controlled barn and fed corn silage with supplemental mineral and vitamin. Initially, it was assumed all animals were pregnant with twins, and the feed rations were formulated using the SheepBytes program (https://www.sheepbytes.ca/) in compliance with National Research Council recommendations (1985). Each ewe received nutrients based on live weight of 70-75 kg (equivalent to 1.51 Mcal net energy maintenance) in early gestation.

Estrus Synchronization and Breeding Management

All ewes were synchronized with progesterone-bearing controlled internal drug release (CIDRs; Zoetis Canada Inc.) 14 days prior to ram turn out for breeding. To install the CIDRs, ewes were first lined in the chute and then the CIDR was inserted into the applicator by folding its wings and the tip of the applicator was gently lubricated to facilitate insertion of the device into the ewe. If the vulva appeared to be dirty, it was cleaned prior to implanting the CIDR. The applicator was

then gently inserted into the vagina to release the CIDR. The applicators were disinfected between each use by dipping in a warm water and iodine solution.

Upon CIDR removal, ewes received pregnant mare serum gonadotropin (NOVORMON™, Syntex S.A., Buenos Aires, Argentina) by intramuscular injection in the rump (1 ml/ewe for the prolific Rideau Arcott breed and 2 ml/ewe for the Suffolk x Dorset crosses).

All ewes, except for the CNT group, were then grouped with the breeding rams at a ratio of no more than 10 ewes per ram. Ram turnout at the Alberta private farm location occurred on November 4th, 2017, with ewes lambing between March 29th and April 5th, 2018. Ram turnout at the Olds College location occurred on October 4th and 11th, 2017 (groups A and B, respectively), with ewes lambing between February 26th and March 28th, 2018. Lambing at each location was observed and recorded by farm staff.

Laparoscopic Reproductive Examination

A subset of the negative controls was examined at day 50 of gestation using laparoscopy to visually observe and approve ovarian health. Animals were restrained using a cradle and anesthetized by intravenous injection of a combined sedative of 0.6 mg/mL xylazine (Vetoquinol Canada Inc., ON, Canada) and 2 mg/mL Ketamine (Vetoquinol Canada Inc., ON, Canada). Once on the cradle, the anesthetized ewe was lifted from its rear, bringing the back two legs up while the head and front two legs are down. Approximately six inches from each teat was clipped and cleaned with a 4% chlorhexidine scrub (Ceva Animal Health Inc., ON, Canada) and 99% isopropyl alcohol. The clipped areas provided a point of entry for the scope on one side and a cannula on the other. A moderate amount of CO_2 was introduced into the abdominal cavity through a trocar going into one of the clipped points. The laparoscope was introduced into the cannula to see the ovaries. The ovaries of all open ewes were observed and approved by a veterinarian as reproductively sound

and not showing any apparent abnormalities. The cannulas were then removed and the skin was stapled to close the two holes. The animals were gently rolled off the cradle and within five minutes they were relieved from the anesthesia. All utensils were maintained and cleaned in a dilute iodine solution (West Penetone Inc., QC, Canada) between each animal examination.

Ultrasound Diagnosis

All bred ewes were trans-abdominally scanned (Sonosite M-Turbo ultrasound machine, FUJIFILM Sonosite Inc., ON, Canada) for pregnancy and litter detection while standing in a chute at day 50 of gestation by an experienced technician for each province. Certified technicians reported pregnancy as open (no detectable fetus present), single (detection of only one fetus), or pregnant with more than one fetus. All ultrasound assessments were reconciled with the actual lambing records from each flock.

Validation Phase Sampling

During the validation phase, ewes were selected from two farms in Alberta (Suffolk and Canadian Arcott crosses at Lakeland College, and Suffolk crosses at a private farm) and two farms in Ontario (Rideau Arcotts and Suffolk crossed with Rideau Arcott at private farm one, and Dorset and Rideau Arcott crosses at private farm two). The combined flock consisted of a total of 243 animals. Based on the discovery phase results, blood was only drawn from all animals at a single timepoint (day 50 of gestation). All ewes were naturally mated to the rams at a ratio of 10:1, none of which were synchronized for estrus. All ewes had their lambing outcome recorded and categorized similar to the discovery phase (i.e., CNT, PRG, SNG, TWN and TRP).

Blood Collection and Processing

Blood samples from all ewes of both phases (discovery and validation) were drawn from the jugular vein. Samples were collected using 21-gauge needles (PrecisionGlide®, USA) and

vacutainers coated with no anticoagulant (BD Vacutainer, USA) for a maximum volume of 10 mL. Blood samples were kept on ice upon collection for a maximum of 30 minutes. Samples were then centrifuged (Beckman Coulter, USA) for 30 minutes at 17,700 rpm at 4 °C. The supernatant serum was then transferred to Eppendorf tubes (Axygen, USA) and snap frozen using liquid nitrogen. Frozen serum samples were labelled and stored at -80 °C until used for metabolomic analyses.

Metabolomics Experiments

All ewe serum samples were analyzed using nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography tandem mass spectrometry (LC-MS/MS). A thorough description of sample preparation and analysis methods for each platform is provided in Goldansaz et al. (2020). In brief, for the NMR analysis, all serum samples were filtered using a 3 kDa ultrafiltration device to remove the macromolecules (i.e., proteins and lipoproteins). A total sample volume of 250 µL (including the serum and buffer solution) was introduced to a 700 MHz Avance III (Bruker, USA) spectrometer equipped with a 5 mm HCN Z-gradient pulsed-field gradient cryoprobe. The 1D ^{1}H-NMR spectra were then collected, processed and analyzed using methods previously described and a modified version of the Bayesil automated NMR analysis software package (Ravanbakhsh et al., 2015). For the LC-MS/MS metabolomic analysis, serum samples were analyzed using an in-house quantitative metabolomics kit (called TMIC Prime) run on an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA) coupled with an AB SCIEX QTRAP® 4000 mass spectrometer (Sciex Canada, Concord, Canada). A detailed description of the methods, kit design, workflow and data analysis is given in Goldansaz et al. (2020).

Statistical Analyses

To conduct a standard categorical analysis and identify the relevant serum PLS biomarkers, we categorized the animals into six different groups based on their pregnancy outcome (i.e., CNT,

PRG, SNG, TWN, TRP, MLP). Metabolomic datasets from the two platforms were pre-processed and normalized using standard methods available via MetaboAnalyst 4.0 (Chong et al., 2019). Metabolites that had >20% missing values were removed from the dataset prior to statistical analyses. Univariate and multivariate statistical analyses, including fold change, student's t-test, volcano plot analysis, and partial least squares discriminant analysis (PLS-DA) were conducted. The PLS-DA plot helped visualize the separation of each animal group based on their corresponding serum metabolome, and its significance was verified using permutation testing (n=1000). The PLS-DA analyses that were significant were also evaluated for the top 15 VIP features, revealing those metabolites that had the most significant contribution to separating the comparison groups. Biomarker evaluation was performed using receiver operating characteristic (ROC) analysis conducted by logistic regression and measuring AU-ROC values. Individual or multiple metabolite profiles with an AU-ROC≥0.70 and which were statistically significant via permutation analysis (n=1000; p-value<0.05) were considered as candidate biomarkers for each trait. The threshold for statistical significance reported in this manuscript is a p-value<0.05 and a Benjamini-Hochberg false discovery rate (or Q-value)<0.05, unless otherwise mentioned. Also, a 0.05<p-value<0.10 is referred to as a tendency while, differences with a p-value>0.10 are referred to as not significant.

Results

The results from our metabolomic studies on sheep PLS are divided into three sections. The first describes the changes detected in serum metabolite levels of ewes during different phases of pregnancy. The second (discovery phase) describes the identification of serum-based PLS biomarkers at different stages of pregnancy through pairwise comparisons of pregnant and non-pregnant ewes, as well as via pairwise comparisons of pregnant ewes with different litter sizes

(based on pregnancy outcome). The third describes validation or replication of the PLS biomarkers identified at day 50 of gestation in the discovery phase on an independent (hold-out) larger cohort of ewes.

Changes in the serum metabolome of ewes during pregnancy. The first objective of this study was to comprehensively and quantitatively characterize the serum metabolome of ewes from seven days pre-breeding to 70 days post-breeding. The Livestock Metabolome Database (LMDB; Goldansaz et al., 2017) currently includes 375 compounds assigned to the sheep metabolome, 300 of which were previously reported and quantified in the serum/plasma metabolome of non-pregnant sheep. Unfortunately, there are no published reports regarding the serum metabolome of sheep during gestation. Given that sheep metabolomic studies are quite scarce, we undertook a targeted, quantitative metabolomic analysis of sheep serum using two analytical platforms, NMR spectroscopy and LC-MS/MS. Using the combination of these two platforms, we were able to identify and quantify 107 metabolites with unique chemical structures in the serum of pregnant ewes over 5 different timepoints for a total of 99 pregnant ewes and 32 non-pregnant ewes (the classification of these metabolites based on each platform is provided in Table 6). Details regarding the most significant longitudinal changes and most differentiating metabolites are described below.

Identifying PLS biomarkers via pairwise comparisons. For the discovery phase of the study, we divided the flocks into six different groups (as defined in the methods). Each of the six groups of ewes were compared (pairwise) at each of the five different timepoints (7 days pre-breeding [-7 day], day 0, 35, 50 and 70 post-breeding). In total 15 different pairwise comparisons were done over five timepoints (75 total comparisons). To simplify the results, we present the outcomes from univariate and multivariate analyses of only those comparison groups that yielded significant candidate biomarkers. These include: 1) CNT versus PRG, 2) CNT versus MLP, 3) SNG versus

TRP, and 4) TWN versus TRP. A detailed summary of the t-test, volcano plot and PLS-DA results are presented in Tables 1, 2 and 3, respectively.

The data presented in these tables clearly show that as ewes progress through gestation, the serum metabolome of pregnant ewes compared to open ewes, as well as pregnant ewes with different litter sizes, significantly diverges. Moreover, within each group, the blood metabolome significantly (p-value<0.05) differed between each timepoint as determined by two-way ANOVA. Over the five timepoints tested, it was clear that day 50 and day 70 yielded the most promising results. In particular, the volcano plot and PLS-DA plot were successful in detecting statistically significant metabolites that differentiated each group within each comparison. T-test results were most significant and abundant for the last two timepoints (days 50 and 70) between the most divergent comparison groups (CNT vs PRG and CNT vs MLP). Based on these data, we decided to focus on identifying serum candidate biomarkers at day 50 and day 70 of gestation.

Longitudinal assessment of significant metabolites during pregnancy. Longitudinal assessment of the t-test results (Table 1) revealed three significant metabolites (acetic acid, urea, and L-arginine) differentiating between pregnant and open ewes at day 50 and day 70 into gestation. All the metabolites that were significantly different by day 50 (according to the t-test) for the CNT vs MLP groups were also significant in the CNT vs PRG comparison, except L-carnitine. Similarly, differentiating metabolites from day 70 (according to the t-test) of the CNT vs MLP groups were all similar to the CNT vs PRG group, except isoleucine. The similarities between these two comparisons were expected since the PRG group is composed of both MLP and SNG ewes.

Longitudinal assessment of the volcano plots (Table 2) among all pairwise comparison groups revealed that acetic acid was significantly different between the CNT vs MLP groups from day 35 of gestation. However, acetic acid only appeared to be significantly different from day 50

for the CNT vs PRG groups. At day 70 post-breeding, choline was significantly different in all comparison groups except the TWN vs TRP groups. We also observed that comparison of CNT against PRG and MLP at later timepoints of gestation shared the largest number of metabolite similarities among other data collections and comparisons.

Longitudinal assessment using PLS-DA and variable importance of projection (VIP; Table 3) showed that L-lysine and acetic acid were two of the 15 most differentiating metabolites throughout all timepoints of gestation (days 0, 35, 50 and 70) in the CNT vs MLP comparison. Three other metabolites (urea, 3-hydroxybutyric acid, and methanol) were also commonly observed in three of the four post-breeding timepoints (days 35, 50 and 70). Moreover, acetic acid and urea were the two highest scoring VIP metabolites on day 50 and day 70 in both CNT vs PRG and CNT vs MLP comparisons. This further confirms the trend we observed in our univariate analyses and underlines how the CNT group, when compared against the PRG and MLP groups, typically shared more metabolic similarities in later pregnancy timepoints.

We further determined temporal trends by performing our statistical analyses within each comparison group at different timepoints. For the CNT vs PRG comparison, one group of significantly altered metabolites at day 50 was identified (acetic acid, L-arginine, SM (OH) C24:1, lysoPC a C26:0, lysoPC a C26:1, tryptophan, C3 [propionylcarnitine], putrescine, trimethylamine N-oxide,), and another group at day 70 was identified (acetic acid, L-arginine, urea, glycine, dimethylamine, dimethyl sulfone, 3-hydroxybutyric acid, sarcosine, L-lysine). These metabolites were consistently identified throughout all statistical analyses.

Temporal comparison of the CNT group against the MLP group at days 0 and 35 identified L-ornithine as a significantly altered metabolite. L-ornithine was found to be significant in all analyses for both timepoints. Acetic acid was another significantly altered metabolite at day 35.

At day 50 of gestation, the metabolites that exhibited the greatest difference included acetic acid, L-arginine, tryptophan and carnosine. At day 70, nine other significantly altered metabolites were identified, including urea, L-arginine, choline, glycine, acetic acid, dimethylamine, formate, 3-hydroxybutyric acid, dimethyl sulfone and acetoacetate. In contrast, we did not identify any temporal pattern using univariate or multivariate statistical analyses of the SNG vs TRP groups or the TWN vs TRP groups.

Candidate biomarkers of ewe pregnancy. To identify candidate biomarkers of ewe pregnancy, we compared the CNT ewes against all other pregnant ewes regardless of their litter size (PRG). To seek further confirmation and examine the extremes in terms of litter size, we removed the SNG ewes from the PRG dataset and also compared the CNT and MLP ewes. The advantage of the latter comparison is that the outcome biomarkers could help inform producers not only if the animal is pregnant but also that the ewe is expected to deliver more than one lamb. A detailed summary of the results is presented in Table 4. We identified no statistically useful serum biomarkers until day 35 of gestation when comparing the CNT group with the PRG group. However, at day 50 of the CNT vs PRG comparison, we identified a panel of five metabolites (methanol, L-carnitine, D-glucose, L-arginine, and urea; AU-ROC=0.76) with a tendency to serve as candidate biomarkers for detecting pregnant ewes. At day 70, we identified a panel of two metabolites for ewe pregnancy that had an AU-ROC of close to 1.0 with very high statistical significance (p-value<0.001). Comparing the CNT and MLP groups, we identified no useful biomarkers at day -7, while the other four timepoints revealed potentially useful biomarkers. The AU-ROC value and statistical significance of the biomarkers improved substantially later in the gestation, i.e., at day 70. Among the different timepoints that we assessed, day 50 had the largest panel of biomarkers, and these biomarkers were identical to the candidate biomarkers found at day

50 of the CNT vs PRG comparison. Given the value of detecting PLS at the earliest timepoint in gestation, we developed a logistic regression equation for the candidate biomarkers found at day 50 using the CNT vs PRG comparison. This equation is given below:

$$\text{logit}(P) = \log(P / (1-P)) = 1.599 + 1.217\ \text{L-arginine} + 2.095\ \text{urea} + 1.222\ \text{L-carnitine} + 0.137\ \text{methanol} - 0.505\ \text{D-glucose} \quad \text{(Equation [Eq.] 1)}$$

where P is the probability of y=1/x with a cut-off of 0.81. Because the concentrations of the metabolites used in the CNT vs PRG comparison were sum normalized, log transformed and Pareto scaled, the metabolite values used in the equation must be adjusted. These adjustments are provided in Table 7. This same logistic regression equation was later used to predict the pregnancy status of ewes in the validation phase.

Candidate biomarkers of ewe litter size. The comparisons that led to the identification of candidate biomarkers of ewe litter size involved looking at the CNT vs MLP groups (revealing pregnant ewes that deliver more than one lamb; explained above), the SNG vs TRP groups (revealing pregnant ewes that deliver a single or more than two lambs) and the TWN vs TRP groups (revealing pregnant ewes that deliver a twin or more than two lambs). A detailed summary of results for this section is presented in Table 4. We identified candidate biomarkers at all five timepoints for the SNG vs TRP comparison. This comparison revealed three to four candidate biomarkers at each timepoint with AU-ROC values varying from a low of 0.74 on day 0 to a high of 0.81 on day 70. All biomarkers were statistically significant except for the markers identified for day 35, which only had a statistical tendency. L-carnitine was the most frequently observed candidate biomarker, appearing at days -7, 35 and 50. Based on the results presented above, since

day 50 of gestation was the earliest timepoint to detect pregnancy, we used the same day-50 timepoint to develop a logistic regression equation for the panel of candidate biomarkers (methionine and L-carnitine) of the SNG vs TRP comparison. This equation is given below:

$$\text{logit}(P) = \log(P / (1-P)) = 0.211 - 4.464 \text{ methionine} + 4.393 \text{ L-carnitine} \quad \text{(Eq. 2)}$$

where P is the probability of y=1/x with a cut-off of 0.70. Because the concentrations of the metabolites used in this study were median normalized, cube root transformed and Pareto scaled, the metabolite values must be adjusted. These adjustments are provided in Table 7.

With regard to the TWN vs TRP group comparison, L-carnitine was also identified as the most frequently recurrent metabolite at all timepoints. For this comparison group, biomarkers at day -7 and day 50 only had a statistical tendency, while other timepoints had statistically significant biomarkers. All AU-ROC values were below 0.80 and most panels consisted of a relatively larger number of metabolites. Following the previous pattern, we used the candidate biomarkers (isobutyric acid, L-lactic acid, L-carnitine, valine, tyrosine, and methanol) identified for the TWN vs TRP comparison groups at day 50 of gestation to develop a logistic regression model as follows:

$$\text{logit}(P) = \log(P / (1-P)) = -0.124 + 0.406 \text{ isobutyric acid} - 0.388 \text{ L-lactic acid} - 0.771 \text{ L-carnitine} + 0.593 \text{ valine} + 0.144 \text{ tyrosine} + 0.683 \text{ methanol} \quad \text{(Eq. 3)}$$

where P is the probability of y=1/x with a cut-off of 0.57. Because the concentrations of the metabolites used in this study were sum normalized, cube root transformed and auto scaled, the metabolite values used in the equation must be adjusted. These adjustments are provided in the

supplementary material (Table 2). The above two equations were later used to predict litter size status of pregnant ewes in the validation phase.

Validation phase. Given that we determined the ideal time to assess PLS in ewes via serum metabolomics was at day 50 post-breeding, the sample collection for the validation phase was conducted only at day 50 of gestation. This section describes the validation of the same panel of day 50 candidate biomarkers, and the prediction of the validation dataset using the logistic regression equations developed in the discovery phase. In conducting this validation phase, we looked at three times as many samples as analyzed in the discovery phase, from commercial flocks located in different regions and under different management practices (in two of the top sheep producing provinces in Canada, Alberta and Ontario).

Validated biomarkers of ewe pregnancy. Statistical analyses of the validation dataset for the five candidate biomarkers of pregnancy (presented previously) actually improved the AU-ROC to ≥0.90 and the p-value to <0.05 (Table 4). Therefore, we confirmed that methanol, L-carnitine, D-glucose, L-arginine, and urea can be robustly used as biomarkers to detect ewe pregnancy at day 50 of gestation. Note that we used the same logistic regression model (Eq. 1) presented for the candidate biomarkers in the discovery phase to predict the pregnancy status of the validation dataset. This regression model was successful in making predictions with a sensitivity of 69% and a specificity of 85%.

Validated biomarkers of ewe litter size. We also validated the panels of candidate biomarkers for litter size in pregnant ewes. The AU-ROC value for candidate biomarkers (methionine and L-carnitine) of SNG vs TRP improved from 0.78 in the discovery phase to 0.84 in the validation set (Figure 2). This was accompanied by improved significance from a p-value<0.05 to a p-value<0.001 (Table 4). Therefore, we confirmed that methionine and L-carnitine can be robustly

used as biomarkers of ewe litter size. We also used the same logistic regression model (Eq. 2) developed in the discovery phase for the panel of candidate biomarkers to distinguish SNG vs TRP in the validation dataset. The regression model was successful in predicting litter size (SNG vs. TRP) with a sensitivity of 56% and a specificity of 91%.

Moreover, the candidate biomarkers (isobutyric acid, L-lactic acid, L-carnitine, valine, tyrosine, and methanol) identified for the TWN vs TRP comparison also reached statistical significance with an improved AU-ROC of 0.81 (Figure 3). These compounds were confirmed as robust biomarkers of ewe litter size. In addition, we used the same logistic regression model (Eq. 3) for the panel of candidate biomarkers of TWN vs TRP comparison groups developed in the discovery phase to predict the validation dataset. This regression model was successful in predicting litter size (TWN vs. TRP) with a sensitivity of 66% and specificity of 85%.

It is noteworthy that the overlap of biomarkers of pregnancy with those of the CNT versus MLP comparison groups indicates that if a ewe tests positive for the above-mentioned panel, not only is she pregnant but she is also expected to carry multiple fetuses. On the other hand, if the animal tests negative, she is not pregnant. To get a more precise measure of the litter size, further evaluation of the pregnant ewe's blood using the other panels of litter size biomarkers will likely be required. Therefore, if a pregnant ewe tests positive for the triplet biomarker panel (methionine, L-carnitine), the ewe is expected to deliver more than two lambs while a negative test does not necessarily indicate that the ewe will deliver a single lamb. On the other hand, pregnant ewes that test negative for biomarkers of twin vs triplet biomarker panel (isobutyric acid, L-lactic acid, L-carnitine, valine, tyrosine, and methanol) are expected to deliver twins.

Discussion

Over the past decade, livestock metabolomics research has gained considerable momentum with the number of papers being published on the subject almost doubling every two years. However, sheep metabolomics is still lagging far behind the research activities for other livestock species such as cattle and pigs. For this reason, we focused on further characterizing the sheep metabolome and identifying candidate biomarkers associated with production traits of high economic value such as residual feed intake, carcass merit (Goldansaz et al., 2020) and reproductive performance. In this study, we examined sheep serum using NMR and LC-MS/MS-based metabolomics to identify robust and useful metabolite biomarkers of PLS. The initial step involved profiling the sheep serum metabolome during the first half of pregnancy. In doing so, we identified and quantified a total of 107 serum metabolites. Although no new sheep serum metabolites were identified (after comparison to the data in the LMDB [Goldansaz et al., 2017]), this study did increase the proportion of quantified sheep serum metabolites in the LMDB from 55% to 93%. Furthermore in LMDB, the total quantified values in the ovine metabolome increased by 44%, while adding 15% more quantified data to LMDB. Data from this experimental work also adds to the reference values obtained from healthy pregnant and non-pregnant sheep in the LMDB. Moreover, our study provides quantitative information about the metabolic dynamics of the ewe serum metabolome from seven days prior to breeding to day 70 of gestation. These data are now publicly accessible in the LMDB (www.lmdb.ca).

The central objective of this study was to identify serum metabolite biomarkers for sheep PLS using high throughput metabolomic platforms. As far as we are aware, this is the first study to identify non-hormonal metabolite biomarkers of both pregnancy and litter size, and the only study to provide logistic regression models to predict pregnancy status in domestic sheep up to

date. It is important to note, however, that there are other compounds or biomarkers that have shown promise for assessing ewe PLS. These include genes, proteins and metabolites, some of which are described below.

Efforts to identify specific gene transcript levels and genetic markers for sheep PLS have been previously described in the literature. For example, changes in the expression levels of the interferon-tau-stimulated gene found in the thymus (Zhang et al., 2018) and endometrium (Kiyma et al., 2016) have been found to signal pregnancy at early gestation. There are also a number of studies on genes responsible for sheep litter size (Abdoli et al., 2016). The Booroola gene, located on ovine chromosome 6, is among the better-recognized genes that has a major impact on ovulation rate and is a major determining factor for the litter size in sheep (Davis et al., 2006). This gene has at least 23 different variants and is located on ovine chromosome six. Certain Booroola variants increase follicle sensitivity to the follicle-stimulating hormone, thereby inducing a faster follicle maturation (Fogarty, 2009). Moradband and colleagues (2011) revealed how heterozygosity in this gene appears to increase the litter size in the Iranian Baluchi sheep breed. Ewes that are homozygous for this gene variant almost double their ovulation rate. However, their lambs have a low survival rate with a lower growth rate and weaning rate (Fogarty, 2009). It is noteworthy that the Booroola gene has not been reported in all sheep breeds. Therefore, this limits its use as a global biomarker or genetic selection tool for increasing sheep litter size. While there is a considerable body of genetic data pertaining to sheep PLS which are used as a selection tool, none of these genes and transcripts have been properly evaluated via ROC curve analysis or sensitivity/specificity analysis to confirm their true utility as biomarkers of PLS.

The Booroola gene is associated with the bone morphogenetic protein receptor 1B (BMPR-1B; Abdoli et al., 2016). Increased blood concentrations of the BMPR-1B protein has been

reported to benefit follicular development, yielding better ovulation and increased litter size (Zhang et al., 2020). A separate study that evaluated proteins in the follicular fluid (FF) of ewes found that the FF of larger follicles compared to smaller follicles had increased glucose and cholesterol concentrations, but lower concentration of triglycerides, lactate, alkaline phosphatase and lactate dehydrogenase (Nandi et al., 2007). These metabolites and proteins appear to be correlated with ovulation rate, suggesting their relevance to prolific ewes and the litter they carry. In another study, Koch and colleagues (2010) used MS-based proteomics to identify 15 signature proteins from the uterine luminal fluid of ewes as indicators of pregnancy and involved with embryonic growth, immune regulation and nutritional needs. As yet, none of these protein markers have been rigorously validated by ROC curve analysis and none are commercially used in sheep PLS testing.

Another example of a protein biomarker in pregnant ewes is the pregnancy-associated glycoprotein (PAG). The PAG is a placental-secreted factor that is detected in maternal serum upon implantation of the fetus onto the endometrium. This protein can be measured as early as 30 days in the gestation (Khan et al., 2020), with increasing concentrations as the ewe pregnancy progresses (Roberts et al., 2017). The pregnancy specific protein B (PSPB) is a form of the PAG that is released by the fetus to maintain the corpus luteum (CL; Ruder et al., 1988). Also, PSPB along with other PAGs increases with increasing number of fetuses carried by the ewe (Pickworth et al., 2020). However, PSPB is a breed-specific compound (Redden and Passavant, 2013) which limits its universal application for all sheep breeds. Generally, PAGs are also positively correlated with maternal serum P4 levels (Roberts et al., 2017). In one study by Karen et al. (2003), measuring blood PAG had 93.5% sensitivity for detecting pregnancy at day 22 of gestation, however, their results were skewed by the abnormally low (17%) pregnancy rate of the flock.

In addition to genetic and protein biomarkers of sheep PLS, a number of metabolite biomarkers have also been explored. Progesterone is a promising example of a hormonal metabolite biomarker that could be used for assessing sheep PLS. Progesterone is predominantly produced by the CL at the beginning of gestation and later (day 50 onwards) is produced by the placenta to maintain the pregnancy (Lonergan et al., 2016; Roberts et al., 2017). The concentration of P4 in ewe blood increases over the course of gestation and has been used by certain research labs as an indicator of pregnancy, as well as placental and fetal wellbeing (Roberts et al., 2017). However, identifying ewe PLS through measurements of P4 concentrations at around days 50-80 of gestation has a sensitivity varying between 65-85% and a specificity between 65-93% (Karen et al., 2006; See et al., 2007). While potentially promising, blood P4 concentrations are not considered sufficiently accurate indicators of non-pregnant ewes (Karen et al., 2003) and are not useful for differentiating ewes based on litter size (See et al., 2007). Another steroid hormone, estradiol, has also been used by researchers for detecting litter size after 50 days into gestation (Sumaryadi and Manalu, 1999). Despite P4 and estradiol being significant reproductive hormones and associated with ewe PLS, the literature lacks sufficient evidence and validation based on ROC analysis or regression modeling to make these hormones truly useful for assessing sheep PLS status (Xia et al., 2013).

Other (non-hormonal) metabolites have also been identified as potentially useful pregnancy markers in other livestock species. A recent publication on pregnant buffaloes identified five milk metabolites detected by LC-MS on day 18 after artificial insemination as candidate biomarkers of pregnancy (de Nicola et al., 2020). Likewise, in beef cattle, four plasma metabolites were detected by NMR at day 40 of gestation (Gómez et al., 2020). These reports suggest that

measurement of non-hormonal metabolites may serve as an indirect means of pregnancy and/or litter size detection in ruminants.

To date, few studies have reported non-hormonal metabolites associated with sheep PLS. Sun and colleagues (2017) used NMR to investigate pregnant ewe metabolism in relation to *in utero* fetal growth at four timepoints from day 50 of gestation onwards. They reported 13 serum metabolites that are associated with protein and lipid metabolism of twin-bearing pregnant ewes. In another study using MS-based analysis of FF and ovarian vein serum in the Han sheep breed (Guo et al., 2018), a total of eight metabolites (glucose 6-phosphate, glucose 1-phosphate, aspartate, asparagine, glutathione oxidized, cysteine-glutathione disulfide, γ-glutamylglutamine, and 2-hydroxyisobutyrate) were significantly associated with ewe litter size. Another recent metabolomic study using LC-MS/MS revealed that sphingolipid and amino acid metabolism is important for maintaining the uterine environment to increase embryo survival rate (La et al., 2020). In addition to these studies, there are a few other reports that measured individual metabolites in pregnant sheep (Huang et al., 2012; Washburn et al., 2015; Kandiel et al., 2016; Cihan et al., 2016). Unfortunately, none of these studies identified or rigorously assessed the reported metabolites as robust PLS biomarkers. Overall, existing data suggests that certain individual genes, proteins and metabolites may be useful for assessing sheep PLS. However, as yet, there has been no metabolomics studies that have attempted to rigorously identify and validate a panel of readily accessible non-hormonal metabolite blood biomarkers for assessing sheep PLS.

A common feature of the serum biomarkers presented in this study is that all are detectable by NMR spectroscopy. While the identification and validation of a set of useful sheep PLS biomarker panels was our primary interest in this study (see Table 4), we also believe it is important to provide some biological context and to suggest how some of these metabolites may play a role

in sheep pregnancy. Indeed, the biological role of some of these metabolites appears to tie in with the reproductive physiology of sheep. However, some metabolites have not previously been identified as having a role in pregnancy, litter size or gestation and so it is difficult to understand their biological context. The following section further discusses the known biological relevance of each metabolite biomarker identified in this study. It also elaborates on the potential impact that these biomarkers may have for the sheep industry.

L-arginine is an essential amino acid that is known to be very relevant to successful pregnancy. We found that at day 50 of gestation, the average L-arginine was significantly (p-value<0.05; Table 1) elevated in pregnant ewes (214±85 μM) relative to non-pregnant controls (174±78 μM). Arginine appears to play a role in a number of physiological pathways related to pregnancy. Luther and colleagues (2009) provided pregnant ewes with L-arginine supplementation and observed enhanced ovarian function along with elevated numbers of viable fetuses. The same study identified a direct positive correlation between L-arginine and P4, leading to improved pregnancy maintenance and early embryonic growth. Our results appear to be consistent with these reports and show that pregnant ewes as well as ewes that delivered more lambs had a higher serum concentration of L-arginine. Furthermore, maternal administration of this amino acid in the later portion of gestation has been shown to increase lamb birth weight, enhance blood flow and increase nutrient transport to the fetus through synthesis of nitric oxide (Thureen et al., 2002; De Boo et al., 2005). L-arginine also improves pancreatic and brown adipose tissue growth during fetal development (Satterfield et al., 2013), and increases post-partum brown fat storage and the survivability of female lambs (McCoard et al., 2013). Serum L-arginine is associated with improved post-partum weaning weight and the weaning rate of lambs (Crane et al., 2016). Administering this amino acid to prolific ewes further improves the lambing rate by nearly 60%,

increases lamb birth weight by over 20% without negatively impacting maternal body weight, and decreases lamb mortality rate at birth by more than 20% (Lassala et al., 2011).

Another metabolite that was identified as a strong biomarker of litter size was urea. We found that at day 50 of gestation, the average urea concentration was significantly (p-value<0.001) lower in pregnant ewes (1823±667 μM) compared to the open ewes (2518±871 μM). Urea is a source of nitrogen for rumen microbes and is produced through the degradation of amino acids. Interestingly, elevated blood concentrations of urea in ewes seems to reduce the conception and pregnancy rate (Raboisson et al., 2017). Likewise, high concentrations of circulating urea have adverse impacts on embryonic development (Bishonga et al., 1996). Our results are in agreement with the literature as we identified that pregnant ewes as well as ewes with a greater litter size have a lower concentration of blood urea compared to non-pregnant ewes.

One of the more interesting biomarkers we identified for litter size was methionine. We found that the average methionine serum concentration was significantly lower (p-value<0.001) with methionine concentrations of (28±9 μM) in pregnant ewes that delivered more than two lambs compared to ewes that delivered just one lamb (33±9 μM). Methionine is an essential amino acid that plays an important role in general animal performance (El-Tahawy and Ismaeil, 2013), as well as the growth and development of lambs in early life (Wang et al., 2018). Methionine is also a methyl group supplier for epigenetic alteration of the DNA, especially in late gestation (Wooldridge et al., 2018). Indeed, Sinclair and associates (2007) reported widespread epigenetic alterations in the progeny, mostly male lambs, resulting from restricted supply of dietary methionine to the pregnant dam. Alterations to the genome induced by metabolites such as methionine are responsible for modification of health-related phenotypes, cell growth, host

immunity, and protein production (Strahl and Allis, 2000; Sinclair et al., 2007; Canani et al., 2011; Moore et al., 2013).

L-lactic acid is another biomarker of litter size that is traditionally associated with muscle metabolism. However, during pregnancy its concentration increases with the progression of gestation (Freetly and Ferrell, 1998). Indeed, we found that the average L-lactic acid concentration was significantly (p-value=0.01) higher (3293±1948 μM) in pregnant ewes that delivered more than two lambs compared to ewes that delivered only two lambs (2432±989 μM). Lactate can be used as an alternative source of energy by the fetal brain (Bissonnette et al., 1991). Therefore, a ewe with a higher number of fetuses is expected to have a higher concentration of serum L-lactic acid.

Valine is another biomarker we found to be associated with ewe litter size, and it decreased with increasing number of lambs. In particular, we found that the average valine serum concentration was significantly (p-value=0.007) higher (219±74 μM) in TWN versus TRP (191±64 μM) pregnant ewes. This metabolite is a branched-chain amino acid that stimulates protein synthesis in the fetal muscle (Kimball and Jefferson, 2004; Regnault et al., 2005). Therefore, ewes that deliver three or more lambs and have an overall higher fetal protein synthesis compared to those that deliver twins are expected to have a higher utilization of this amino acid and lower concentration in the serum. Branched-chain amino acids are also integral to the immune system by supporting the growth of lymphocytes and natural killer cells to remove viral infections (Calder, 2006). Pregnant ewes are more prone to immune challenges and an increased number of fetuses increases immune vulnerability of the ewe (Jamieson et al., 2006; Downs et al., 2018). Therefore, ewes that have the largest litter size, i.e., triplets vs twins, are expected to draw more valine from the maternal serum, which aligns with our results.

Comparison to Ultrasonography

The current gold standard for sheep PLS assessment is ultrasonography. Ultrasound is mostly used to determine pregnancy status (open vs pregnant). However, certain experienced ultrasound operators can detect the number of fetuses in pregnant ewes as early as approximately 40-45 days of pregnancy and onwards (based on industry observations in Canada). In fact, our field observations indicate that most Canadian ultrasound technicians identify litter size as one fetus or more than one. Ultrasound scanning is relatively rapid (2-5 min/ewe) and costs CAD$5-8/ewe (depending on the location of the farm, travel required for the operator to reach the farm, and the number of ewes being scanned). All sheep used in this study were characterized via ultrasound analysis by trained technicians at day 50 of pregnancy.

Using records from 166 ewes with complete data from ultrasound scanning and corresponding pregnancy outcome, we determined that the sensitivity of ultrasound was 55%, the specificity was 70% and the AU-ROC of using ultrasonography for pregnancy detection was 0.65. With regard to ultrasonography results for litter size, we found that for distinguishing SNG vs TRP, the sensitivity was 51% while the specificity was 18%. With regard to distinguishing TWN vs TRP, the sensitivity of ultrasonography was 43% while the specificity was 18%. It is noteworthy that the consistency of ultrasound prediction varied between farms mainly due to the expertise and experience of the technician who tended to underestimate singles and triplets while overestimating twins. Comparing our metabolomics results to these ultrasound measurements, as seen from Table 5, the serum metabolite markers we developed were better than ultrasonography by 24% in terms of AU-ROC, 20% better in terms of sensitivity, and 18% better in terms of specificity for detecting ewe pregnancy. Likewise, if we compare our predictive biomarker panels for detecting litter size against ultrasonography, we find that our metabolite panels performed 9-35% better in terms of

sensitivity and nearly 80% better in terms of specificity for predicting litter size. These results indicate serum metabolite measurements are significantly more accurate than ultrasound in detecting and assessing sheep PLS in this study.

Clearly, in order for any alternative tool, such as blood marker tests identified here to compete with ultrasound for sheep PLS assessment, it would have to be either cheaper, more accurate, more convenient or able to detect PLS at earlier gestational timepoints. Based on our current data, the metabolite panels we have identified are clearly more accurate in this study. However, could they compete with the cost of ultrasound? Recall that ultrasound tests cost between CAD$5-8 per ewe, for those producers who can access ultrasound technicians. Currently metabolite tests consisting of three or four metabolites conducted on MS instruments can be done for as little as CAD$5 per sample (excluding shipping costs). These costs can be reduced further if testing were to be optimized or more widespread. If the metabolite tests could be converted to a handheld device (such as a lateral flow assay or a simple colorimetric test) for pen side testing, then both the lower cost (perhaps as little as $3 a test) and improved convenience would make these sorts of blood tests very appealing to the producers. It is also clear that these biomarkers have a better performance when it comes to predicting larger litter sizes in pregnant ewes. Even if we assume that these metabolite biomarkers perform comparably to the ultrasound, the cost of the blood test would not vary (as it does for ultrasound scanning) based on the flock size and geographical location of the farm. This would permit farms with smaller flocks and farms located in remote areas to benefit from blood-based PLS detection. Certainly, if serum markers could be found effective much earlier in gestation (say at day 25 or 35) with a sensitivity or specificity that is comparable to ultrasound, then the potential of a blood test for sheep PLS would be that much greater.

Industry Impact

Ewe reproductive efficiency, lamb performance and overall economic productivity of a lamb production enterprise are all key factors in determining flock profitability. Single born lambs tend to have large birth weights with more dystocia issues while triplets tend to have low birthweights with low survivability (Juengel et al., 2018). Ewes giving birth to triplets, or even more lambs, have increased risks of pregnancy toxemia and hypocalcaemia, which results in high mortality rates in ewes. Such PLS management and health issues come with increased costs of production. Ewe nutrition in the last 8 weeks of gestation is critical as it impacts lamb survivability. Typically, 95% of singles, 79% of twins and only 67% of triplet born lambs survive the first week of life. Using this information, we can calculate the potential economic impact of improved PLS management on sheep farms across Canada. In doing so, we assumed some variation in breed type, seasonal effects and a 15% cull rate (Statistics Canada, 2020), knowing that 437,000 Canadian ewes are exposed to breeding in a year with 20% of these expected to bear three or more lambs in one litter. If we further assume that a handheld blood test with our biomarkers would have a detection accuracy of 80% and would cost approximately CAD$3/ewe, then the following calculation can be done. We estimate that ~87,000 ewes will potentially yield 9% more lambs at weaning (with the litter size increasing from an average 1.9 lambs per ewe to 2.07 lambs per ewe) at an extra cost of CAD$961,400 for nutrition expenses (equivalent to CAD$11/ewe/year). These lambs are expected to be ~21 kg at weaning and worth CAD$5.50/kg (conservatively totaling to CAD$115/lamb; reflecting current Alberta prices with expected variation in breed type, condition, age, season, sale date, etc.). Hence, Canadian lamb sales could increase by up to CAD$2 million/yr if we could reliably identify those prolific ewes at 50 days gestation and sort them into management groups for more targeted feeding. By detecting and culling open ewes, or rebreeding them, and

improving the nutrition of ewes that deliver more viable and healthier lambs, we anticipate flock profitability could be increased by CAD$2 for every CAD$1 invested in ewe nutrition – particularly if accurate, low-cost sheep PLS management could be fully implemented. In addition, ewe health and feed related costs would be reduced by adjusting feed based on pregnancy requirements and preventing blind feeding of all animals with the same ration. Moreover, epigenetics and nutrigenomics studies (Sinclair et al., 2007; Wooldridge et al., 2018) have proven that adjusting maternal feed based on pregnancy requirements programs the progeny to be healthier and physiologically more sound than the average lamb.

Future Prospects

We have shown that targeted, quantitative metabolomics technologies can be used to discover and validate serum metabolite biomarkers of sheep pregnancy and litter size. Using a large cohort of samples collected from multiple commercial flocks across Canada, we successfully identified four panels of biomarkers that can determine ewe PLS with good accuracy and precision. The performance of these markers appears to exceed that seen with ultrasound measurements within the context of this experiment. Therefore, we believe that if these biomarkers could be further optimized (for high throughput off-site assays) or translated to hand-held or pen-side tests (similar to the urine-based pregnancy detection kit for women), they could be used to routinely assess PLS in Canadian sheep flocks. Currently we are working on developing a pen-side kit, using the panel of five biomarkers identified and validated in this study, to detect ewe pregnancy 50 days into gestation. If producers require the exact number of the litter size, a second test incorporating the two panels of biomarkers reported here could also be developed. In conclusion, translating these results for on-farm, pen-side use could significantly improve reproduction management and profitability of sheep breeding enterprises.

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Disclosures

All data are publicly available online at the Livestock Metabolome Database (www.lmdb.ca). The authors confirm no conflicts of interest that may affect their ability to objectively present research or data.

Literature Cited

1. Abdoli, R., P. Zamani, S. Mirhoseini, N. Ghavi Hossein-Zadeh, and S. Nadri. 2016. A review on prolificacy genes in sheep. Repro. Dom. Anim. 51:631-637. https://doi.org/10.1111/rda.12733.
2. Bielli, A., R. Pérez, G. Pedrana, J. T. B. Milton, Á. Lopez, M. A. Blackberry, G. Duncombe, H. Rodriguez-Martinez, and G. B. Martin. 2002. Low maternal nutrition during pregnancy reduces the number of Sertoli cells in the newborn lamb. Repro. Fert. Develop. 14:333-337. https://doi.org/10.1071/RD02046.
3. Bishonga, C., J. J. Robinson, T. G. McEvoy, P. Findlay, R. P. Aitken, and I. Robertson. 1996. Excess dietary urea intake in ewes and its effect on ovulation rate and embryo development. Japanese J. Vet. Res. 44:139-151. http://doi.org/10.14943/jjvr.44.3.139.
4. Bissonnette, J. M., A. R. Hohimer, and C. R. Chao. 1991. Unidirectional transport of glucose and lactate into brain of fetal sheep and guinea-pig. Exper. Physio. 76:515-523. doi: 10.1113/expphysiol.1991.sp003517.
5. Bloomfield, F. H., M. H. Oliver, P. Hawkins, A. C. Holloway, M. Campbell, P. D. Gluckman, J. E. Harding, and J. R. G. Challis. 2004. Periconceptional undernutrition in sheep accelerates maturation of the fetal hypothalamic-pituitary-adrenal axis in late gestation. Endocri. 145:4278e85. https://doi.org/10.1210/en.2004-0424.
6. Calder, P. C. 2006. Branched-chain amino acids and immunity. J. Nutri. 136:288S–293S. https://doi.org/10.1093/jn/136.1.288S.
7. Canani, R. B., M. Di Costanzo, L. Leone, G. Bedogni, and P. Brambilla. 2011. Epigenetic mechanisms elicited by nutrition in early life. Nutr. Res. Rev. 24:198-205. DOI: 10.1017/S0954422411000102.

8. Cihan, H., E. M. Temizel, Z. Yilmaz, and Y. Ozarda. 2016. Serum iron status and its relation with haematological indexes before and after parturition in sheep. Kafkas. Uni. Vet. Fak. Derg. 22:679-683. DOI: 10.9775/kvfd.2016.15103.
9. Crane, A. R., R. R. Redden, M. L. Van Emon, T. L. Neville, L. P. Reynolds, J. S. Caton, and C. S. Schauer. 2016. Impacts of supplemental arginine on the reproductive performance of fall lambing ewes. J. Anim. Sci. 94:3540–3549. https://doi.org/10.2527/jas.2016-0379.
10. Davis, G. H., L. Balakrishnan, I. K. Ross, T. Wilson, S. M. Galloway, B. M. Lumsden, J. P. Hanrahan, M. Mullen, X. Z. Mao, G. L. Wang, and Z. S. Zhao. 2006. Investigation of the Booroola (FecB) and Inverdale (FecX(I)) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. Anim. Repro. Sci. 92:87–96. https://doi.org/10.1016/j.anireprosci.2005.06.001.
11. De Boo, H., P. L. Van Zijl, D. E. C. Smith, W. Kulik, H. N. Lafeber, and J. E. Harding. 2005. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr. Res. 58:270–277. https://doi.org/10.1203/01.PDR.0000169977.48609.55.
12. de Nicola, D., F. Vinale, A. Salzano, G. d'Errico, A. Vassetti, N. D'Onofrio, M. L. Balestrieri, and G. Neglia. 2020. Milk Metabolomics reveals potential biomarkers for early prediction of pregnancy in buffaloes having undergone artificial insemination. Animals. 10:758. https://doi.org/10.3390/ani10050758.
13. Downs, C. J., B. V. Boan, T. D. Lohuis, and K. M. Stewart. 2018. Investigating Relationships between Reproduction, Immune Defenses, and Cortisol in Dall Sheep. Front. Immun. 9:105. https://doi.org/10.3389/fimmu.2018.00105.

14. Du, M., J. Tong, J. Zhao, K. R. Underwood, M. Zhu, S. P. Ford, and P. W. Nathanielsz. 2010. Fetal programming of skeletal muscle development in ruminant animals. J. Anim. Sci. 88:E51–E60. https://doi.org/10.2527/jas.2009-2311.

15. El-Tahawy, A. S., and P. A. Ismaeil. 2013. Methionine-supplemented diet increases the general performance and value of Rahmani Lambs. Iranian J. App. Anim. Sci. 3:513–520. https://www.sid.ir/en/journal/ViewPaper.aspx?id=325859.

16. Fogarty, N. M. 2009. A review of the effects of the Booroola gene (FecB) on sheep production. Sm. Rumin. Res. 85:75-84. https://doi.org/10.1016/j.smallrumres.2009.08.003.

17. Fontanesi, L. 2016. Metabolomics and livestock genomics: Insights into a phenotyping frontier and its applications in animal breeding. Anim Front. 6:73-79. https://doi.org/10.2527/af.2016-0011.

18. Freetly, H. C., and C. L. Ferrell. 1998. Net flux of glucose, lactate, volatile fatty acids, and nitrogen metabolites across the portal-drained viscera and liver of pregnant ewes. J. Anim. Sci. 76:3133–3145. https://doi.org/10.2527/1998.76123133x.

19. Ghafouri-Kesbi, F., and M. P. Eskandarinasab. 2008. An evaluation of maternal influences on growth traits: the Zandi sheep breed of Iran as an example. J. Anim. Feed Sci. 17:519–529. https://pdfs.semanticscholar.org/8a2a/744641208357f7e63f12959888588bf44792.pdf

20. Goldansaz, S. A., A. C. Guo, T. Sajed, M. A. Steele, G. S. Plastow, and D. S. Wishart. 2017. Livestock metabolomics and the livestock metabolome: A systematic review. PLoS ONE. 12:e0177675. https://doi.org/10.1371/journal.pone.0177675.

21. Goldansaz, S. A., S. Markus, M. Berjanskii, M. Rout, A. C. Guo, Z. Wang, G. Plastow, and D. S. Wishart. 2020. Candidate serum metabolite biomarkers of residual feed intake and carcass merit in sheep. J. Anim. Sci. 98:skaa298. https://doi.org/10.1093/jas/skaa298.

22. Gómez, E., M. Muñoz, J. Gatien, S. Carrocera, D. Martín-González, and P. Salvetti. 2020. Metabolomic identification of pregnancy-specific biomarkers in blood plasma of BOS TAURUS beef cattle after transfer of in vitro produced embryos. J. Prot. 225:103883. https://doi.org/10.1016/j.jprot.2020.103883.

23. Guo, X., X. Wang, R. Di, Q. Liu, W. Hu, X. He, J. Yu, X. Zhang, J. Zhang, K. Broniowska, and W. Chen. 2018. Metabolic effects of FecB gene on follicular fluid and ovarian vein serum in sheep (Ovis aries). Int. J. Mol. Sci. 19:539. https://doi.org/10.3390/ijms19020539.

24. Haibel, G. K. 1990. Use of ultrasonography in reproductive management of sheep and goat herds. The Veterinary Clinics of North America. Food Anim. Prac. 6:597-613. DOI: 10.1016/s0749-0720(15)30835-5.

25. Huang, L., C. Hsieh, K. Chang, and Y. Tain. 2012. Roles of nitric oxide and asymmetric dimethylarginine in pregnancy and fetal programming. Int. J. Mol. Sci. 13:14606-14622. https://doi.org/10.3390/ijms131114606.

26. Ishwar, A. K. 1995. Pregnancy diagnosis in sheep and goats: a review. Sm. Rumin. Res. 17:37-44. https://doi.org/10.1016/0921-4488(95)00644-Z.

27. Jamieson, D. J., R. N. Theiler, and S. A. Rasmussen. 2006. Emerging infections and pregnancy. Emerg. Infect. Dis. 12:1638-1643. doi: 10.3201/eid1211.060152.

28. Jones, A. K., and S. A. Reed. 2017. Benefits of ultrasound scanning during gestation in the small ruminant. Sm. Rumin. Res. 149:163-171. https://doi.org/10.1016/j.smallrumres.2017.02.008.

29. Jones, A. K., R. E. Gately, K. K. McFadden, S. A. Zinn, K. E. Govoni, and S. A. Reed. 2016. Transabdominal ultrasound for detection of pregnancy, fetal and placental landmarks, and fetal

age before Day 45 of gestation in the sheep. Therio. 85:939-945.e1. https://doi.org/10.1016/j.theriogenology.2015.11.002.

30. Juengel, J. L., G. H. Davis, R. Wheeler, K. G. Dodds, and P. D. Johnston. 2018. Factors affecting differences between birth weight of littermates (BWTD) and the effects of BWTD on lamb performance. Anim. Repro. Sci. 191:34-43. https://doi.org/10.1016/j.anireprosci.2018.02.002.
31. Kandiel, M. M. M., H. M. El-Khaiat, and K. Gh. M. Mahmoud. 2016. Changes in some hematobiochemical and hormonal profile in Barki sheep with various reproductive statuses. Sm. Rumin. Res. 136:87-95. https://doi.org/10.1016/j.smallrumres.2016.01.011.
32. Karen, A., J. Beckers, J. Sulon, N. M. de Sousa, K. Szabados, J. Reczigel, and O. Szenci. 2003. Early pregnancy diagnosis in sheep by progesterone and pregnancy-associated glycoprotein tests. Therio. 59:1941-1948. https://doi.org/10.1016/S0093-691X(02)01289-X.
33. Karen, A., B. El Amiri, J. Beckers, J. Sulon, M. A. M. Taverne, and O. Szenci. 2006. Comparison of accuracy of transabdominal ultrasonography, progesterone and pregnancy-associated glycoproteins tests for discrimination between single and multiple pregnancy in sheep. Therio. 66:314-322. DOI: 10.1016/j.theriogenology.2005.11.017.
34. Khan, D., H. Khan, A. Nazir, M. T. Tunio, M. Tahir, M. S. Khan, and R. U. Khan. 2020. Early pregnancy diagnosis using pregnancy-associated glycoproteins in the serum of pregnant ruminants. Pakistan J. Zool. 52:785-788. DOI: http://dx.doi.org/10.17582/journal.pjz/20190527080541.
35. Kimball, S. R., and L. S. Jefferson. 2004. Regulation of global and specific mRNA translation by oral administration of branched-chain amino acids. Biochem. Biophys. Res. Com. 313:423-427. https://doi.org/10.1016/j.bbrc.2003.07.014.

36. Kiyma, Z., M. Kose, M. O. Atli, C. Ozel, M. Hitit, G. Sen, M. Kaya, M. S. Kaya, E. Kurar, S. Ali Kayis, and A. Guzeloglu. 2016. Investigation of interferon-tau stimulated genes (ISGs) simultaneously in the endometrium, corpus luteum (CL) and peripheral blood leukocytes (PBLs) in the preluteolytic stage of early pregnancy in ewes. Sm. Rumin. Res. 140:1-6. https://doi.org/10.1016/j.smallrumres.2016.05.007.

37. Koch, J. M., J. Ramadoss, and R. R. Magness. 2010. Proteomic profile of uterine luminal fluid from early pregnant ewes. J. Prot. Res. 9:3878-3885. https://doi.org/10.1021/pr100096b.

38. La, Y., J. Tang, X. Guo, L. Zhang, S. Gan, X. Zhang, J. Zhang, W. Hu, and M. Chu. 2020. Proteomic analysis of sheep uterus reveals its role in prolificacy. J. Prot. 210:103526. https://doi.org/10.1016/j.jprot.2019.103526.

39. Lassala, A., F. W. Bazer, T. A. Cudd, S. Datta, D. H. Keisler, M. C. Satterfield, T. E. Spencer, and G. Wu. 2011. Parenteral administration of L-arginine enhances fetal survival and growth in sheep carrying multiple fetuses. J. Nutri. 141:849–855. https://doi.org/10.3945/jn.111.138172.

40. Lonergan, P., N. Forde, and T. Spencer. 2016. Role of progesterone in embryo development in cattle. Reprod. Fertil. Dev. 28:66e74. https://doi.org/10.1071/RD15326.

41. Luther, J. S., E. J. Windorski, J. S. Caton, G. Wu, J. D. Kirsch, K. A. Vonnahme, L. P. Reynolds, and C. S. Schauer. 2009. Effects of arginine supplementation on reproductive performance in Rambouillet ewes. Sheep Res. Rep. 50:11-13. https://www.ag.ndsu.edu/Hettingerrec/sheep/individual-articles-from-2009-sheep-research-report/Effects%20of%20arginine%20supplementation%20on%20reproductive%20performance.pdf

42. Moore, L. D., T. Le, and G. Fan. 2013. DNA methylation and its basic function. Neuropsychopharmacology. 38:23-38. https://doi.org/10.1038/npp.2012.112.

43. McCoard, S., F. Sales, N. Wards, Q. Sciascia, M. Oliver, J. Koolaard, and D. van der Linden. 2013. Parenteral administration of twin-bearing ewes with L-arginine enhances the birth weight and brown fat stores in sheep. Spr. Plu. 2:684. https://doi.org/10.1186/2193-1801-2-684.

44. Moradband, F., G. E. Rahimi, and M. Gholizadeh. 2011. Association of polymorphisms in fecundity genes of GDF9, BMP15 and BMP15-1B with litter size in Iranian Baluchi sheep. Asian-Aus. J. Anim. Sci. 24:1179-1183. DOI: https://doi.org/10.5713/ajas.2011.10453.

45. Nandi, S., V. G. Kumar, B. M. Manjunatha, and P. S. Gupta. 2007. Biochemical composition of ovine follicular fluid in relation to follicle size. Dev. Grow. Differ. 49:61–66. https://doi.org/10.1111/j.1440-169X.2007.00901.x.

46. Nutrient Requirements of Sheep. Sixth Revised Edition. Subcommittee on Sheep Nutrition. Committee on Animal Nutrition. Board on Agriculture, National Research Council (NRC). National Academy Press. Washington. D.C. (1985).

47. Parkins, J. J., J. Fraser, N. S. Ritchie, and R. G. Hemingway. 1974. Urea as a protein source for ewes with twin lambs in late pregnancy and early lactation. Anim. Sci. 19:321-329. doi:10.1017/S0003356100022893.

48. Raboisson, D., A. Albaaj, G. Nonne, and G. Foucras. 2017. High urea and pregnancy or conception in dairy cows: A meta-analysis to define the appropriate urea threshold. J. Dairy Sci. 100:7581-7587. https://doi.org/10.3168/jds.2016-12009.

49. Ravanbakhsh, S., P. Liu, T. C. Bjorndahl, R. Mandal, J. R. Grant, M. Wilson, R. Eisner, I. Sinelnikov, X. Hu, C. Luchinat, R. Greiner, and D. S. Wishart. 2015. Accurate, fully-

automated NMR spectral profiling for metabolomics. PLoS ONE. 10(5): e0124219. doi: 10.1371/journal.pone.0124219.

50. Regnault, T. R. H., J. E. Friedman, R. B. Wilkening, R. V. Anthony, and W. W. Hay. 2005. Fetoplacental transport and utilization of amino acids in IUGR — a review. Placenta. 26:S52-S62. https://doi.org/10.1016/j.placenta.2005.01.003.
51. Roberts, J. N., K. J. May, and A. Veiga-Lopez. 2017. Time-dependent changes in pregnancy-associated glycoproteins and progesterone in commercial crossbred sheep. Therio. 89:271-279. https://doi.org/10.1016/j.theriogenology.2016.10.029.
52. Rosales-Nieto, C. A., R. Ehrhardt, A. Mantey, B. Makela, and A. Veiga-Lopez. 2021. Preconceptional diet manipulation and fetus number can influence placenta endocrine function in sheep. Dom. Anim. Endo. 74:106577. https://doi.org/10.1016/j.domaniend.2020.106577.
53. Satterfield, M. C., K. A. Dunlap, D. H. Keisler, F. W. Bazer, and G. Wu. 2013. Arginine nutrition and fetal brown adipose tissue development in nutrient-restricted sheep. Amin. Aci. 45:489–499. https://doi.org/10.1007/s00726-011-1168-8.
54. See, K., A. J. Bailer, E. J. Behnke, R. S. Baker, K. E. Clark, and P. T. Russell. 2007. Predicting pregnancy status and fetal number in time-dated pregnant ewes using serum progesterone and ultrasound. Res. J. Anim. Sci. 1:65-71. https://medwelljournals.com/abstract/?doi=rjnasci.2007.65.71.
55. Sinclair, K. D., C. Allegrucci, R. Singh, D. S. Gardner, S. Sebastian, J. Bispham, A. Thurston, J. F. Huntley, W. D. Rees, C. A. Maloney, and R. G. Lea. 2007. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. PNAS. 104:19351-19356. https://doi.org/10.1073/pnas.0707258104.

56. Strahl, B. D., and C. D. Allis. 2000. The language of covalent histone modifications. Nature. 403:41-45. https://doi.org/10.1038/47412.

57. Sumaryadi, M., and W. Manalu. 1999. Prediction of litter size based on hormones and blood metabolites concentrations during pregnancy in Javanese thin-tail ewes. Asian-Aus. J. Anim. Sci. 12:682-688. https://doi.org/10.5713/ajas.1999.682.

58. Sun, L., Yixuan Guo, Y. Fan, H. Nie, R. Wang, and F. Wang. 2017. Metabolic profiling of stages of healthy pregnancy in Hu sheep using nuclear magnetic resonance (NMR). Therio. 92:121-128. https://doi.org/10.1016/j.theriogenology.2017.01.025.

59. Susmel, P., and E. Piasentier. 1992. Assessment of pregnancy in Bergamasca ewes by analysis of plasma progesterone. Sm. Rumin. Res. 8:325-332. https://doi.org/10.1016/0921-4488(92)90213-N.

60. Thureen, P., K. Baron, P. Fennessey, and W. W. Hay. 2002. Ovine placental and fetal arginine metabolism at normal and increased maternal plasma arginine concentrations. Pediatr. Res. 51:464–471. https://doi.org/10.1203/00006450-200204000-00011.

61. Umer, S., S. J. Zhao, A. Sammad, B. W. Sahlu, Y. Pang, and H. Zhu. 2019. AMH: Could it be used as a biomarker for fertility and superovulation in domestic animals? Genes. 10:1009. https://doi.org/10.3390/genes10121009.

62. Wang, J., K. Cui, T. Ma, F. Zhang, S-Q. Wang, Y. Tu, Q-Y. Diao, and N-F. Zhang. 2018. Effects of dietary methionine deficiency followed by replenishment on the growth performance and carcass characteristics of lambs. Anim. Pro. Sci. 59:243-248. https://doi.org/10.1071/AN16643.

63. Washburn, S. E., M. A. Caudill, O. Malysheva, A. J. MacFarlane, N. A. Behan, B. Harnett, L. MacMillan, T. Pongnopparat, J. T. Brosnan, and M. E. Brosnan. 2015. Formate metabolism in

fetal and neonatal sheep. Am. J. Physiol. Endocrinol. Metab. 308:E921–E927. https://doi.org/10.1152/ajpendo.00046.2015.

64. Wooldridge, A. L., R. J. Bischof, H. Liu, G. K. Heinemann, D. S. Hunter, L. C. Giles, R. A. Simmons, Y. C. Lien, W. Lu, J. D. Rabinowitz, and K. L. Kind. 2018. Late-gestation maternal dietary methyl donor and cofactor supplementation in sheep partially reverses protection against allergic sensitization by IUGR. Am. J. Physio. Reg. Integ. Comp. Physio. 314:R22-R33. https://doi.org/10.1152/ajpregu.00549.2016.
65. Xia, J., D. Broadhurst, M. Wilson, and D. Wishart. 2013. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics. 9:280-299. https://doi.org/10.1007/s11306-012-0482-9.
66. Zaher, H. A., S. A. Alawaash, and A. A. Swelum. 2020. Effects of season and breed on the reproductive performance of sheep. J. Anim. Repro. Biotech. 35:149–154. https://doi.org/10.12750/JARB.35.2.149.
67. Zhang, L., J. Xue, Q. Wang, W. Lv, H. Mi, Y. Liu, and L. Yang. 2018. Changes in expression of ISG15, progesterone receptor and progesterone-induced blocking factor in ovine thymus during early pregnancy. Therio. 121:153-159. https://doi.org/10.1016/j.theriogenology.2018.08.018.
68. Zhang, X., L. Zhang, W. Sun, X. Lang, J. Wu, C. Zhu, J. Jia, J. Jin, Y. La, and D. P. Casper. 2020. Study on the correlation between BMPR1B protein in sheep blood and reproductive performance. J. Anim. Sci. 98:skaa100. https://doi.org/10.1093/jas/skaa100.

Tables

Table 1. Student's t-test of four comparison groups from the discovery dataset. Statistical analysis using t-test revealed significant (p-value<0.05) serum metabolites of each comparison at five timepoints during the discovery phase. NS = Not Significant; CNT = control open ewes; PRG = pregnant ewes; SNG = pregnant ewes that delivered one lamb; TWN = pregnant ewes that delivered two lambs; TRP = pregnant ewes that delivered more than two lambs. Day -7 refers to seven days prior to initiation of gestation and day 0 is the start of pregnancy.

T-test					
	Day -7	Day 0	Day 35	Day 50	Day 70
CNT vs PRG	NS	NS	NS	acetic acid, urea, SM (OH) C24:1, lysoPC a C26:0, lysoPC a C26:1, tryptophan, C3 (propionylcarnitine), carnosine, alpha-aminoadipic acid, putrescine, trimethylamine N-oxide, lysoPC a C18:2, hippuric acid, lysoPC a C14:0, L-arginine, lysoPC a C16:1	urea, glycine, L-arginine, dimethylamine, formate, dimethyl sulfone, choline, acetic acid, 3-hydroxybutyric acid, acetoacetate, L-alanine, sarcosine, isobutyric acid, L-lysine, creatinine, pyruvic acid, D-mannose, L-serine
CNT vs MLP	NS	kynurenine, L-ornithine	NS	urea, acetic acid, SM (OH) C24:1, lysoPC a C26:0, L-arginine, C3 (propionylcarnitine), L-carnitine, tryptophan, lysoPC a C26:1, carnosine, putrescine	urea, L-arginine, choline, glycine, acetic acid, dimethylamine, formate, 3-hydroxybutyric acid, dimethyl sulfone, acetoacetate, isobutyric acid, L-alanine, sarcosine, pyruvic acid, L-lysine, isoleucine
SNG vs TRP	NS	NS	L-acetylcarnitine	methionine	NS
TWN vs TRP	NS	NS	NS	valine, L-lactic acid, Isobutyric acid	NS

Table 2. Volcano plot univariate analysis of four comparison groups from the discovery dataset. Statistical analysis using volcano plot revealed significant (p-value<0.05) serum metabolites of each comparison at five timepoints during the discovery phase. Metabolite noted with ^ has a tendency (p-value<0.10). NS = Not Significant; CNT = control open ewes; PRG = pregnant ewes; SNG = pregnant ewes that delivered one lamb; TWN = pregnant ewes that delivered two lambs; TRP = pregnant ewes that delivered more than two lambs. Day -7 refers to seven days prior to initiation of gestation and day 0 is the start of pregnancy.

Volcano Plot					
	Day -7	Day 0	Day 35	Day 50	Day 70
CNT vs PRG	citric acid	SM C20:2, trans-hydroxyProline, kynurenine, total dimethylarginine	acetone, total dimethylarginine, sarcosine, isobutyric acid, taurine, C3 (propionylcarnitine), methanol, putrescine	acetic acid, SM (OH) C24:1, lysoPC a C26:0, lysoPC a C26:1, tryptophan, C3 (propionylcarnitine), putrescine, trimethylamine N-oxide, L-arginine, lysoPC a C16:1	urea, glycine, L-arginine, dimethylamine, formate, dimethyl sulfone, choline, acetic acid, 3-hydroxybutyric acid, acetoacetate, sarcosine, L-lysine, acetone, dimethylglycine
CNT vs MLP	citric acid^	L-ornithine, kynurenine, trans-hydroxyProline, SM C20:2, total dimethylarginine	acetone, L-ornithine, total dimethylarginine, isobutyric acid, taurine, trans-hydroxyProline, methanol, aspartic acid, C3 (propionylcarnitine), acetic acid, sarcosine, 3-hydroxyisovaleric acid	acetic acid, SM (OH) C24:1, lysoPC a C26:0, L-arginine, C3 (propionylcarnitine), tryptophan, lysoPC a C26:1, carnosine, putrescine, lysoPC a C18:2, lysoPC a C16:1, lysoPC a C14:0, methionine-sulfoxide, spermidine, trimethylamine N-oxide	urea, L-arginine, choline, glycine, acetic acid, dimethylamine, formate, 3-hydroxybutyric acid, dimethyl sulfone, acetoacetate, sarcosine
SNG vs TRP	isobutyric acid	NS	L-acetylcarnitine	acetyl-ornithine, kynurenine, methionine	choline, L-ornithine, ethanol

TWN vs TRP	ethanol	C3 (propionylcarnitine), serotonin	trans-hyrdoxyproline, kynurenine, hypoxanthine, acetone, formate, SM C20:2, lysoPC a C26:1	SM C20:2, valine, L-lactic acid, Isobutyric acid	L-ornithine, 3-methyl-2-oxovaleric acid, ethanol

Table 3. Partial least squares discriminant analysis (PLS-DA) analysis of four comparison groups from the discovery dataset. Multivariate statistical analysis of the discovery dataset using PLS-DA revealed top 15 metabolites that significantly (p-value<0.05) differentiate between the two comparison groups at each timepoint. NS = Not Significant; CNT = control open ewes; PRG = pregnant ewes; SNG = pregnant ewes that delivered one lamb; TWN = pregnant ewes that delivered two lambs; TRP = pregnant ewes that delivered more than two lambs. Day -7 refers to seven days prior to initiation of gestation and day 0 is the start of pregnancy.

PLS-DA VIP					
	Day -7	Day 0	Day 35	Day 50	Day 70
CNT vs PRG	NS	NS	putrescine, butyrate, sarcosine, L-ornithine, acetone, total dimethylarginine, ethanol, L-lysine, C3 (propionylcarnitine), taurine, methanol, trimethylamine N-oxide, isobutyric acid, aspartic acid, 3-hydroxyisovaleric acid	acetic acid, urea, SM (OH) C24:1, lysoPC a C26:0, lysoPC a C26:1, tryptophan, C3 (propionylcarnitine), carnosine, alpha-aminoadipic acid, putrescine, trimethylamine N-oxide, lysoPC a C18:2, hippuric acid, lysoPC a C14:0, L-arginine	urea, glycine, acetic acid, L-arginine, dimethyl sulfone, 3-hydroxybutyric acid, ethanol, L-lactic acid, L-lysine, sarcosine, dimethylamine, D-glucose, tyrosine, L-alanine, betaine
CNT vs MLP	Tendency	urea, L-ornithine, L-lysine, acetoacetate, acetic acid, glycine, kynurenine, 3-hydroxybutyric acid, trans-hydroxyProline, total dimethylarginine, SM C16:0, taurine, L-threonine, methanol, butyrate	acetic acid, L-ornithine, L-lysine, methanol, taurine, trimethylamine N-oxide, acetone, citric acid, sarcosine, ethanol, isobutyric acid, C0 (Carnitine), aspartic acid, butyrate, total dimethylarginine	acetic acid, urea, L-arginine, tryptophan, carnosine, 3-hydroxybutyric acid, dimethyl sulfone, trimethylamine N-oxide, L-lysine, L-carnitine, lysoPC a C18:2, L-ornithine, hippuric acid, C0	urea, dimethylamine, L-arginine, glycine, dimethyl sulfone, choline, acetic acid, formate, 3-hydroxybutyric acid, L-alanine, isobutyric acid, acetoacetate, isoleucine, L-lysine, pyruvic acid

				(Carnitine), methanol	
SNG vs TRP	NS	NS	NS	NS	Tendency
TWN vs TRP	NS	NS	NS	NS	NS

Table 4. Receiver Operating Characteristics (ROC) analysis of the comparison groups in the discovery and validation datasets. Candidate biomarkers were evaluated during all five timepoints of the discovery phase and day 50 of gestation was the best timepoint to reveal candidate biomarkers of ewe PLS. Therefore, biomarker analysis was pursued for only day 50 of gestation in the validation phase. The panel of metabolites that reached an area-under-the-curve (AU-ROC) of at least 0.65 or were significant (p-value<0.05) were considered as candidate biomarkers in the discovery phase and were confirmed as biomarkers if the AU-ROC and p-value improved in the validation analysis. NS = Not Significant; NA = biomarker not available; CNT = control open ewes; PRG = pregnant ewes; SNG = pregnant ewes that delivered one lamb; TWN = pregnant ewes that delivered two lambs; TRP = pregnant ewes that delivered more than two lambs. Day -7 refers to seven days prior to initiation of gestation and day 0 is the start of pregnancy

ROC						
	Discovery Phase					Validation Phase
	Day -7	Day 0	Day 35	Day 50	Day 70	Day 50
CNT vs PRG	NA	NA	NA	methanol, L-carnitine, D-glucose, L-arginine, urea	urea, glycine	methanol, L-carnitine, D-glucose, L-arginine, urea
	NA	NA	NA	AU-ROC=0.76 p<0.10	AU-ROC=0.98 p<0.001	AU-ROC=0.90 p<0.05
CNT vs MLP	NA	L-ornithine, choline	acetone, L-ornithine, C0, total dimethylarginine	methanol, L-carnitine, D-glucose, L-arginine, urea	choline, urea, L-arginine, glycine	methanol, L-carnitine, D-glucose, L-arginine, urea
	NA	AU-ROC=0.79 p<0.05	AU-ROC=0.73 p<0.05	AU-ROC=0.76 p<0.05	AU-ROC=0.97 p<0.01	AU-ROC=0.93 p<0.001
SNG vs TRP	choline, L-carnitine, L-phenylalanine	C4, L-threonine, trans-hydroxyproline	L-acetylcarnitine, L-carnitine, trans-hydroxyproline	methionine, L-carnitine	choline, D-glucose, L-phenylalanine	methionine, L-carnitine
	AU-ROC=0.80 p<0.05	AU-ROC=0.74 p<0.05	AU-ROC=0.76 p<0.10	AU-ROC=0.78 p<0.05	AU-ROC=0.81 p<0.05	AU-ROC=0.84 p<0.001
TWN vs TRP	hypoxanthine, L-phenylalanine, choline,	serotonin, C3	hypoxanthine, trans-	isobutyric acid, L-lactic acid, L-carnitine,	hypoxanthine,	isobutyric acid, L-lactic acid,

	L-carnitine, creatinine		hydroxyproline, kynurenine	valine, tyrosine, methanol	L-phenylalanine, L-carnitine, isobutyric acid	L-carnitine, valine, tyrosine, methanol
	AU-ROC=0.77 p<0.10	AU-ROC=0.74 p<0.05	AU-ROC=0.75 p<0.05	AU-ROC=0.66 p<0.10	AU-ROC=0.77 p<0.05	AU-ROC=0.81 p<0.05

Table 5. Performance comparison of metabolomic biomarkers and ultrasonography. Sensitivity and specificity and the ability to predict sheep PLS is compared between ultrasonography and regression models of blood metabolite biomarkers. Most biomarker panels offer a higher sensitivity and specificity than that of ultrasound diagnosis of PLS. The values calculated for ultrasound are for detecting pregnancy status (CNT vs PRG) and whether the pregnant ewes carry a single fetus or more (SNG vs MLP) while, the biomarker panels also identify the specific number of the litter (i.e., SNG, TWN, TRP).

	Ultrasonography CNT vs PRG	Ultrasonography SNG vs MLP	CNT vs PRG	SNG vs TRP	TWN vs TRP
Sensitivity	0.56	0.87	0.69	0.56	0.66
Specificity	0.70	0.53	0.85	0.91	0.85
AU-ROC	0.65	0.68	0.76	0.82	0.80

Table 6. Serum metabolome associated with sheep pregnancy. Metabolites include those identified and quantified by NMR and LC-MS/MS from serum of healthy sheep assessed for pregnancy and litter size. Metabolite IDs identified by ^ refer to an isomer of that lipid. Note that total dimethylarginine does not have a LMDB ID since it consists of the sum of two metabolites (symmetrical and asymmetric dimethylarginine).

Platform	Metabolite	LMDB ID	ClassyFire Chemical Classification
NMR	1-Methylhistidine	LMDB00001	Carboxylic acids and derivatives
	2-Hydroxybutyric acid	LMDB00003	Hydroxy acids and derivatives
	2-Hydroxyisovalerate	LMDB01096	Fatty Acyl derivatives
	3-Hydroxybutyric acid	LMDB00144	Hydroxy acids and derivatives
	3-Hydroxyisovaleric acid	LMDB00238	Fatty Acyl derivatives
	3-Methyl-2-oxovaleric acid	LMDB01097	Keto acids and derivatives
	Acetic acid	LMDB00014	Carboxylic acids and derivatives
	Acetoacetate	LMDB00026	Keto acids and derivatives
	Acetone	LMDB00352	Organooxygen compounds
	L-Arginine	LMDB00171	Carboxylic acids and derivatives
	L-Asparagine	LMDB00075	Carboxylic acids and derivatives
	Betaine	LMDB00015	Carboxylic acids and derivatives
	Butyrate	LMDB00013	Fatty Acyl derivatives
	Choline	LMDB00041	Organonitrogen compounds
	Citric acid	LMDB00040	Carboxylic acids and derivatives
	Creatine	LMDB00029	Carboxylic acids and derivatives
	Creatinine	LMDB00180	Carboxylic acids and derivatives
	Dimethylamine	LMDB00037	Organonitrogen compounds
	Dimethyl sulfone	LMDB00459	Sulfonyl compounds
	Dimethylglycine	LMDB00039	Carboxylic acids and derivatives
	D-Mannose	LMDB00076	Organooxygen compounds
	Ethanol	LMDB00044	Organooxygen compounds
	Formate	LMDB00060	Carboxylic acids and derivatives
	Glucose	LMDB00048	Organooxygen compounds
	Glycerol	LMDB00055	Organooxygen compounds
	Glycine	LMDB00049	Carboxylic acids and derivatives
	Hippuric acid	LMDB00227	Benzene and substituted benzene derivatives

	Hypoxanthine	LMDB00067	Imidazopyrimidines
	Isobutyric acid	LMDB00357	Carboxylic acids and derivatives
	Isoleucine	LMDB00077	Carboxylic acids and derivatives
	L-Acetylcarnitine	LMDB00091	Fatty Acyl derivatives
	L-Alanine	LMDB00070	Carboxylic acids and derivatives
	L-Carnitine	LMDB00027	Organonitrogen compounds
	L-Glutamic acid	LMDB00063	Carboxylic acids and derivatives
	L-Glutamine	LMDB00202	Carboxylic acids and derivatives
	L-Histidine	LMDB00080	Carboxylic acids and derivatives
	L-Lactic acid	LMDB00084	Hydroxy acids and derivatives
	L-Leucine	LMDB00215	Carboxylic acids and derivatives
	L-Ornithine	LMDB00099	Carboxylic acids and derivatives
	L-Phenylalanine	LMDB00069	Carboxylic acids and derivatives
	L-Proline	LMDB00071	Carboxylic acids and derivatives
	L-Serine	LMDB00083	Carboxylic acids and derivatives
	L-Threonine	LMDB00074	Carboxylic acids and derivatives
	L-Lysine	LMDB00081	Carboxylic acids and derivatives
	Malonic acid	LMDB00217	Carboxylic acids and derivatives
	Methanol	LMDB00358	Organooxygen compounds
	Methionine	LMDB00221	Carboxylic acids and derivatives
	Oxoglutaric acid	LMDB00094	Keto acids and derivatives
	Pyruvic acid	LMDB00112	Keto acids and derivatives
	Sarcosine	LMDB00124	Carboxylic acids and derivatives
	Tyrosine	LMDB00068	Carboxylic acids and derivatives
	Urea	LMDB00131	Organic carbonic acids and derivatives
	Valine	LMDB00271	Carboxylic acids and derivatives
LC-MS/MS	SM (OH) C14:1	LMDB00624	Sphingolipids
	SM C16:0	LMDB00524	Sphingolipids
	SM C16:1	LMDB00656	Sphingolipids
	SM (OH) C16:1	LMDB00780	Sphingolipids
	SM C18:0	LMDB00569	Sphingolipids
	SM C18:1	LMDB01208	Sphingolipids
	SM C20:2	LMDB00626	Sphingolipids
	SM (OH) C22:1	LMDB00627	Sphingolipids
	SM (OH) C22:2	LMDB00628	Sphingolipids
	SM (OH) C24:1	LMDB00630	Sphingolipids
	Acetylornithine	LMDB00430	Carboxylic acids and derivatives
	Alpha-aminoadipic acid	LMDB00168	Carboxylic acids and derivatives

	Asymmetric dimethylarginine (ADMA)	LMDB00344	Carboxylic acids and derivatives
	C0 (Carnitine)	LMDB00027	Organonitrogen compounds
	C14:1 (tetradecenoylcarnitine)	LMDB01011	Fatty Acyl derivatives
	C2 (Acetylcarnitine)	LMDB00091	Fatty Acyl derivatives
	C3 (Propionylcarnitine)	LMDB00253	Fatty Acyl derivatives
	C4 (butyrylcarnitine)	LMDB00374	Fatty Acyl derivatives
	C5 (Valerylcarnitine)	LMDB00581	Fatty Acyl derivatives
	Carnosine	LMDB00010	Peptides
	Citrulline	LMDB00274	Carboxylic acids and derivatives
	Kynurenine	LMDB00214	Organooxygen compounds
	L-Aspartic acid	LMDB00085	Carboxylic acids and derivatives
	lysoPC a C14:0	LMDB00525	Glycerophospholipids
	lysoPC a C16:0	LMDB00526	Glycerophospholipids
	lysoPC a C16:1	LMDB00527	Glycerophospholipids
	lysoPC a C17:0	LMDB00571	Glycerophospholipids
	lysoPC a C18:0	LMDB00528	Glycerophospholipids
	lysoPC a C18:1	LMDB00409	Glycerophospholipids
	lysoPC a C18:2	LMDB00530	Glycerophospholipids
	lysoPC a C20:3	LMDB00533	Glycerophospholipids
	lysoPC a C20:4	LMDB00534	Glycerophospholipids
	lysoPC a C26:0	LMDB00653	Glycerophospholipids
	lysoPC a C26:1	LMDB01226	Glycerophospholipids
	Methionine sulfoxide	LMDB00373	Carboxylic acids and derivatives
	PC aa C32:2	LMDB01211^	Glycerophospholipids
	PC aa C36:0	LMDB01212 ^	Glycerophospholipids
	PC ae C36:0	LMDB01210 ^	Glycerophospholipids
	PC aa C36:6	LMDB01110 ^	Glycerophospholipids
	PC aa C38:0	LMDB01111 ^	Glycerophospholipids
	PC aa C38:6	LMDB01122 ^	Glycerophospholipids
	PC aa C40:1	LMDB01119 ^	Glycerophospholipids
	PC aa C40:2	LMDB01125 ^	Glycerophospholipids
	PC aa C40:6	LMDB01140 ^	Glycerophospholipids
	PC ae C40:6	LMDB00599	Glycerophospholipids
	Putrescine	LMDB00329	Organonitrogen compounds
	Serotonin	LMDB00120	Indoles and derivatives
	Spermidine	LMDB00311	Organonitrogen compounds
	Spermine	LMDB00310	Organonitrogen compounds
	Taurine	LMDB00115	Organic sulfonic acids and derivatives

	Total dimethylarginine	N/A	Carboxylic acids and derivatives
	trans-Hydroxyproline (t4-OH-Pro)	LMDB00230	Carboxylic acids and derivatives
	Trimethylamine N-oxide	LMDB00278	Organonitrogen compounds
	Tryptophan	LMDB00279	Indoles and derivatives

Table 7. Biomarker concentrations adjusted for calculation in the logistic regression. Raw concentration of each metabolite (indicated in [ ]) is converted based on the following formula and the resulting value is used in the corresponding logistic regression.

	CNT vs PRG	SNG vs TRP	TWN vs TRP
Methanol	Log_2([methanol]/4901.36) – 7.13)/1.08	N/A	Log_2([methanol]/2261.69) + 0.25)/0.07
L-carnitine	Log_2([L-carnitine]/3733.21) – 6.76)/0.56	Log_2([L-carnitine]/39.70) + 0.98)/0.10	Log_2([L-carnitine]/1961.53) + 0.0.26)/0.03
D-glucose	Log_2([D-glucose]/384197.32) – 6.76)/0.57	N/A	N/A
L-arginine	Log_2([L-arginine]/21202.62) – 6.85)/0.81	N/A	N/A
Urea	Log_2([urea]/205076.40) – 6.80)/0.61	N/A	N/A
Methionine	N/A	Log_2([methionine]/30.22) + 0.98)/0.12	N/A
Isobutyric Acid	N/A	N/A	Log_2([isobutyric acid]/669.83) + 0.26)/0.03
L-lactic acid	N/A	N/A	Log_2([L-lactic acid]/145410.12) + 0.26)/0.04
Valine	N/A	N/A	Log_2([valine]/10719.58) + 0.26)/0.03
Tyrosine	N/A	N/A	Log_2([tyrosine]/3242.95) + 0.26)/0.05

Figures

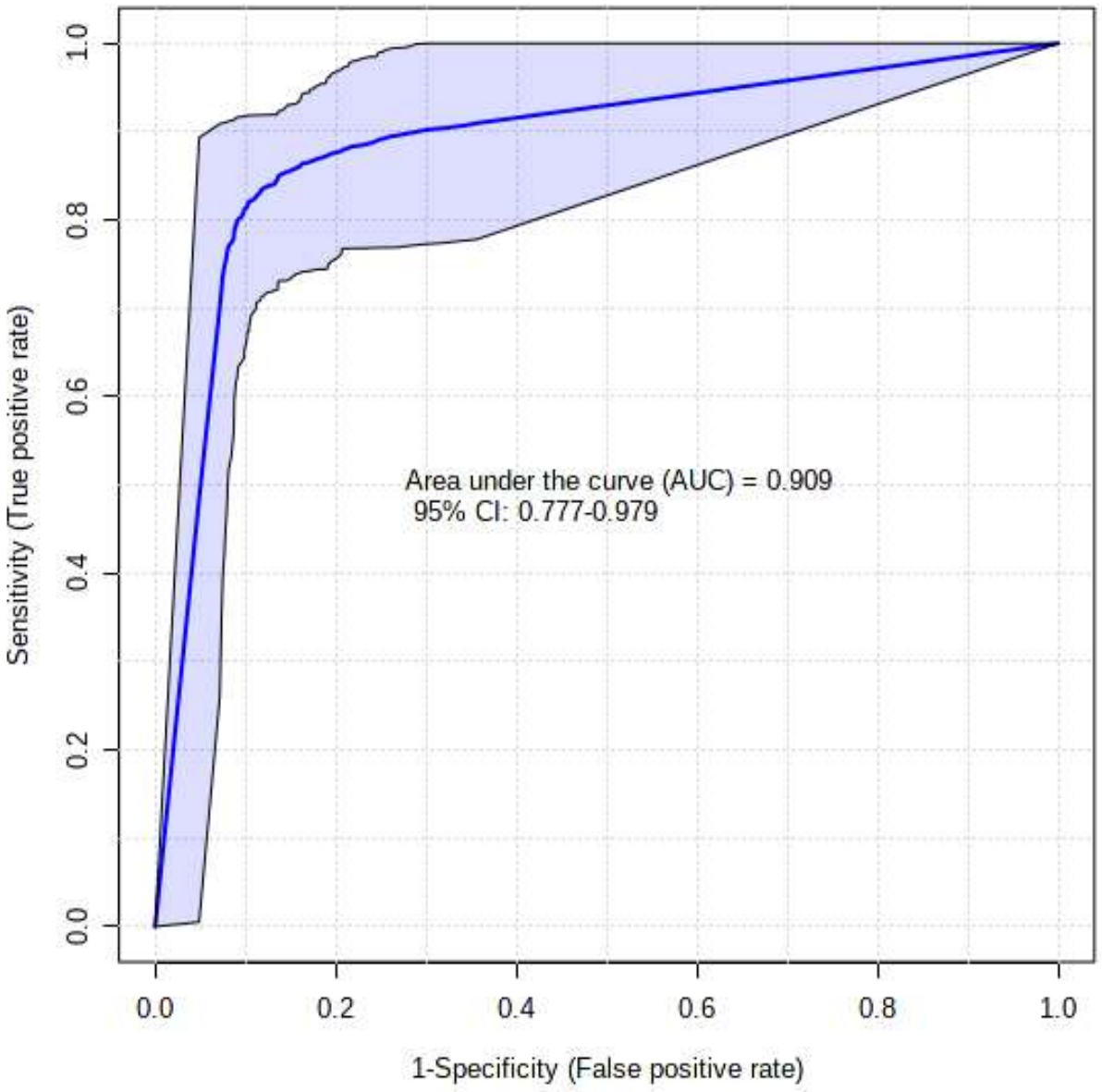

Figure 1. Receiver Operating Characteristics (ROC) curve of biomarkers of sheep pregnancy. The panel of five metabolites (methanol, L-carnitine, D-glucose, L-arginine, urea) from the CNT vs PRG comparison were selected as significant (p-value<0.05) biomarkers of sheep pregnancy.

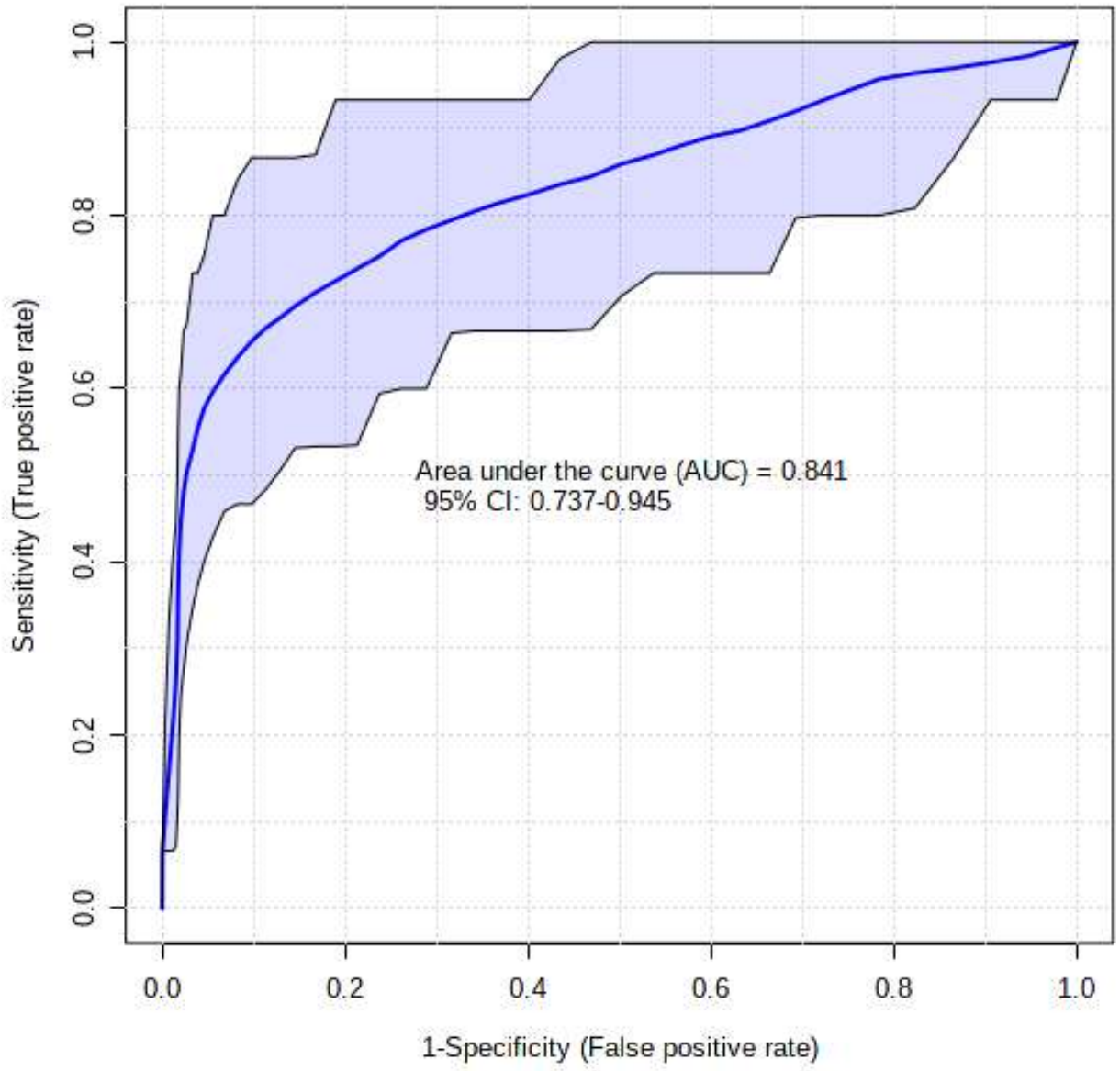

Figure 2. Receiver Operating Characteristics (ROC) curve of biomarkers of pregnant ewes with a single or more than two lambs. The comparison of SNG vs TRP groups identified methionine and L-carnitine as significant (p-value<0.001) biomarkers that would identify ewes that carry a single lamb or those that carry more than two lambs.

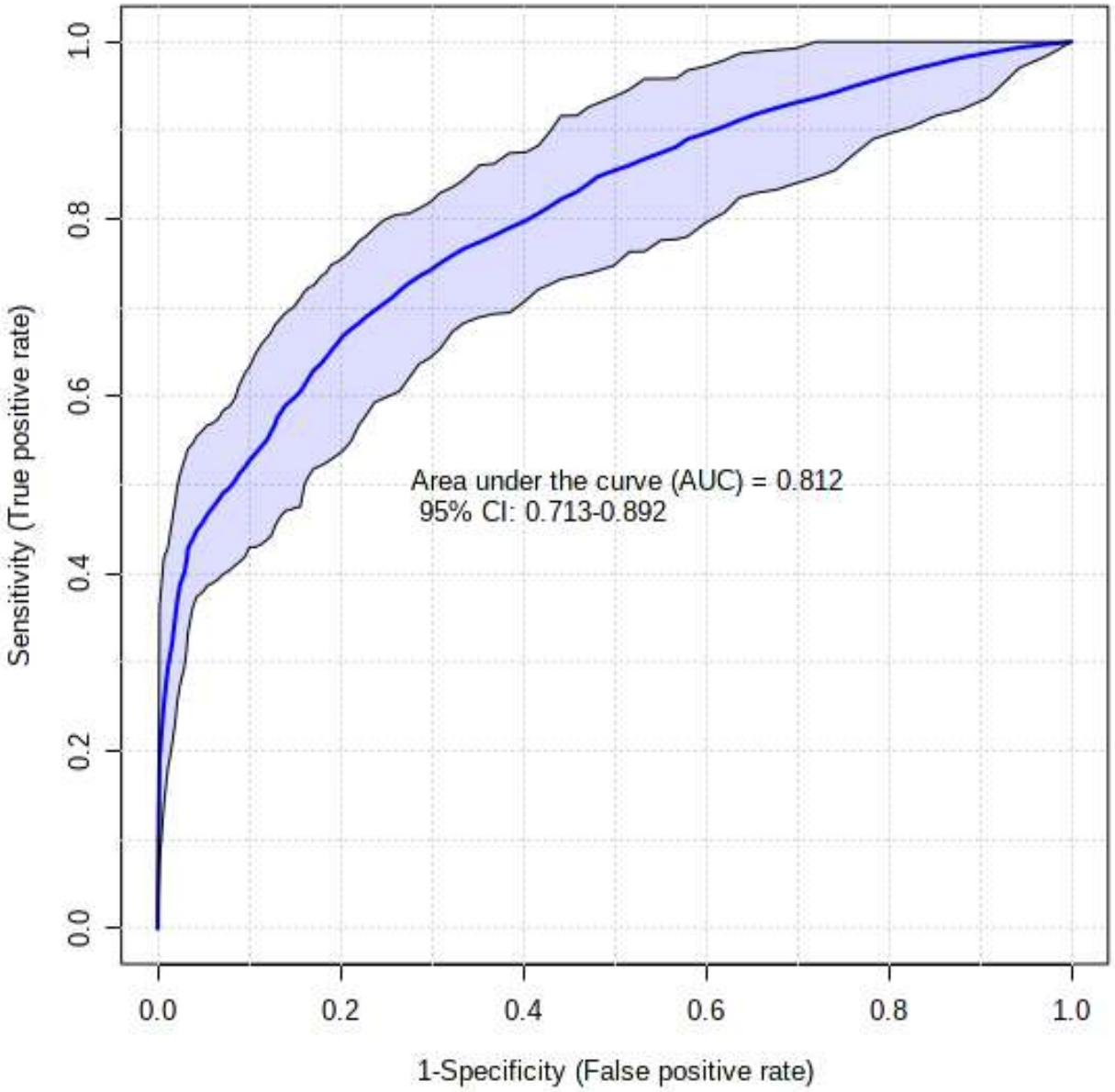

Figure 3. Receiver Operating Characteristics (ROC) curve of biomarkers of pregnant ewes with twin or triplet lambs. A panel of six metabolites (isobutyric acid, L-lactic acid, L-carnitine, valine, tyrosine, methanol) from comparing TWN vs TRP groups were identified as significance (p-value<0.05) biomarkers of pregnant ewes that carry multiple lambs.

Chapter 5: Conclusion

The Canadian sheep industry is worth more than $750 million GDP a year. While still a relatively small segment of the livestock industry (about 4% of the total Canadian meat market), the sheep industry is expected to grow by about 3.5% per year for the next 5 years. As with nearly all livestock operations, profitability and viability in sheep farming are greatly impacted by three key production traits: 1) feed efficiency, 2) meat production and 3) reproductive performance of the maternal flock. Animal feed contributes up to 85% of the cost of production for sheep producers. Farm revenue on the other hand is based on the number of viable lambs born and weaned and the carcass meat yield from each animal. The challenge for sheep producers is to increase production efficiency by maximizing yield while maintaining or minimizing input. This often means identifying or selecting for ewes (or rams) that are most feed-efficient, fertile and able to deliver viable lambs. Being able to predict these traits early on in a sheep's lifecycle provides the opportunity for producers to make feed adjustments, select or cull animals and improve their overall operational efficiency. As highlighted in the first chapter of this thesis, a number of newly emerging technologies (genomics, proteomics and metabolomics) offer sheep farmers the opportunity to identify or predict the most fertile ewes and the most feed-efficient lambs. Among all of these "omics" technologies, I believe that metabolomics offers some of the most exciting and affordable approaches to help producers optimize their operations and realize significant economic benefits. Therefore, the central hypothesis behind this thesis is that *metabolomics can be used to identify and quantify blood biomarkers of key economic and production traits in sheep*.

To test this hypothesis I set out to complete three specific objectives: (1) comprehensively identify and quantify the blood metabolome of sheep, (2) identify and quantify blood biomarkers of residual feed intake (RFI) and carcass merit in lambs, and (3) identify and quantify predictive blood biomarkers of pregnancy and litter size (PLS) in sheep. To further formulate the hypothesis

and achieve these objectives, I spent a number of months developing a multidisciplinary collaboration and consulting with a team of scientists and sheep producers to develop the research plan. My research objectives were chosen and refined based on initial interactions and feedback from sheep industry leaders and sheep organizations in both Alberta and across Canada. In particular, I sought feedback about industry expectations and production priorities that fostered increasing revenue and decreasing production costs for sheep producers. Based on this feedback, the key traits that were determined to be most important to producers were: 1) RFI (as a measure of feed efficiency), 2) muscle:bone ratio (MBR), 3) carcass yield grade (YG), as two measures of carcass merit, and 4) PLS detection before 60 days into gestation. These four traits all have substantial influence on sheep production profitability.

These research ideas and objectives were then framed into grant applications that involved a number of universities and colleges across Canada [University of Alberta (Edmonton, AB), University of Calgary (Calgary, AB), University of Guelph (Guelph, ON), Laval University (Quebec, QC), Olds College (Olds, AB), and Lakeland College (Vermillion, AB)], as well as research centers in Alberta (The Metabolomics Innovation Center, Livestock Gentec), and provincial partners (Alberta Agriculture and Forestry). Because of the close involvement of the Canadian sheep industry, we also received significant (financial and technical) support from sheep organizations around the country, including provincial organizations such as the Alberta Lamb Producers, the Alberta Sheep Breeders' Association, the Ontario Sheep Farmers, and national organizations such as the National Sheep Network and the Canadian Sheep Breeders' Association. As part of this support, we gained access to multiple private sheep flocks in the provinces of Alberta and Ontario to collect >1400 blood samples.

Prior to undertaking the experimental portion of this project, I conducted an extensive literature review on sheep metabolomics in an effort to determine what was known about the sheep metabolome. This was aimed at completing objective one (comprehensively identify and quantify the blood metabolome of sheep). In the course of conducting this literature review, I realized that to fully understand what was known about the sheep metabolome, I would also have to contextualize it with what was known about the metabolomes of other livestock species (cattle, pigs, horses, goats, etc.). As a result, I expanded my original objective from only reviewing/characterizing the sheep metabolome to reviewing/characterizing the metabolomes of all major livestock species. However, I quickly found out that much of the information on livestock metabolomes and livestock metabolomics was very diffuse and poorly consolidated. Therefore, I decided to consolidate this information into an open access, electronically accessible database called the Livestock Metabolome Database (LMDB – http://www.lmdb.ca). Chapter two of this thesis describes the methods used to assemble the data and the software used to put the database online. In assembling this database, I reviewed nearly 150 publications in peer-reviewed journals and extracted data on nearly 1100 metabolites or metabolite species. It was through this work that I also identified a number of trends and gaps in the knowledge of the sheep metabolome. In particular, I concluded that application of metabolomics in sheep research is rather outdated and the sheep serum metabolome has been poorly investigated. I also found that the use of metabolomics to explore biomarkers of important production traits in sheep, such as RFI, carcass merit and PLS is generally lacking in the literature. Another gap observed from this review was the limited scope of metabolite detection in the few sheep metabolomics publications. The majority of the studies reported only a handful of metabolites from the sheep metabolome or used only a single analytical platform to characterize the metabolome. Indeed, there were no published studies

at the time that used more than one analytical platform to explore the sheep serum metabolome. More importantly, the majority of studies lacked quantified data and just reported the presence or absence of a metabolite. This analysis essentially identified a number of opportunities to fully characterize the sheep metabolome (especially the serum metabolome) using experimental methods. This led to the work described in chapter three of this thesis.

The experimental work described in chapter three allowed me to further address objective one (comprehensively identify and quantify the blood metabolome of sheep), while at the same time completing objective two (identify and quantify predictive blood biomarkers of RFI and carcass merit in lambs). In working towards these two objectives, I used four analytical platforms that are relatively popular in metabolomics studies. These include nuclear magnetic resonance (NMR) spectroscopy, direct injection tandem mass spectrometry (DI-MS/MS), liquid chromatography tandem mass spectrometry (LC-MS/MS) and inductively coupled plasma mass spectrometry (ICP-MS). The NMR method is particularly useful for detecting sugars, alcohols, polyols and volatile compounds. The DI-MS/MS method is useful for detecting and quantifying lipids and acylcarnitines. The LC-MS/MS method is best for detecting organic acids, amino acids and biogenic amines, while ICP-MS is best for the detection and quantification of metal ions. Analyzing the serum samples with these instruments allowed us to explore different categories of metabolites and report a wide range of concentrations.

Residual feed intake and carcass merit are two traits that contribute substantially to profitability of sheep production. Because animal feed is the biggest contributor to production costs, any route that can reduce feed requirements will obviously improve farm profitability. Carcass yield and quality are other factors that play a role in determining the price obtained for feedlot lambs and, therefore, overall farm income. There is more than one way to manage animal

feed and identifying/selecting an animal's feed efficiency level. Direct measurement of RFI requires lengthy trials, expensive equipment, and technical knowledge to process the data. On the other hand, direct measurement of carcass quality requires ultrasound, a method that is expensive and rarely practiced by sheep producers in Alberta. Another way of quantifying carcass merit is post-mortem evaluation of the casrcass which obviously defeats the purpose of selection for future breeding. As a result, indirect measurement of these traits using readily accessible blood biomarkers in live animals would be preferred. Therefore, I used the analytical instruments mentioned above to profile the serum metabolome of sheep categorized based on RFI and carcass merit. From these studies, I discovered a panel of three serum metabolites that can serve as candidate biomarkers for detecting sheep RFI (isopropyl alcohol, aminoadipic acid, and acetone). Additionally, I discovered two panels of candidate serum biomarkers for detecting MBR and carcass YG (lysoPC a C26:1 for MBR and total dimethylarginine, citric acid, hypoxanthine, hippuric acid, asymmetric dimethylarginine, l-phenylalanine, and SM C16:1 for YG). These results were published in the Journal of Animal Science.

Chapter four of my thesis focused on objective three (identify and quantify predictive blood biomarkers of PLS in sheep). Pregnancy and the number of lambs a ewe delivers are other key aspects that determines a sheep producer's income. The number of lambs born and weaned is a determining factor in replacing the maternal flock and creating revenue by selling the top ram-lambs as seedstock, backgrounding the ram-lambs in the feedlot and retaining/selling the ewe-lambs as replacements. Increasing lambing rate is, in part, dependent on detecting pregnancy at early stages of gestation. The sheep industry is currently limited to using ultrasonography for direct detection of ewe pregnancy between 40-90 days into gestation. This requires specialized equipment, certification and specialized training and, perhaps, booking appointments with the

service providers several weeks in advance. Moreover, the accuracy of detecting litter size via ultrasound relies mostly on the experience of the operator and the timepoint in pregnancy detection. Indirect measurement of PLS using blood biomarkers could offer a cheaper and faster alternative. While plasma progesterone can be used, there are no commercially available kits for measuring this hormone in sheep and no serum-based markers for sheep PLS have been identified. Therefore, I designed a longitudinal study and collected blood from pregnant ewes at five timepoints from seven days prior to conception until 70 days into gestation. These samples were taken from different breeds, in different farms with varying management practices, so that the resulting biomarkers would have global application. I used the same analytical platforms (NMR, DI-MS/MS and LC-MS/MS) and statistical methods to analyze these serum samples as done in chapter three. Through this work, I was able to identify a panel of five candidate serum biomarkers (methanol, L-carnitine, D-glucose, L-arginine and urea) for detecting pregnancy at day 50 of gestation. At the same timepoint, I also identified two other panels of biomarkers that could detect how many lambs a pregnant ewe would deliver (methionine and L-carnitine to detect pregnant ewes that deliver a single lamb or more than two lambs, and methionine, isobutyric acid, L-lactic acid, L-carnitine, valine, tyrosine, and methanol to detect pregnant ewes that deliver two or more lambs). I then validated the initial discovery biomarkers using a cohort that was three times larger and confirmed 50 days post-conception is the earliest point in time to detect sheep PLS using metabolite blood biomarkers. These biomarkers are independent of breed, environmental conditions and management system.

In both studies described in chapters three and four, I chose to analyze serum (obtained from blood) because it is a readily accessible biofluid that is ideal for investigating biomarkers of different traits. This is because blood uniformly bathes all organs in the body and therefore

provides a global metabolic picture of all physiological activities within the body. Blood collection from sheep is a relatively straightforward procedure and blood can be drawn at large volumes within the boundaries of animal care codes. This is an important consideration when conducting metabolomics analysis by NMR or MS-based instrument. The lack of adequate sample volume is often a bottleneck in metabolomics, especially comprehensive, quantitative, multi-platform metabolomic studies that use NMR, LC-MS/MS, DI-MS/MS and ICP-MS – as done in this thesis. I typically collected 7-10 mL of blood from each animal, which yields 40-50% serum (3-5 mL). This supplied me with adequate sample size to allow using a small quantity for instrument optimization and the remaining amount for metabolomic analysis. More precisely, depending on methods used to run the samples, I used about 500 µL of serum for NMR, about 200 µL for DI-MS/MS and LC-MS/MS, and less than 500 µL for ICP-MS

By successfully completing these projects, I was able to prove the hypothesis that *metabolomics can be used to identify and quantify blood biomarkers of key economic and production traits in sheep*. Furthermore, I was able to meet all three objectives set out for this thesis. With regard to objective one, I conducted a comprehensive assessment of the sheep serum metabolome by gathering the published data from the literature and conducting a detailed experimental analysis of sheep serum samples using NMR, LC-MS/MS, DI-MS/MS and ICP-MS. From the experimental works presented in chapters three and four, I identified 161 unique metabolites along with their concentrations in sheep serum. As a result of this experimental work, the number of known serum metabolites in the sheep metabolome expanded by 50%. I also revealed, for the first time, the kinetics of sheep serum metabolome over the course of the first 70 days of pregnancy. Data on the sheep serum metabolome is now housed in the LMDB, which is an open access database for the public. As part of objective two and three, I also identified and

quantified novel serum biomarkers of sheep RFI, carcass merit, and PLS. The biomarkers for the first two traits require further validation, while the serum biomarkers of sheep PLS have been validated. In particular, based on the data from the sheep PLS study, 50 days post-conception appears to be the earliest point in time to simultaneously detect biomarkers of sheep PLS.

I believe these results illustrate the potential that metabolomics offers to livestock researchers and producers alike, particularly with regard to revealing predictive biomarkers of economically important traits in livestock. I believe this work has significantly expanded what is known in the field of livestock metabolomics and it has shown that metabolomics can be productively applied to sheep research with promising applications for sheep production.

Future Research

The work I started could proceed in a number of directions, some of which I would hope to undertake myself in the near term. One obvious research activity that could be pursued is an update of the LMDB. This resource was last comprehensively updated in 2017 and a great number of high-quality livestock metabolomics papers have appeared in the past three years. Indeed, a quick check of PubMed for the words “livestock” and “metabolomics” shows that livestock metabolomics research is accelerating, comparing 16 papers published on the subject in 2016 to 51 publications in 2020. I expect the rate of publications in this area to grow exponentially. I believe an updated LMDB could serve as a convenient route to standardize and consolidate the most recent published livestock metabolome data in a centralized, online repository.

With regard to my work on sheep RFI and carcass quality, there are at least two obvious routes to follow. One is to validate the candidate biomarkers using a sample size that is at least three times larger than the initial cohort. The second is to investigate how early in the sheep life cycle the biomarkers can be distinguished. Currently, the Canadian sheep industry appears to be

largely unaware about RFI as a measure of feed efficiency, therefore, educating sheep producers on this topic and uncovering ways to measure RFI is another route to follow. I am currently working with the provincial and national sheep organizations to plan and deliver seminars, courses and (virtual) tours for Canadian sheep producers to expand their knowledge and provide a toolkit on efficient feed (and other) management practices. In addition, very little research has been done to improve carcass yield and quality in Canadian sheep flocks while similar research on beef and dairy cattle is relatively advanced. Therefore, the wealth of experience and results published from other large ruminant (i.e., cattle) studies and previous metabolomics studies could pave the way for more detailed research on sheep RFI and carcass merit in the future.

With regard to my work on sheep PLS, I believe that since the biomarkers have been validated, translating the results for field application is the most appropriate future path. The metabolite biomarkers of sheep PLS reported in this thesis resulted from vigorous statistical analysis of nearly 1200 serum samples collected over five timepoints in two experimental phases, from sheep on six commercial flocks in two different provinces. Overall, the results appear to be robust and high performing. Furthermore, it would be interesting to see if these markers could be further validated in other countries (such as New Zealand or Australia) where the sheep industry is much larger. Therefore, it seems reasonable to introduce these biomarkers for use in the Canadian sheep industry (and possibly elsewhere). I believe the markers identified from this project could be translated or commercialized via three routes: 1) a laboratory kit which is designed to run in a centralized lab, 2) a handheld device that can do real-time pen-side detection, or 3) an in-line system that could be incorporated into the milking machines or other facilities pre-installed on the farm. The first two options are most feasible, as not all producers have pre-installed facilities, like a milking system on their farm, and not all equipment can easily take-in an add-on

feature. In terms of simplicity, the easiest and fastest route to bring this test “online” is to have a centralized laboratory analyze blood samples sent from producers using a PLS detection kit. In terms of cost and convenience, the best route is to develop a handheld device that the producers can use at their discretion. Such a device would perhaps use a lateral flow system to separate serum, a set of enzyme-based colorimetric assays and a simple color sensor to detect and quantify the metabolites. We envision this device requiring a few drops of blood to simultaneously analyze pregnancy and the number of fetuses a pregnant ewe is carrying. I have already initiated efforts towards developing this handheld device and am hopeful that such a system will be available in the near future.

Bibliography

1. Abarghuei, M. J., Y. Rouzbehan, A. Z. M. Salem, and M. J. Zamiri. 2014. Nitrogen balance, blood metabolites and milk fatty acid composition of dairy cows fed pomegranate-peel extract. Livest. Sci. 164:72-80. https://doi.org/10.1016/j.livsci.2014.03.021.
2. Abbiss, H., J. P. A. Gummer, M. Francki, and R. D. Trengove. 2020. Untargeted liquid chromatography-mass spectrometry-based metabolomics analysis of wheat grain. J. Vis. Exp. 157:10.3791/60851. doi:10.3791/60851.
3. Abdoli, R., P. Zamani, S. Mirhoseini, N. Ghavi Hossein-Zadeh, and S. Nadri. 2016. A review on prolificacy genes in sheep. Repro. Dom. Anim. 51:631-637. https://doi.org/10.1111/rda.12733.
4. Abreu, A. C., L. M. Aguilera-Sáez, A. Peña, M. García-Valverde, P. Marín, D. L. Valera, and I. Fernández. 2018. NMR-based metabolomics approach to study the influence of different conditions of water irrigation and greenhouse ventilation on zucchini crops. J. Agricul. Food. Chem. 66:8422-8432. DOI: 10.1021/acs.jafc.8b02590.
5. Alves, R. D. A. M., A. D. Dane, A. Harms, K. Strassburg, R. Maleki Seifar, L. B. Verdijk, S. Kersten, R. Berger, T. Hankemeier, and R. J. Vreeken. 2015. Global profiling of the muscle metabolome: method optimization, validation and application to determine exercise-induced metabolic effects. Metabolomics. 11:271-285. https://doi.org/10.1007/s11306-014-0701-7.
6. Andreas, N. J., M. J. Hyde, M. Gomez-Romero, M. A. Lopez-Gonzalvez, A. Villaseñor, A. Wijeyesekera, C. Barbas, N. Modi, E. Holmes, and I. Garcia-Perez. 2015. Multiplatform characterization of dynamic changes in breast milk during lactation. Electrophoresis. 36:2269–2285. https://doi.org/10.1002/elps.201500011.

7. Araújo, C. A. S. C., F. A. M. L. Rodrigues, A. H. H. Minervino, B. F. Trivelatto, L. F. D. Reis, C. S. Mori, and E. L. Ortolani. 2013. Clinical evaluation of nervous ketosis induced by isopropanol in sheep. Braz. J. Jet. Jes. Anim. Sci. 50:493-496. https://doi.org/10.11606/issn.1678-4456.v50i6p493-496.
8. Arthur, P. F., and R. M. Herd. 2005. Efficiency of feed utilization by livestock — Implications and benefits of genetic improvement. Can. J. Anim. Sci. 85(3): 281-290. https://doi.org/10.4141/A04-062.
9. Beauchemin, K. A. 2009. Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture. Vet. Sci. Nutr. Nat. Res. 4:35. doi: 10.1079/PAVSNNR20094035.
10. Barrera-Redondo, J., D. Piñero, and L. E. Eguiarte. 2020. Genomic, transcriptomic and epigenomic tools to study the domestication of plants and animals: a field guide for beginners. Fron. Gen. 11:742. https://doi.org/10.3389/fgene.2020.00742.
11. Basarab, J. A., M. A. Price, J. L. Aalhus, E. K. Okine, W. M. Snelling, and K. L. Lyle. 2003. Residual feed intake and body composition in young growing cattle. Can. J. Anim. Sci. 83:189-204. https://doi.org/10.4141/A02-065.
12. Basarab, J. A., K. A. Beauchemin, V. S. Baron, K. H. Ominski, L. L. Guan, S. P. Miller, and J. J. Crowley. 2013. Residual feed intake: An indirect approach for reducing GHG emissions. Greenhouse Gases & Animal Agriculture Conference. Dublin. Ireland. https://www1.agric.gov.ab.ca/$Department/deptdocs.nsf/ba3468a2a8681f69872569d60073fde1/d98f9f23f6b0e1ae87257cd00001c5ed/$FILE/rfi-ghg-ggaa.pdf
13. Benítez, R., Y. Núñez, and C. Óvilo. 2017. Nutrigenomics in farm animals. J. Invest. Genomics. 4:00059. DOI:10.15406/jig.2017.04.00059.

14. Berlinguer, F., C. Porcu, G. Molle, A. Cabiddu, M. Dattena, M. Gallus, V. Pasciu, S. Succu, F. D. Sotgiu, P. Paliogiannis, S. Sotgia, A. A. Mangoni, A. Gonzalez-Bulnes, C. Carru, and A. Zinellu. 2020. Circulating concentrations of key regulators of nitric oxide production in undernourished sheep carrying single and multiple fetuses. Animals. 10:65. https://doi.org/10.3390/ani10010065.

15. Bertram, H. C., and L. M. A. Jakobsen. 2018. Nutrimetabolomics: integrating metabolomics in nutrition to disentangle intake of animal-based foods. Metabolomics. 14:34. https://doi.org/10.1007/s11306-018-1322-3.

16. Bielli, A., R. Pérez, G. Pedrana, J. T. B. Milton, Á. Lopez, M. A. Blackberry, G. Duncombe, H. Rodriguez-Martinez, and G. B. Martin. 2002. Low maternal nutrition during pregnancy reduces the number of sertoli cells in the newborn lamb. Repro. Fert. Develop. 14:333-337. https://doi.org/10.1071/RD02046.

17. Bishonga, C., J. J. Robinson, T. G. McEvoy, P. Findlay, R. P. Aitken, and I. Robertson. 1996. Excess dietary urea intake in ewes and its effect on ovulation rate and embryo development. Japanese J. Vet. Res. 44:139-151. http://doi.org/10.14943/jjvr.44.3.139.

18. Bissonnette, J. M., A. R. Hohimer, and C. R. Chao. 1991. Unidirectional transport of glucose and lactate into brain of fetal sheep and guinea-pig. Exper. Physio. 76:515-523. doi: 10.1113/expphysiol.1991.sp003517.

19. Bloomfield, F. H., M. H. Oliver, P. Hawkins, A. C. Holloway, M. Campbell, P. D. Gluckman, J. E. Harding, and J. R. G. Challis. 2004. Periconceptional undernutrition in sheep accelerates maturation of the fetal hypothalamic-pituitary-adrenal axis in late gestation. Endocri. 145:4278e85. https://doi.org/10.1210/en.2004-0424.

20. Bouatra, S., F. Aziat, R. Mandal, A. C. Guo, M. R. Wilson, C. Knox, T. C. Bjorndahl, R. Krishnamurthy, F. Saleem, P. Liu, and Z. T. Dame. 2013. The human urine metabolome. PLoS One. 8:e73076. https://doi.org/10.1371/journal.pone.0073076.

21. Bussche, J. V., M. Marzorati, D. Laukens, and L. Vanhaecke. 2015. Validated high resolution mass spectrometry-based approach for metabolomic fingerprinting of the human gut phenotype. Anal. Chem. 87:10927–10934. https://doi.org/10.1021/acs.analchem.5b02688.

22. Caboni, P., C. Manis, I. Ibba, M. Contu, V. Coroneo, and P. Scano. 2017. Compositional profile of ovine milk with a high somatic cell count: A metabolomics approach. Int. Dairy J. 69:33-39. https://doi.org/10.1016/j.idairyj.2017.02.001.

23. Caboni, P., A. Murgia, A. Porcu, C. Manis, I. Ibba, M. Contu, P. Scano. 2019. A metabolomics comparison between sheep's and goat's milk. Food Res. Int. 119:869-875. https://doi.org/10.1016/j.foodres.2018.10.071.

24. Caldeira, R. M., A. T. Belo, C. C. Santos, M. I. Vazques, and A. V. Portugal. 2007. The effect of body condition score on blood metabolites and hormonal profiles in ewes. Sm. Rumin. Res. 68:233-241. https://doi.org/10.1016/j.smallrumres.2005.08.027.

25. Calder, P. C. 2006. Branched-chain amino acids and immunity. J. Nutri. 136:288S–293S. https://doi.org/10.1093/jn/136.1.288S.

26. Cammack, K. M., K. A. Leymaster, T. G. Jenkins, M. K. Nielsen. 2005. Estimates of genetic parameters for feed intake, feeding behavior, and daily gain in composite ram lambs. J. Anim. Sci. 83:777–785. https://doi.org/10.2527/2005.834777x.

27. Canani, R. B., M. Di Costanzo, L. Leone, G. Bedogni, and P. Brambilla. 2011. Epigenetic mechanisms elicited by nutrition in early life. Nutr. Res. Rev. 24:198-205. DOI: 10.1017/S0954422411000102.

28. Castejón, D., J. M. García-Segura, R. Escudero, A. Herrera, and M. I. Cambero. 2015. Metabolomics of meat exudate: Its potential to evaluate beef meat conservation and aging. Anal. Chim. Acta. 901:1–11. https://doi.org/10.1016/j.aca.2015.08.032.

29. Chapinal, N., M. E. Carson, S. J. LeBlanc, K. E. Leslie, S. Godden, M. Capel, J. E. P. Santos, M. W. Overton, and T. F. Duffield. 2012. The association of serum metabolites in the transition period with milk production and early-lactation reproductive performance. J. Dairy Sci. 95:1301-1309. https://doi.org/10.3168/jds.2011-4724.

30. Chen, X., C. Xie, L. Sun, J. Ding, and H. Cai. 2015. Longitudinal metabolomics profiling of Parkinson's disease-related α-synuclein A53T transgenic mice. PLoS One. 10:e0136612. https://doi.org/10.1371/journal.pone.0136612.

31. Chong, J., D. S. Wishart, and J. Xia. 2019. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. Cur. Prot. Bioinf. 68:e86. doi: 10.1002/cpbi.86.

32. Cihan, H., E. M. Temizel, Z. Yilmaz, and Y. Ozarda. 2016. Serum iron status and its relation with haematological indexes before and after parturition in sheep. Kafkas. Uni. Vet. Fak. Derg. 22:679-683. DOI: 10.9775/kvfd.2016.15103.

33. Ceccarelli, G., E. Pozzo, F. Scorletti, L. Benedetti, G. Cusella, et al. 2015. Molecular signature of amniotic fluid derived stem cells in the fetal sheep model of myelomeningocele. J. Pedia. Surg. 50:1521-1527, ISSN 0022-3468, https://doi.org/10.1016/j.jpedsurg.2015.04.014.

34. Cevallos-Cevallos, J. M., M. D. Danyluk, and J. I. Reyes-De-Corcuera. 2011. GC-MS based metabolomics for rapos simultaneous detection of Escherichia coli O157:H7, Salmonella Typhimurium, Salmonella Muenchen, and Salmonella Hartford in ground beef and chicken. J. Food Sci. 76:M238-246. https://doi.org/10.1111/j.1750-3841.2011.02132.x.

35. Clark, M., J. D. Murray, and E. A. Maga. 2014. Assessing unintended effects of a mammary-specific transgene at the whole animal level in host and non-target animals. Transgenic Res. 23:245–256. https://doi.org/10.1007/s11248-013-9768-6.

36. Clarke, E. D., M. E. Rollo, K. Pezdirc, C. E. Collins, and R. L. Haslam. 2020. Urinary biomarkers of dietary intake: a review. Nutr. Rev. 78:364-381. doi:10.1093/nutrit/nuz048.

37. Clemmons, B. A., R. I. Mihelic, R. C. Beckford, J. B. Powers, E. A. Melchior, Z. D. McFarlane, E. R. Cope, M. M. Embree, J. T. Mulliniks, S. R. Campagna, B. H. Voy, and P. R. Myer. 2017. Serum metabolites associated with feed efficiency in black angus steers. Metabolomics. 13:147. https://doi.org/10.1007/s11306-017-1282-z.

38. Cockrum, R. R., R. H. Stobart, S. L. Lake, and K. M. Cammack. 2013. Phenotypic variation in residual feed intake and performance traits in rams. Sm. Rumin. Res. 113:313-322. https://doi.org/10.1016/j.smallrumres.2013.05.001.

39. Connolly, S., A. Dona, L. Wilkinson-White, D. Hamblin, M. D'Occhio, and L. A. González. 2019. Relationship of the blood metabolome to subsequent carcass traits at slaughter in feedlot Wagyu crossbred steers. Sci. Rep. 9:15139. https://doi.org/10.1038/s41598-019-51655-2.

40. Corona, G., F. Rizzolio, A. Giordano, and G. Toffoli. 2012. Pharmaco-metabolomics: an emerging "omics" tool for the personalization of anticancer treatments and identification of new valuable therapeutic targets. J. Cell Physiol. 227:2827-2831. https://doi.org/10.1002/jcp.24003.

41. Crane, A. R., R. R. Redden, M. L. Van Emon, T. L. Neville, L. P. Reynolds, J. S. Caton, and C. S. Schauer. 2016. Impacts of supplemental arginine on the reproductive performance of fall lambing ewes. J. Anim. Sci. 94:3540–3549. https://doi.org/10.2527/jas.2016-0379.

42. Crews, D. H. 2005. Genetics of efficient feed utilization and national cattle evaluation: a review. Genet. Mol. Res. 4:152-165. https://europepmc.org/article/med/16110437.

43. Daley, C. A., A. Abbott, P. S. Doyle, G. A. Nader, and S. Larson. 2010. A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. Nutr. J. 9:10. https://doi.org/10.1186/1475-2891-9-10.

44. Davis, P. L., L. A. Dal Cortivo, and J. Maturo. 1984. Endogenous isopropanol: forensic and biochemical implications. J. Anal. Toxicol. 8:209-212. doi:10.1093/jat/8.5.209.

45. Davis, G. H., L. Balakrishnan, I. K. Ross, T. Wilson, S. M. Galloway, B. M. Lumsden, J. P. Hanrahan, M. Mullen, X. Z. Mao, G. L. Wang, and Z. S. Zhao. 2006. Investigation of the Booroola (FecB) and Inverdale (FecX(I)) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. Anim. Repro. Sci. 92:87–96. https://doi.org/10.1016/j.anireprosci.2005.06.001.

46. De Boo, H., P. L. Van Zijl, D. E. C. Smith, W. Kulik, H. N. Lafeber, and J. E. Harding. 2005. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr. Res. 58:270–277. https://doi.org/10.1203/01.PDR.0000169977.48609.55.

47. De Moraes Pontes, J. G., F. B. De Santana, R. W. Portela, V. Azevedo, R. J. Poppi, and L. Tasic. 2017. Biomarkers of the Caseous Lymphadenitis in sheep by NMR-based metabolomics. Metabolomics. 7:2. DOI: 10.4172/2153-0769.1000190.

48. de Nicola, D., F. Vinale, A. Salzano, G. d'Errico, A. Vassetti, N. D'Onofrio, M. L. Balestrieri, and G. Neglia. 2020. Milk metabolomics reveals potential biomarkers for early prediction of pregnancy in buffaloes having undergone artificial insemination. Animals. 10:758. https://doi.org/10.3390/ani10050758.

49. Deng, M., X. Zhang, J. Luo, H. Liu, W. Wen, H. Luo, J. Yan, and Y. Xiao. 2020. Metabolomics analysis reveals differences in evolution between maize and rice. Plant J. 103:1710-1722. doi:10.1111/tpj.14856.

50. Denkert, C., J. Budczies, T. Kind, W. Weichert, P. Tablack, J. Sehouli, S. Niesporek, D. Konsgen, M. Dietel, and O. Fiehn. 2006. Mass spectrometry-based metabolic profiling reveals different metabolite patterns in invasive ovarian carcinomas and ovarian borderline tumors. Cancer Res. 66:10795–10804. DOI: 10.1158/0008-5472.CAN-06-0755.

51. Djoumbou Feunang, Y., R. Eisner, C. Knox, L. Chepelev, J. Hastings, G. Owen, E. Fahy, C. Steinbeck, S. Subramanian, E. Bolton, R. Greiner, and D. S. Wishart. 2016. ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. J. Cheminform. 8:61. doi:10.1186/s13321-016-0174-y.

52. Dogan, B., A. Karaer, G. Tuncay, N. Tecellioglu, and A. Mumcu. 2020. High-resolution 1H-NMR spectroscopy indicates variations in metabolomics profile of follicular fluid from women with advanced maternal age. J. Assist. Reprod. Genet. 37:321–330. https://doi.org/10.1007/s10815-020-01693-x

53. Downs, C. J., B. V. Boan, T. D. Lohuis, and K. M. Stewart. 2018. Investigating relationships between reproduction, immune defenses, and cortisol in Dall sheep. Front. Immun. 9:105. https://doi.org/10.3389/fimmu.2018.00105.

54. Du, M., J. Tong, J. Zhao, K. R. Underwood, M. Zhu, S. P. Ford, and P. W. Nathanielsz. 2010. Fetal programming of skeletal muscle development in ruminant animals. J. Anim. Sci. 88:E51–E60. https://doi.org/10.2527/jas.2009-2311.

55. Duggan, G. E., D. S. Hittel, C. W. Sensen, A. M. Weljie, H. J. Vogel, and J. Shearer. 2011. Metabolomic response to exercise training in lean and diet-induced obese mice. J. Appl. Physiol. 110:1311-1318. https://doi.org/10.1152/japplphysiol.00701.2010.

56. Dunn, W. B. 2008. Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes. Phys. Biol. 5:011001. doi:10.1088/1478-3975/5/1/011001.

57. Ehret, A., D. Hochstuhl, N. Krattenmacher, J. Tetens, M. S. Klein, W. Gronwald, and G. Thaller. 2015. Short communication: Use of genomic and metabolic information as well as milk performance records for prediction of subclinical ketosis risk via artificial neural networks. J. Dairy Sci. 98:322-329. https://doi.org/10.3168/jds.2014-8602.

58. Ellison, M. J., G. C. Conant, W. R. Lamberson, K. J. Austin, E. van Kirk, H. C. Cunningham, D. C. Rule, and K. M. Cammack. 2019. Predicting residual feed intake status using rumen microbial profiles in ewe lambs. J. Anim. Sci. 97:2878–2888. https://doi.org/10.1093/jas/skz170

59. El-Tahawy, A. S., and P. A. Ismaeil. 2013. Methionine-supplemented diet increases the general performance and value of Rahmani lambs. Iranian J. App. Anim. Sci. 3:513–520. https://www.sid.ir/en/journal/ViewPaper.aspx?id=325859.

60. Escalona, E. E., J. Leng, A. C. Dona, C. A. Merrifield, E. Holmes, C. J. Proudman, and J. R. Swann. 2015. Dominant components of the Thoroughbred metabolome characterized by 1H-nuclear magnetic resonance spectroscopy: A metabolite atlas of common biofluids. Equine Vet. J. 47:721-730. https://doi.org/10.1371/journal.pone.0246290.

61. Escudero, E., L. Mora, M. C. Aristoy, and F. Toldrá. 2011. Possible biological markers of the time of processing of dry-cured ham. Meat. Sci. 89:536-539. https://doi.org/10.1016/j.meatsci.2011.05.002.

62. Emwas, A.-H., R. Roy, R.T. McKay, L. Tenori, E. Saccenti, et al. 2019. NMR spectroscopy for metabolomics research. Metabolites. 9:123. https://doi.org/10.3390/metabo9070123.

63. FAO statistical pocketbook: World food and agriculture. 2015. ww.fao.org/publications.

64. FAO. 2017. The future of food and agriculture – trends and challenges. Rome, Italy: FAO. http://www.fao.org/3/y4252e/y4252e07.htm.

65. FAO. 2018. http://www.fao.org/faostat/en/#data/QL.

66. FAOSTAT. http://www.fao.org/faostat/en/#data/QA/visualize.

67. Farrell, L. J., P. R. Kenyon, P. R. Tozer, T. Ramilan, and L. M. Cranston. 2020. Quantifying sheep enterprise profitability with varying flock replacement rates, lambing rates, and breeding strategies in New Zealand. Agri. Sys. 184:102888. https://doi.org/10.1016/j.agsy.2020.102888.

68. Fiehn, O. 2002. Metabolomics – the link between genotypes and phenotypes. Plant Mol. Biol. 48:155-171. https://doi.org/10.1007/978-94-010-0448-0_11.

69. Fogarty, N. M. 2009. A review of the effects of the Booroola gene (FecB) on sheep production. Sm. Rumin. Res. 85:75-84. https://doi.org/10.1016/j.smallrumres.2009.08.003.

70. Fontanesi, L. 2016. Metabolomics and livestock genomics: Insights into a phenotyping frontier and its applications in animal breeding. Anim. Front. 6:73-79. https://doi.org/10.2527/af.2016-0011.

71. Foroutan, A., C. Fitzsimmons, R. Mandal, H. Piri-Moghadam, J. Zheng, A. Guo, C. Li, L. L. Guan, and D. S. Wishart. 2020. The Bovine Metabolome. Metabolites. 10:233. https://doi.org/10.3390/metabo10060233.

72. Foroutan, A., S. A. Goldansaz, M. Lipfert, and D. S. Wishart. 2019. Protocols for NMR analysis in livestock metabolomics. In: Bhattacharya S. (eds) Metabolomics. Methods in Molecular Biology, vol 1996. Humana, New York, NY. https://doi.org/10.1007/978-1-4939-9488-5_23.

73. Freetly, H. C., and C. L. Ferrell. 1998. Net flux of glucose, lactate, volatile fatty acids, and nitrogen metabolites across the portal-drained viscera and liver of pregnant ewes. J. Anim. Sci. 76:3133–3145. https://doi.org/10.2527/1998.76123133x.

74. Ganna, A., T. Fall, S. Salihovic, W. Lee, C. D. Broeckling, J. Kumar, S. Hägg, M. Stenemo, P. K. Magnusson, J. E. Prenni, and L. Lind. 2016. Large-scale non-targeted metabolomic profiling in three human population-based studies. Metabolomics. 12: 4. https://doi.org/10.1007/s11306-015-0893-5.

75. Garza Hernandez, D., S. Mucha, G. Banos, K. Kaseja, K. Moore, N. Lambe, J. Yates, and L. Bunger. 2018. Analysis of single nucleotide polymorphisms variation associated with important economic and computed tomography measured traits in Texel sheep. Animal. 12: 915-922. doi:10.1017/S1751731117002488.

76. Ghafouri-Kesbi, F., and M. P. Eskandarinasab. 2008. An evaluation of maternal influences on growth traits: the Zandi sheep breed of Iran as an example. J. Anim. Feed Sci. 17:519–529. https://pdfs.semanticscholar.org/8a2a/744641208357f7e63f12959888588bf44792.pdf

77. Ghazi, N., M. Arjmand, Z. Akbari, A. O. Mellati, H. Saheb-Kashaf, and Z. Zamani. 2016. 1H NMR-based metabolomics approaches as non-invasive tools for diagnosis of endometriosis. Int. J. Reprod. Biomed. (Yazd). 14:1–8.

78. Gilany, K., R. S. Moazeni-Pourasil, N. Jafarzadeh, and E. Savadi-Shiraz. 2014. Metabolomics fingerprinting of the human seminal plasma of asthenozoospermic patients. Mol. Repro. Dev. 81:84-86. https://doi.org/10.1002/mrd.22284.

79. Giskeødegård, G. F., T. S. Madssen, L. R. Euceda, M-B. Tessem, S. A. Moestue, and T. F. Bathen. 2018. NMR-based metabolomics of biofluids in cancer. NMR in Biom. 32:e3927. https://doi.org/10.1002/nbm.3927.

80. Godfrey, L. V., and J. B. Glass. 2011. Research on nitrification and related processes, Part A. Methods in Enzymology. 486:608 page.

81. Goldansaz, S. A., A. C. Guo, T. Sajed, M. A. Steele, G. S. Plastow, and D. S. Wishart. 2017. Livestock metabolomics and the livestock metabolome: A systematic review. PLoS ONE. 12:e0177675. https://doi.org/10.1371/journal.pone.0177675.

82. Goldansaz, S. A., S. Markus, M. Berjanskii, M. Rout, A. C. Guo, Z. Wang, G. Plastow, and D. S. Wishart. 2020. Candidate serum metabolite biomarkers of residual feed intake and carcass merit in sheep. J. Anim. Sci. 98:skaa298. https://doi.org/10.1093/jas/skaa298.

83. Gómez, E., P. Salvetti, J. Gatien, S. Carrocera, D. Martín-González, and M. Muñoz. 2020. Blood plasma metabolomics predicts pregnancy in Holstein cattle transferred with fresh and vitrified/warmed embryos produced in vitro. J. Prote. Res. 19:1169-1182. https://doi.org/10.1021/acs.jproteome.9b00688.

84. Gómez, E., M. Muñoz, J. Gatien, S. Carrocera, D. Martín-González, and P. Salvetti. 2020. Metabolomic identification of pregnancy-specific biomarkers in blood plasma of BOS

TAURUS beef cattle after transfer of in vitro produced embryos. J. Prot. 225:103883. https://doi.org/10.1016/j.jprot.2020.103883.

85. Gray, D. W., M. D. Welsh, S. Doherty, F. Mansoor, O. P. Chevallier, C. T. Elliott, and M. H. Mooney. 2015. Identification of systemic immune response markers through metabolomics profiling of plasma from calves given an intra-nasally delivered respiratory vaccine. Vet. Res. 46:7. https://doi.org/10.1186/s13567-014-0138-z.

86. Grill, L., F. Ringdorfer, R. Baumung, and B. Fuerst-Waltl. 2015. Evaluation of ultrasound scanning to predict carcass composition of Austrian meat sheep. Sm. Rumin. Res. 123:260-268. https://doi.org/10.1016/j.smallrumres.2014.12.005.

87. Guidetti, P., and R. Schwarcz. 2003. Determination of α-aminoadipic acid in brain, peripheral tissues, and body fluids using GC/MS with negative chemical ionization. Brain. Res. Mol. Brain. Res. 118:132-139. https://doi.org/10.1016/j.molbrainres.2003.08.004.

88. Guo, Y. S., and J. Z. Tao. 2018. Metabolomics and pathway analyses to characterize metabolic alterations in pregnant dairy cows on D 17 and D 45 after AI. Sci. Rep. 8:5973. https://doi.org/10.1038/s41598-018-23983-2.

89. Guo, C., Y. Xue, Y. Yin, H. Xuan, F. Hu, and S. Mao. 2020. Urine metabolome alterations in malnutrition and the impact of glycerol or rumen-protected choline chloride supplementation in advanced pregnant ewes. Brit. J. Nutri. 123:1258-1268. doi:10.1017/S000711452000063X.

90. Guo, X., X. Wang, R. Di, Q. Liu, W. Hu, X. He, J. Yu, X. Zhang, J. Zhang, K. Broniowska, and W. Chen. 2018. Metabolic effects of FecB gene on follicular fluid and ovarian vein serum in sheep (Ovis aries). Int. J. Mol. Sci. 19:539. https://doi.org/10.3390/ijms19020539.

91. Haibel, G. K. 1990. Use of ultrasonography in reproductive management of sheep and goat herds. The Veterinary Clinics of North America. Food Anim. Prac. 6:597-613. DOI: 10.1016/s0749-0720(15)30835-5.

92. Hailemariam, D., R. Mandal, F. Saleem, S. M. Dunn, D. S. Wishart, and B. N. Ametaj. 2014. Identification of predictive biomarkers of disease state in transition dairy cows. J. Dairy Sci. 97:2680–2693. https://doi.org/10.3168/jds.2013-6803.

93. Hailemariam, D., G. Manafiazar, J. A. Basarab, P. Stothard, F. Miglior, G. Plastow, and Z. Wang. 2020. Comparative analyses of enteric methane emissions, dry matter intake and milk somatic cell count in different residual feed intake categories of dairy cows. Can. J. Anim. Sci. https://doi.org/10.1139/CJAS-2019-0085.

94. Halket, J. M., D. Waterman, A. M. Przyborowska, R. K. P. Patel, P. D. Fraser, and P. M. Bramley. 2005. Chemical derivatization and mass spectral libraries in metabolite profiling by GC–MS and LC– MS/MS. J. Exp. Botany. 56:219-243. https://doi.org/10.1093/jxb/eri069.

95. Hanuš, O., P. Roubal, M. Vyletělová, T. Yong, M. Bjelka, and A. Dufek. 2011. The relations of some milk indicators of energy metabolism in cow, goat and sheep milk. Sci. Agri. Bohem. 42:102-112. https://www.cabdirect.org/cabdirect/abstract/20123035379.

96. Harshman, S. W., R. L. Pitsch, N. M. Schaeublin, Z. K. Smith, K. E. Strayer, M. S. Phelps, A. V. Qualley, D. W. Cowan, S. D. Rose, M. L. O'Connor, J. J. Eckerle, T. Das, A. K. Barbey, A. J. Strang, and J. A. Martin. 2019. Metabolomic stability of exercise-induced sweat. J. Chrom. B. 1126–1127:121763. https://doi.org/10.1016/j.jchromb.2019.121763.

97. Heasman, L., L. Clarke, K. Firth, T. Stephenson, and M. E. Symonds. 1998. Influence of restricted maternal nutrition in early to mid gestation on placental and fetal development at

term in Sheep. Pediatr. Res. 44:546–551. https://doi.org/10.1203/00006450-199810000-00013.

98. Heitmann, R. N., D. J. Dawes, and S. C. Sensenig. 1987. Hepatic ketogenesis and peripheral ketone body utilization in the ruminant. J. Nutr. 117:1174–1180. https://doi.org/10.1093/jn/117.6.1174.

99. Henze, P., K. Bickhardt, H. Fuhrmann, and H. P. Sallmann. 1998. Spontaneous pregnancy toxaemia (ketosis) in sheep and the role of insulin. Zentralbl Veterinarmed A. 45:255-66. doi:10.1111/j.1439-0442.1998.tb00825.x.

100. Herd, R. M., and S. C. Bishop. 2000. Genetic variation in residual feed intake and its association with other production traits in British Hereford cattle. Livest. Prod. Sci. 63:111-119. https://doi.org/10.1016/S0301-6226(99)00122-0.

101. Herd, R. M., V. H. Oddy, and E. C. Richardson. 2004. Biological basis for variation in residual feed intake in beef cattle. 1. Review of potential mechanisms. Aust. J. Exp. Agr. 44:423-430. https://doi.org/10.1071/EA02220.

102. Herd, R. M., and P. F. Arthur. 2009. Physiological basis of residual feed intake. J. Anim. Sci. 87:E64–E71. https://doi.org/10.2527/jas.2008-1345.

103. Holmgren, L., and D. Feuz. 2015. 2015 costs and returns for a 200 cow, cow-calf operation. All Curr. Pub. 711. http://digitalcommons.usu.edu/extension_curall/711.

104. Hosking, J., J. Pinkney, A. Jeffery, O. Cominetti, L. Da Silva, S. Collino, M. Kussmann, J. Hager, and F. Martin. 2019. Insulin resistance during normal child growth and development is associated with a distinct blood metabolic phenotype (Earlybird 72). Pediatr. Diabetes. 20:832– 841. https://doi.org/10.1111/pedi.12884.

105. Houle, D., D. R. Govindaraju, and S. Omholt. 2010. Phenomics: the next challenge. Nat. Rev. Genet. 11:855-866. https://doi.org/10.1038/nrg2897.

106. Huang, L., C. Hsieh, K. Chang, and Y. Tain. 2012. Roles of nitric oxide and asymmetric dimethylarginine in pregnancy and fetal programming. Int. J. Mol. Sci. 13:14606-14622. https://doi.org/10.3390/ijms131114606.

107. Ibrahim, A. H. M. 2019. Estimation of co-variance components, genetic parameters and genetic trends of VIA scan carcass traits of New Zealand Romney lambs. Sm. Rumin. Res. 178:130-136. https://doi.org/10.1016/j.smallrumres.2019.08.016.

108. Imhasly, S., H. Naegeli, S. Baumann, M. von Bergen, A. Luch, H. Jungnickel, S. Potratz, and C. Gerspach. 2014. Metabolomic biomarkers correlating with hepatic lipidosis in dairy cows. BMC. Vet. Res. 10:122. https://doi.org/10.1186/1746-6148-10-122.

109. Ishwar, A. K. 1995. Pregnancy diagnosis in sheep and goats: a review. Sm. Rumin. Res. 17:37-44. https://doi.org/10.1016/0921-4488(95)00644-Z.

110. Jackson, T., J. Heard, and B. Malcolm. 2014. System changes to a lamb farm in south-west Victoria: some pre-experimental modelling. AFBM. J. 11:1–18. https://search.informit.org/doi/10.3316/informit.883492148347757.

111. Jalali, A., A. Hatamie, T. Safarpour, A. Khajeamiri, T. Safa, and F. Buazar. 2016. Impact of pharmaceutical impurities in Ecstasy tablets: gas chromatography-mass spectrometry study. Iran. J. Pharm. Res. 15:221-229. https://search.informit.org/doi/10.3316/informit.883492148347757.

112. Jamieson, D. J., R. N. Theiler, and S. A. Rasmussen. 2006. Emerging infections and pregnancy. Emerg. Infect. Dis. 12:1638-1643. doi: 10.3201/eid1211.060152.

113. Jewison, T., V. Neveu, J. Lee, C. Knox, P. Liu, R. Mandal, R. Krishnamurthy, I. Sinelnikov, and M. Wilson. 2012. YMDB: The Yeast Metabolome Database. Nucleic Acids Res. 40:D815-20. https://doi.org/10.1093/nar/gkr916.

114. Johnson, C. H., J. Ivanisevic, and G. Siuzdak. 2016. Metabolomics: beyond biomarkers and towards mechanisms. Nat. Rev. Mol. Cell Biol. 17:451–459. https://doi.org/10.1038/nrm.2016.25.

115. Jones, H. E., R. M. Lewis, M. J. Young, and B. T. Wolf. 2002. The use of X-ray computer tomography for measuring the muscularity of live sheep. Anim. Sci. 75:387–399. DOI: https://doi.org/10.1017/S1357729800053169.

116. Jones, S. D. M., W. M. Robertson, M. A. Price, and T. Coupland. 1996. The prediction of saleable meat yield in lamb carcasses. Can. J. Anim. Sci. 76:49-53. https://doi.org/10.4141/cjas96-007.

117. Jones, A. K., and S. A. Reed. 2017. Benefits of ultrasound scanning during gestation in the small ruminant. Sm. Rumin. Res. 149:163-171. https://doi.org/10.1016/j.smallrumres.2017.02.008.

118. Jones, A. K., R. E. Gately, K. K. McFadden, S. A. Zinn, K. E. Govoni, and S. A. Reed. 2016. Transabdominal ultrasound for detection of pregnancy, fetal and placental landmarks, and fetal age before day 45 of gestation in the sheep. Therio. 85:939-945.e1. https://doi.org/10.1016/j.theriogenology.2015.11.002.

119. Jones, D. P., Y. Park, and T. R. Ziegler. 2012. Nutritional metabolomics: progress in addressing complexity in diet and health. Annu. Rev. Nutr. 32:183-202. https://doi.org/10.1146/annurev-nutr-072610-145159.

120. Jones, A. K., R. E. Gately, T. D. Kellogg, S. A. Zinn, K. E. Govoni, S. A. Reed. 2018. Evaluation of the Nova Vet Meter for sheep-side monitoring of β-hydroxybutyric acid (BHBA) and description of ewe BHBA during late gestation in three flocks from the northeastern U.S. Res. Vet. Sci. 118:491-497. https://doi.org/10.1016/j.rvsc.2018.05.002.

121. Jorge-Smeding, E., G. Renand, D. Centeno, M. Pétéra, S. Durand, S. Polakof, and G. Cantalapiedra-Hijar. 2019. Metabolomics reveals changes in urea cycle associated to residual feed intake in growing heifers. Ene. Pro. Metab. Nutr. 138:231-232. https://doi.org/10.3920/978-90-8686-891-9_50.

122. Juengel, J. L., G. H. Davis, R. Wheeler, K. G. Dodds, and P. D. Johnston. 2018. Factors affecting differences between birth weight of littermates (BWTD) and the effects of BWTD on lamb performance. Anim. Repro. Sci. 191:34-43. https://doi.org/10.1016/j.anireprosci.2018.02.002.

123. Kadkhodaei Elyaderani, M., A. Malek, M. Ali, M. Rostami, M. Aberomand, and A. Kheirollah. 2013. Inhibitory effect of asymmetric dimethylarginine and NG-Monomethyl-L-arginine methyl ester on nitric oxide synthase activity. J. Gorgan Uni. Med. Sci. 15:84-92. https://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=97760037&site=eds-live&scope=site.

124. Kaliel, D. A. 2004. Insights into managing winter feed costs in Alberta cow/calf operations. URL: http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/econ9538/$FILE/winterfeed.pdf.

125. Kandiel, M. M. M., H. M. El-Khaiat, and K. Gh. M. Mahmoud. 2016. Changes in some hematobiochemical and hormonal profile in Barki sheep with various reproductive statuses. Sm. Rumin. Res. 136:87-95. https://doi.org/10.1016/j.smallrumres.2016.01.011.

126. Karen, A., J. Beckers, J. Sulon, N. M. de Sousa, K. Szabados, J. Reczigel, and O. Szenci. 2003. Early pregnancy diagnosis in sheep by progesterone and pregnancy-associated glycoprotein tests. Therio. 59:1941-1948. https://doi.org/10.1016/S0093-691X(02)01289-X.

127. Karen, A., B. El Amiri, J. Beckers, J. Sulon, M. A. M. Taverne, and O. Szenci. 2006. Comparison of accuracy of transabdominal ultrasonography, progesterone and pregnancy-associated glycoproteins tests for discrimination between single and multiple pregnancy in sheep. Therio. 66:314-322. DOI: 10.1016/j.theriogenology.2005.11.017.

128. Karisa, B. K., J. Thomson, Z. Wang, C. Li, Y. R. Montanholi, S. P. Miller, S. S. Moore, and G. S. Plastow. 2014. Plasma metabolites associated with residual feed intake and other productivity performance traits in beef cattle. Liv. Sci. 165:200–211. https://doi.org/10.1016/j.livsci.2014.03.002.

129. Karisa, B. K., J. Thomson, Z. Wang, H. L. Bruce, G. S. Plastow, and S. S. Moore. 2013a. Candidate genes and biological pathways associated with carcass quality traits in beef cattle. Can. J. Anim. Sci. 93:295-306. https://doi.org/10.4141/cjas2012-136.

130. Karisa, B., S. Moore, and G. Plastow. 2013b. Complicated relationships: a review of biological interaction networks and pathways in animal science. Springer Sci. Rev. 1:73–83. DOI 10.1007/s40362-013-0005-8.

131. Kelly, A. K., M. McGee, D. H. Crews, A. G. Fahey, A. R. Wylie, and D. A. Kenny. 2010. Effect of divergence in residual feed intake on feeding behaviour, blood metabolic variables, and body composition traits in growing heifers. J. Anim. Sci. 88(1): 109-123. https://doi.org/10.2527/jas.2009-2196.

132. Kenyon, P. R., and H. T. Blair. 2014. Foetal programming in sheep – Effects on production. Sm. Rumin. Res. 118:16-30. https://doi.org/10.1016/j.smallrumres.2013.12.021.

133. Khan, D., H. Khan, A. Nazir, M. T. Tunio, M. Tahir, M. S. Khan, and R. U. Khan. 2020. Early pregnancy diagnosis using pregnancy-associated glycoproteins in the serum of pregnant ruminants. Pakistan J. Zool. 52:785-788. DOI: http://dx.doi.org/10.17582/journal.pjz/20190527080541.

134. Kim, S., J. Kim, E. J. Yun, and K. H. Kim. 2016. Food metabolomics: from farm to human. Curr. Opin. Biotechnol. 37:16-23. https://doi.org/10.1016/j.copbio.2015.09.004.

135. Kimball, S. R., and L. S. Jefferson. 2004. Regulation of global and specific mRNA translation by oral administration of branched-chain amino acids. Biochem. Biophys. Res. Com. 313:423-427. https://doi.org/10.1016/j.bbrc.2003.07.014.

136. Kind, T., H. Tsugawa, T. Cajka, Y. Ma, Z. Lai, S. S. Mehta, G. Wohlgemuth, D. Barupal, M. Showalter, M. Arita, and O. Fiehn. 2018. Identification of small molecules using accurate mass MS/MS search. Mass. Spec. Rev. 37:513–532. https://doi.org/10.1002/mas.21535.

137. Kirchberg, F. F., V. Grote, D. Gruszfeld, P. Socha, R. Closa-Monasterolo, J. Escribano, E. Verduci, B. Mariani, J. Langhendries, P. Poncelet, B. Koletzko, C. Hellmuth, and European Childhood Obesity Trial Study Group. 2019. Are all breast-fed infants equal? Clustering metabolomics data to identify predictive risk clusters for childhood obesity. J. Pediatr. Gastroenterol. Nutr. 68:408-415. doi:10.1097/MPG.0000000000002184.

138. Kiyma, Z., M. Kose, M. O. Atli, C. Ozel, M. Hitit, G. Sen, M. Kaya, M. S. Kaya, E. Kurar, S. Ali Kayis, and A. Guzeloglu. 2016. Investigation of interferon-tau stimulated genes (ISGs) simultaneously in the endometrium, corpus luteum (CL) and peripheral blood leukocytes (PBLs) in the preluteolytic stage of early pregnancy in ewes. Sm. Rumin. Res. 140:1-6. https://doi.org/10.1016/j.smallrumres.2016.05.007.

139. Klein, M. S., N. Buttchereit, S. P. Miemczyk, A. K. Immervoll, C. Louis, S. Wiedemann, W. Junge, G. Thaller, P. J. Oefner, and W. Gronwald. 2012. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. J. Proteome Res. 11:1373–1381. https://doi.org/10.1021/pr201017n.

140. Knott, S. A., L. J. Cummins, F. R. Dunshea, and B. J. Leury. 2008. The use of different models for the estimation of residual feed intake (RFI) as a measure of feed efficiency in meat sheep. Anim. Feed Sci. Technol. 143:242–255. https://doi.org/10.1016/j.anifeedsci.2007.05.013.

141. Knott, S. A., L. J. Cummins, F. R. Dunshea, and B. J. Leury. 2010. Feed efficiency and body composition are related to cortisol response to adrenocorticotropin hormone and insulin-induced hypoglycemia in rams. Domest. Anim. Endocrinol. 39:137-46. doi: 10.1016/j.domaniend.2010.03.003.

142. Koch, R. M., L. A. Swiger, D. Chambers, and K. E. Gregory. 1963. Efficiency of feed use in beef cattle. J. Anim. Sci. 22:486–494. https://doi.org/10.2527/jas1963.222486x.

143. Koch, J. M., J. Ramadoss, and R. R. Magness. 2010. Proteomic profile of uterine luminal fluid from early pregnant ewes. J. Prot. Res. 9:3878-3885. https://doi.org/10.1021/pr100096b.

144. Koczula, K. M., and A. Gallotta. 2016. Lateral flow assays. Ess. Biochem. 60:111–120. doi: 10.1042/EBC20150012.

145. Kordalewska, M., and M. J. Markuszewski. 2015. Metabolomics in cardiovascular diseases. J. Pharm. Biomed. Anal. 113:121-136. https://doi.org/10.1016/j.jpba.2015.04.021.

146. Kosmides, A. K., K. Kamisoglu, S. E. Calvano, S. A. Corbett, and I. P. Androulakis. 2013. Metabolomic fingerprinting: Challenges and opportunities. Crit. Rev. Biomed. Eng. 41:205–221. DOI: 10.1615/CritRevBiomedEng.2013007736.

147. Kuhn, C., R. Weikard, and P. Widmann. 2014. Metabolomics: a pathway for improved understanding of genetic modulation of mammalian growth and tissue deposition. Proc. 10th World Cong. Genet. App. Livest. Prod. 1-6. http://wcgalp.org/system/files/proceedings/2014/metabolomics-pathway-improved-understanding-genetic-modulation-mammalian-growth-and-tissue.pdf.

148. La, Y., J. Tang, X. Guo, L. Zhang, S. Gan, X. Zhang, J. Zhang, W. Hu, and M. Chu. 2020. Proteomic analysis of sheep uterus reveals its role in prolificacy. J. Prot. 210:103526. https://doi.org/10.1016/j.jprot.2019.103526.

149. Laeger, T., C. C. Metges, and B. Kuhla. 2010. Role of β-hydroxybutyric acid in the central regulation of energy balance. Appetite. 54:450-455. https://doi.org/10.1016/j.appet.2010.04.005.

150. Lassala, A., F. W. Bazer, T. A. Cudd, S. Datta, D. H. Keisler, M. C. Satterfield, T. E. Spencer, and G. Wu. 2011. Parenteral administration of L-arginine enhances fetal survival and growth in sheep carrying multiple fetuses. J. Nutri. 141:849–855. https://doi.org/10.3945/jn.111.138172.

151. LeBlanc, S. J., K. E. Leslie, and T. F. Duffield. 2005. Metabolic predictors of displaced abomasum in dairy cattle. J. Dairy Sci. 88:159-170. https://doi.org/10.3168/jds.S0022-0302(05)72674-6.

152. Leymaster, K. A. 1991. Straightbred comparison of a composite population and the Suffolk breed for performance traits of sheep. J. Anim. Sci. 69:993-999. https://doi.org/10.2527/1991.693993x.

153. Li, B., P. M. VanRaden, E. Guduk, J. R. O'Connell, D. J. Null, E. E. Connor, M. J. VandeHaar, R. J. Tempelman, K. A. Weigel, and J. B. Cole. 2020a. Genomic prediction of residual feed intake in US Holstein dairy cattle. J. Dairy Sci. 103:2477-2486. https://doi.org/10.3168/jds.2019-17332.

154. Li, H., Q. Yu, T. Li, L. Shao, M. Su, H. Zhou, and J. Qu. 2020b. Rumen microbiome and metabolome of Tibetan sheep (Ovis aries) reflect animal age and nutritional requirement. Front. Vet. Sci. 7:609. doi: 10.3389/fvets.2020.00609.

155. Li, J., C. Tang, Q. Zhao, Y. Yang, F. Li, Y. Qin, X. Liu, X. Yue, and J. Zhang. 2020c. Integrated lipidomics and targeted metabolomics analyses reveal changes in flavor precursors in psoas major muscle of castrated lambs. Food Chem. 333:127451. https://doi.org/10.1016/j.foodchem.2020.127451.

156. Liang, X., X. Bi, M. Kamruzzaman, and H. Sano. 2019. Effect of Chinese herbal medicine on kinetics of plasma phenylalanine, tyrosine and whole body protein synthesis in sheep. Anim. Sci. J. 90:533–538. https://doi.org/10.1111/ asj.13180.

157. Lockwood, A., J. Trompf, S. Hancock, L. Kubeil, A. Thompson, and J. Young. 2020. Factors influencing the optimum mob size of ewes at lambing and the economic benefit of lambing ewes in smaller mobs to increase lamb survival across southern Australia. Agri. Sys. 184:102916. https://doi.org/10.1016/j.agsy.2020.102916.

158. Lonergan, P., N. Forde, and T. Spencer. 2016. Role of progesterone in embryo development in cattle. Reprod. Fertil. Dev. 28:66e74. https://doi.org/10.1071/RD15326.

159. López-Hernández, Y., J. J. Oropeza-Valdez, J. O. Blanco-Sandate, A. S. Herrera-Van Oostdam, J. Zheng, et al. 2020. The urinary metabolome of healthy newborns. Metabolites. 10:165. https://doi.org/10.3390/metabo10040165.

160. Lu, J., E. Antunes Fernandes, A. E. Páez Cano, J. Vinitwatanakhun, S. Boeren, T. van Hooijdonk, A. van Knegsel, J. Vervoort, and K. A. Hettinga. 2013. Changes in milk proteome and metabolome associated with dry period length, energy balance, and lactation stage in postparturient dairy cows. J. Proteome Res. 12:3288-3296. https://doi.org/10.1021/pr4001306.

161. Luo, X., J. Meng, X. Chen, L. Cheng, S. Yan, L. Gao, M. Xue, and Y. Yang. 2020. Metabolomics-based study reveals the effect of lead (Pb) in the culture environment on Whitmania pigra. Sci. Rep. 10:4794. https://doi.org/10.1038/s41598-020-61745-1.

162. Luther, J. S., E. J. Windorski, J. S. Caton, G. Wu, J. D. Kirsch, K. A. Vonnahme, L. P. Reynolds, and C. S. Schauer. 2009. Effects of arginine supplementation on reproductive performance in Rambouillet ewes. Sheep Res. Rep. 50:11-13. https://www.ag.ndsu.edu/Hettingerrec/sheep/individual-articles-from-2009-sheep-research-report/Effects%20of%20arginine%20supplementation%20on%20reproductive%20performance.pdf

163. McCoard, S., F. Sales, N. Wards, Q. Sciascia, M. Oliver, J. Koolaard, and D. van der Linden. 2013. Parenteral administration of twin-bearing ewes with L-arginine enhances the birth weight and brown fat stores in sheep. Spr. Plu. 2:684. https://doi.org/10.1186/2193-1801-2-684.

164. Macfarlane, J. M., R. M. Lewis, G. C. Emmans, M. J. Young, and G. Simm. 2006. Predicting carcass composition of terminal sire sheep using X-ray computed tomography. Anim. Sci. 82:289–300. DOI: 10.1079/ASC200647.

165. Manafiazar, G., L. Goonewardene, F. Miglior, D. Crews, J. Basarab, E. Okine, and Z. Wang. 2016. Genetic and phenotypic correlations among feed efficiency, production and selected conformation traits in dairy cows. Animal. 10:381-389. https://doi.org/10.1017/S1751731115002281.

166. Manafiazar, G., J. A. Basarab, L. McKeown, J. Stewart-Smith, V. Baron, M. D. MacNeil, and G. Plastow. 2017. Optimizing feed intake recording and feed efficiency estimation to increase the rate of genetic gain for feed efficiency in beef cattle. Can. J. Anim. Sci. 97:456-465. https://doi.org/10.1139/cjas-2016-0118.

167. Maniscalco, M., D. Paris, D. Melck, N. Chiariello, F. Di Napoli, M. Manno, I. Iavicoli, and A. Motta. 2018. Biomonitoring of workers using nuclear magnetic resonance-based metabolomics of exhaled breath condensate: A pilot study. Toxico. Let. 298:4-12. https://doi.org/10.1016/j.toxlet.2018.10.018.

168. Mahdavi, V., M. M. Farimani, F. Fathi, and A. Ghassempour. 2015. A targeted metabolomics approach toward understanding metabolic variations in rice under pesticide stress. Anal. Biochem. 478:65-72. https://doi.org/10.1016/j.ab.2015.02.021.

169. Mahdavi, V., F. Ghanati, and A. Ghassempour. 2016. Integrated pathway-based and network-based analysis of GC-MS rice metabolomics data under diazinon stress to infer affected biological pathways. Anal. Biochem. 494:31-36. https://doi.org/10.1016/j.ab.2015.10.017.

170. May, D. H., S. L. Navarro, I. Ruczinski, J. Hogan, Y. Ogata, Y. Schwarz, L. Levy, T. Holzman, M. W. McIntosh, and J. W. Lampe. 2013. Metabolomic profiling of urine: response to a randomized, controlled feeding study of select fruits and vegetables, and application to an observational study. Br. J. Nutr. 110:1760-1770. doi:10.1017/S000711451300127X.

171. Maierle, C. L. 2018. Effect of terminal sire breed on feed efficiency in Katahdin crossbred feedlot-finished lambs. J. Anim. Sci. 96:67-68. https://search.proquest.com/openview/695f811ee7e34c2f1cf45be78b9c914e/1?pq-origsite=gscholar&cbl=49113.

172. Marie-Etancelin, C., D. Francois, J. L. Weisbecker, D. Marcon, C. Moreno-Romieux, F. Bouvier, and F. Tortereau. 2019. Detailed genetic analysis of feeding behaviour in Romane lambs and links with residual feed intake. J. Anim. Breed. Genet. 136:174-182. doi:10.1111/jbg.12392.

173. Mathew, S., S. Krug, T. Skurk, A. Halama, A. Stank, A. Artati, C. Prehn, J. A. Malek, G. Kastenmüller, W. Römisch-Margl, and J. Adamski. 2014. Metabolomics of Ramadan fasting: an opportunity for the controlled study of physiological responses to food intake. J. Transl. Med. 12:161. https://doi.org/10.1186/1479-5876-12-161.

174. Melzer, N., D. Wittenburg, S. Hartwig, S. Jakubowski, U. Kesting, L. Willmitzer, J. Lisec, N. Reinsch, and D. Repsilber. 2012. Investigating associations between milk metabolite profiles and milk traits of Holstein cows. J. Dairy Sci. 96:1521-1534. https://doi.org/10.3168/jds.2012-5743.

175. Melzer, N., D. Wittenburg, and D. Repsilber. 2013. Integrating milk metabolite profile information for the prediction of traditional milk traits based on SNP information for Holstein cows. PLOS One. 8:e70256. https://doi.org/10.1371/journal.pone.0070256.

176. Metzler-Zebeli, B. U., R. Ertl, D. Klein, and Q. Zebeli. 2015. Explorative study of metabolic adaptations to various dietary calcium intakes and cereal sources on serum metabolome and hepatic gene expression in juvenile pigs. Metabolomics. 11:545–558. https://doi.org/10.1007/s11306-014-0714-2.

177. McKeating, D. R., J. J. Fisher, and A. V. Perkins. 2019. Elemental Metabolomics and Pregnancy Outcomes. Nutrients. 11:73. doi:10.3390/nu11010073.

178. McLaughlin, M. J., D. R. Parker, and J. M. Clarke. 1999. Metals and micronutrients - food safety issues. Field Crop. Res. 60:143-163. https://doi.org/10.1016/S0378-4290(98)00137-3.

179. Meyer, A. M., R. A.Vraspir, M. J. Ellison, and K. M. Cammack. 2015. The relationship of residual feed intake and visceral organ size in growing lambs fed a concentrate- or forage-based diet. Livest. Sci. 176:85-90. https://doi.org/10.1016/j.livsci.2015.03.019.

180. Merrifield, C. A., M. Lewis, S. P. Claus, O. P. Beckonert, M. Dumas, S. Duncker, S. Kochhar, S. Rezzi, J. C. Lindon, M. Bailey, and E. Holmes. 2011. A metabolic system-wide characterization of the pig: a model for human physiology. Mol. Biosyst. 7:2577-2588. https://doi.org/10.1039/C1MB05023K.

181. Mickiewicz, B., B. J. Heard, J. K. Chau, M. Chung, D. A. Hart, N. G. Shrive, C. B. Frank, and H. J. Vogel. 2015. Metabolic profiling of synovial fluid in a unilateral Ovine model of anterior cruciate ligament reconstruction of the knee suggests biomarkers for early osteoarthritis. J. Orthop. Res. 33:71-77. https://doi.org/10.1002/jor.22743.

182. Minai-Tehrani, A., N. Jafarzadeh, and K. Gilany. 2015. Metabolomics: a state-of-the-art technology for better understanding of male infertility. Andrologia. 48: 609-616. doi:10.1111/and.12496.

183. Mirsaeidi, M., M. M. Banoei, B. W. Winston, and D. E. Schraufnagel. 2015. Metabolomics: applications and promise in mycobacterial disease. Ann. Am. Thorac. Soc. 12:1278-1287. https://doi.org/10.1513/AnnalsATS.201505-279PS.

184. Moco, S., S. Collino, S. Rezzi, and F. J. Martin. 2013. Metabolomics perspectives in pediatric research. Pediatr. Res. 73:570-576. https://doi.org/10.1038/pr.2013.1.

185. Mohamad, N., R. I. Ismet, M. Rofiee, Z. Bannur, T. Hennessy, M. Selvaraj, A. Ahmad, F. Nor, T. A. Rahman, K. M. Isa, and A. Ismail. 2015. Metabolomics and partial least square discriminant analysis to predict history of myocardial infarction of self-claimed healthy subjects: validity and feasibility for clinical practice. J. Clin. Bioinform. 5:3. https://doi.org/10.1186/s13336-015-0018-4.

186. Monteiro, M. S., M. Carvalho, M. L. Bastos, and P. Guedes de Pinho. 2013. Metabolomics analysis for biomarker discovery: Advances and challenges. Curr. Med. Chem. 20: 257-271. https://www.ingentaconnect.com/content/ben/cmc/2013/00000020/00000002/art00006.

187. Moore, E. R., J. Kirwan, M. K. Doherty, and P. D. Whitfield. 2007. Biomarker discovery in animal health and disease: the application of post-genomics technologies. Biomark. Insights. 2:185-196. https://doi.org/10.1177/117727190700200040.

188. Moore, S. S., F. D. Mujibi, and E. L. Sherman. 2009. Molecular basis for residual feed intake in beef cattle. J. Anim. Sci. 87:E41–E47. https://doi.org/10.2527/jas.2008-1418.

189. Moore, L. D., T. Le, and G. Fan. 2013. DNA methylation and its basic function. Neuropsychopharmacology. 38:23-38. https://doi.org/10.1038/npp.2012.112.

190. Moradband, F., G. E. Rahimi, and M. Gholizadeh. 2011. Association of polymorphisms in fecundity genes of GDF9, BMP15 and BMP15-1B with litter size in Iranian Baluchi sheep. Asian-Aus. J. Anim. Sci. 24:1179-1183. DOI: https://doi.org/10.5713/ajas.2011.10453.

191. Morales-Martinez, M. A., C. Arce-Recinos, M. M. Mendoza-Taco, C. Luna-Palomera, M. A. Ramirez-Bautista, Á. T. Piñeiro-Vazquez, R. Vicente-Perez, L. O. Tedeschi, and A. J. Chay-Canul. 2020. Developing equations for predicting internal body fat in Pelibuey sheep using ultrasound measurements. Sm. Rumin. Res. 183:106031. https://doi.org/10.1016/j.smallrumres.2019.106031.

192. Morris, S. T. 2009. Economics of sheep production, Sm. Rumin. Res. 86:59-62. https://doi.org/10.1016/j.smallrumres.2009.09.019.

193. Morrow, A. L., A. J. Lagomarcino, K. R. Schibler, D. H. Taft, Z. Yu, B. Wang, M. Altaye, M. Wagner, D. Gevers, D. V. Ward, and M. A. Kennedy. 2013. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. Microbiome. 1:13. https://doi.org/10.1186/2049-2618-1-13.

194. Muir, S. K., N. Linden, M. Knight, R. Behrendt, and G. Kearney. 2018. Sheep residual feed intake and feeding behaviour: are 'nibblers' or 'binge eaters' more efficient. Anim. Pro. Sci. 58:1459–1464. doi:10.1071/AN17770.

195. Mung, D., and L. Li. 2019. Chemical isotope labeling liquid chromatography mass spectrometry for investigating acute dietary effects of cow milk consumption on human urine metabolome. J. Food. Drug. Anal. 27:565-574. doi:10.1016/j.jfda.2018.10.007.

196. Muro-Reyes, A., H. Gutierrez-Banuelos, L. H. Diaz-Garcia, F. J. Gutierrez-Pina, L. M. Escareno-Sanchez, R. Banuelos-Valenzuela, C. A. Medina-Flores, and A. Corral Luna. 2011. Potential environmental benefits of residual feed intake as strategy to mitigate methane emissions in sheep. J. Anim. Vet. Adv. 10:1551-1556. https://www.cabdirect.org/cabdirect/abstract/20113185769.

197. Nandi, S., V. G. Kumar, B. M. Manjunatha, and P. S. Gupta. 2007. Biochemical composition of ovine follicular fluid in relation to follicle size. Dev. Grow. Differ. 49:61–66. https://doi.org/10.1111/j.1440-169X.2007.00901.x.

198. Nielsen, K. L., M. L. Hartvigsen, M. S. Hedemann, H. N. Lærke, K. Hermansen, and K. E. B. Knudsen. 2014. Similar metabolic responses in pigs and humans to breads with different

contents and compositions of dietary fibers: a metabolomics study. Am. J. Clin. Nutr. 99:941–949. https://doi.org/10.3945/ajcn.113.074724.

199. Nishimura, R. N., D. Santos, S. T. Fu, and B. E. Dwyer. 2000. Induction of cell death by L-alpha-aminoadipic acid exposure in cultured rat astrocytes: relationship to protein synthesis. Neurotoxicology. 21:313–320. https://europepmc.org/article/med/10894121.

200. Norris, J. L., and R. M. Caprioli. 2013. Analysis of tissue specimens by matrix-assisted laser desorption/ionization imaging mass spectrometry in biological and clinical research. Chem. Rev. 113:2309–2342. https://doi.org/10.1021/cr3004295.

201. Norton, M. 2005. Factors affecting beef and cattle producer prices movements. Mont. Lab. Rev. 128:32. https://heinonline.org/HOL/LandingPage?handle=hein.journals/month128&div=52&id=&page=.

202. Novais, F. J., P. R. L. Pires, P. A. Alexandre, R. A. Dromms, A. H. Iglesias, J. B. S. Ferraz, M. P. Styczynski, and H. Fukumasu. 2019. Identification of a metabolomic signature associated with feed efficiency in beef cattle. BMC Genomics. 20:8. doi:10.1186/s12864-018-5406-2.

203. NRC (National Research Council). 2007. Nutrient requirements of small ruminants: Sheep, goats, cervids and new world camelids. National Academy Press. Washington, D.C.

204. Nkrumah, J. D., E. K. Okine, G. W. Mathison, K. Schmid, C. Li, J. A. Basarab, M. A. Price, Z. Wang, and S. S. Moore. 2005. Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle. J. Anim. Sci. 84:145–153. https://doi.org/10.2527/2006.841145x.

205. Nutrient requirements of sheep. Sixth Revised Edition. Subcommittee on Sheep Nutrition. Committee on Animal Nutrition. Board on Agriculture, National Research Council (NRC). National Academy Press. Washington. D.C. (1985).

206. Obata, T., S. Witt, J. Lisec, N. Palacios-Rojas, I. Florez-Sarasa, S. Yousfi, J. L. Araus, J. E. Cairns, and A. R. Fernie. 2015. Metabolite profiles of maize leaves in drought, heat, and combined stress field trials reveal the relationship between metabolism and grain yield. Plant Physiol. 169:2665–2683. DOI: https://doi.org/10.1104/pp.15.01164.

207. Oliver, S. G., M. K. Winson, D. B. Kell, and F. Baganz. 1998. Systematic functional analysis of the yeast genome. Trends Biotech. 16:373-378. https://doi.org/10.1016/S0167-7799(98)01214-1.

208. Osorio, M., A. Moloney, L. Brennan, and F. Monahan. 2012. Authentication of beef production systems using a metabolomic-based approach. Animal. 6:167-172. doi:10.1017/S1751731111001418.

209. Osorio, M. T., G. Downey, A. P. Moloney, F. T. Röhrle, G. Luciano, O. Schmidt, and F. J. Monahan. 2013. Beef authentication using dietary markers: Chemometric selection and modelling of significant beef biomarkers using concatenated data from multiple analytical methods. Food Chem. 141:2795–2801. https://doi.org/10.1016/j.foodchem.2013.05.118.

210. Paganoni, B., G. Rose, C. Macleay, and C. Jones. 2017. More feed efficient sheep produce less methane and carbon dioxide when eating high-quality pellets. J. Anim. Sci. 95:3839–3850. doi:10.2527/jas2017.1499.

211. Paisley, S., and K. Cammack. 2010. How far will that pound of feed take you? UW examines feed efficiency. University of Wyoming. College of Agriculture and Natural

Resources. Available: http://www.uwyo.edu/uwexpstn/publications/reflections/reflections-2010-web.pdf.

212. Pallister, T., M. Jackson, T. C. Martin, C. A. Glastonbury, A. Jennings, M. Beaumont, R. P. Mohney, K. S. Small, A. MacGregor, C. J. Steves, A. Cassidy, T. D. Spector, C. Menni, and A. M. Valdes. 2017. Untangling the relationship between diet and visceral fat mass through blood metabolomics and gut microbiome profiling. Int. J. Obes. 41:1106–1113. https://doi.org/10.1038/ijo.2017.70.

213. Palma, M., T. Scanlon, T. Kilminster, J. Milton, C. Oldham, J. Greeff, M. Matzapetakis, and A. M. Almeida. 2016. The hepatic and skeletal muscle ovine metabolomes as affected by weight loss: a study in three sheep breeds using NMR-metabolomics. Sci. Rep. 6:39120. https://doi.org/10.1038/srep39120.

214. Park, Y. W., M. Juarez, M. Ramosc, and G. F. W. Haenlein. 2007. Physico-chemical characteristics of goat and sheep milk. Small Rum. Res. 68:88–113. https://doi.org/10.1016/j.smallrumres.2006.09.013.

215. Parkins, J. J., J. Fraser, N. S. Ritchie, and R. G. Hemingway. 1974. Urea as a protein source for ewes with twin lambs in late pregnancy and early lactation. Anim. Sci. 19:321-329. doi:10.1017/S0003356100022893.

216. Parveen, I., J. M. Moorby, M. D. Fraser, G. G. Allison, and J. Kopka. 2007. Application of gas chromatography-mass spectrometry metabolite profiling techniques to the analysis of heathland plant diets of sheep. J. Agric. Food Chem. 55:1129-1138. https://doi.org/10.1021/jf062995w.

217. Patil, R. D., M. J. Ellison, S. M. Wolff, C. Shearer, A. M. Wright, R. R. Cockrum, K. J. Austin, W. R. Lamberson, K. M. Cammack, and G. C. Conant. 2018. Poor feed efficiency in

sheep is associated with several structural abnormalities in the community metabolic network of their ruminal microbes. J. Anim. Sci. 96:2113–2124. https://doi.org/10.1093/jas/sky096.

218. Paula, E. F. E. de, D. F. de Souza, A. L. G. Monteiro, M. H. de A. Santana, S. Gilaverte, P. Rossi Junior, and R. Locatelli Dittrich. 2013. Residual feed intake and hematological and metabolic blood profiles of Ile de France lambs. Rev. Bras. Zoot. 42:806-812. https://dx.doi.org/10.1590/S1516-35982013001100007.

219. Pearson H. 2007. Meet the human metabolome. Nature. 446:8. https://doi.org/10.1038/446008a.

220. Penner, G. B., M. A. Steele, J. R. Aschenbach, and B. W. McBride. 2011. RUMINANT NUTRITION SYMPOSIUM: Molecular adaptation of ruminal epithelia to highly fermentable diets. J. Anim. Sci. 89:1108–1119. https://doi.org/10.2527/jas.2010-3378.

221. Pérez, P., M. Maino, G. Tomic, E. Mardones, and J. Pokniak. 2002. Carcass characteristics and meat quality of Suffolk Down suckling lambs. Sm. Rumin. Res. 44:233-240. https://doi.org/10.1016/S0921-4488(02)00076-7.

222. Peters, R. J. B., A. A. M. Stolker, J. G. J. Mol, A. Lommen, E. Lyris, Y. Angelis, A. Vonaparti, M. Stamou, C. Georgakopoulos, and M. W. F. Nielen. 2010. Screening in veterinary drug analysis and sports doping control based on full-scan, accurate-mass spectrometry. Tren. Anal. Chem. 29:1250-1268. https://doi.org/10.1016/j.trac.2010.07.012.

223. Pickworth, C. L., J. Sheffield, and R. K. Poole. 2020. Pregnancy specific protein B concentration for the prediction of lambing rate in sheep. Sm. Rumin. Res. 84:106052. https://doi.org/10.1016/j.smallrumres.2020.106052.

224. Pinu, F. R., S. A. Goldansaz, and J. Jaine. 2019. Translational metabolomics: current challenges and future opportunities. Metabolites. 9:108. doi:10.3390/metabo9060108.

225. Psychogios, N., D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend, and D. S. Wishart. 2011. The human serum metabolome. PLoS ONE. 6:e16957. doi:10.1371/journal.pone.0016957.

226. Raboisson, D., A. Albaaj, G. Nonne, and G. Foucras. 2017. High urea and pregnancy or conception in dairy cows: A meta-analysis to define the appropriate urea threshold. J. Dairy Sci. 100:7581-7587. https://doi.org/10.3168/jds.2016-12009.

227. Rådjursöga, M., H. M. Lindqvist, A. Pedersen, B. G. Karlsson, D. Malmodin, L. Ellegård, and A. Winkvist. 2018. Nutritional metabolomics: postprandial response of meals relating to vegan, lacto-ovo vegetarian, and omnivore diets. Nutrients. 10:1063. doi:10.3390/nu10081063.

228. Rajaei Sharifabadi, H., M. J. Zamiri, E. Rowghani, W. G. Bottje. 2012. Relationship between the activity of mitochondrial respiratory chain complexes and feed efficiency in fat-tailed Ghezel lambs. J. Anim. Sci. 90:1807–1815, https://doi.org/10.2527/jas.2011-4791.

229. Ravanbakhsh, S., P. Liu, T. C. Bjorndahl, R. Mandal, J. R. Grant, M. Wilson, R. Eisner, I. Sinelnikov, X. Hu, C. Luchinat, R. Greiner, and D. S. Wishart. 2015. Accurate, fully-automated NMR spectral profiling for metabolomics. PLoS ONE. 10(5): e0124219. doi: 10.1371/journal.pone.0124219.

230. Razzaq, A., B. Sadia, A. Raza, M. K. Hameed, and F. Saleem. 2019. Metabolomics: A way forward for crop improvement. Metabolites. 9:303. https://doi.org/10.3390/metabo9120303.

231. Redden, R. R., L. M. M. Surber, B. L. Roeder, B. M. Nichols, J. A. Paterson, and R. W. Kott. 2011. Residual feed efficiency established in a post-weaning growth test may not result

in more efficient ewes on the range. Sm. Rumin. Res. 96:155–159. https://doi.org/10.1016/j.smallrumres.2010.12.007.

232. Redden, R. R., L. M. M. Surber, A. V. Grove, and R. W. Kott. 2013. Growth efficiency of ewe lambs classified into residual feed intake groups and pen fed a restricted amount of feed. Sm. Rum. Res. 114:214–219. https://doi.org/10.1016/j.smallrumres.2013.07.002.

233. Redden, R. R., L. M. M. Surber, A. V. Grove, and R. W. Kott. 2014. Effects of residual feed intake classification and method of alfalfa processing on ewe intake and growth. J. Anim. Sci. 92:830–835. https://doi.org/10.2527/jas.2013-6768.

234. Regal, P., C. Nebot, M. Díaz-Bao, R. Barreiro, A. Cepeda, and C. Fente. 2011. Disturbance in sex-steroid serum profiles of cattle in response to exogenous estradiol: a screening approach to detect forbidden treatments. Steriods. 76:365-375. https://doi.org/10.1016/j.steroids.2010.12.005.

235. Regnault, T. R. H., J. E. Friedman, R. B. Wilkening, R. V. Anthony, and W. W. Hay. 2005. Fetoplacental transport and utilization of amino acids in IUGR — a review. Placenta. 26:S52-S62. https://doi.org/10.1016/j.placenta.2005.01.003.

236. Richardson, E. C., R. M. Herd, V. H. Oddy, J. M. Thompson, J. A. Archer and P. F. Arthur. 2001. Body composition and implications for heat production and Angus steer progeny of parents selected for and against residual feed intake. Anim. Pro. Sci. 41:1065–1072. https://doi.org/10.1071/EA00095.

237. Richardson, E. C., and R. M. Herd. 2004. Biological basis for variation in residual feed intake in beef cattle. 2. Synthesis of results following divergent selection. Aus. J. Exp. Ag. 44:431-440. https://doi.org/10.1071/EA02221.

238. Rincon-Delgado, R. M., H. Gutierrez, E. D. Perez-Vszq, A. Muro-Reyes, L. H. Diaz-Garci, R. Banuelos-V, F. J. Gutierrez, C. A. Medina-Flo, L. M. Escareno-S, J. I. Aguilera-S, M. A. Lopez-Carl, and C. F. Arechiga-F. 2011. Relationship of residual feed intake on specific hematological and biochemical parameters in Rambouillet sheep. Agri. J. 6:87-91. doi:10.3923/aj.2011.87.91.

239. Roberts, J. N., K. J. May, and A. Veiga-Lopez. 2017. Time-dependent changes in pregnancy-associated glycoproteins and progesterone in commercial crossbred sheep. Therio. 89:271-279. https://doi.org/10.1016/j.theriogenology.2016.10.029.

240. Robinson, D. L., J. P. Goopy, A. J. Donaldson, R. T. Woodgate, V. H. Oddy, and R. S. Hegarty. 2014. Sire and liveweight affect feed intake and methane emissions of sheep confined in respiration chambers. Animal. 8:1935–1944. https://doi.org/10.1017/S1751731114001773.

241. Rosales-Nieto, C. A., R. Ehrhardt, A. Mantey, B. Makela, and A. Veiga-Lopez. 2021. Preconceptional diet manipulation and fetus number can influence placenta endocrine function in sheep. Dom. Anim. Endo. 74:106577. https://doi.org/10.1016/j.domaniend.2020.106577.

242. Royal Bank of Canada. 2019. https://thoughtleadership.rbc.com/farmer-4-0-how-the-coming-skills-revolution-can-transform-agriculture/.

243. Reinehr, T., B. Wolters, C. Knop, N. Lass, C. Hellmuth, U. Harder, W. Peissner, S. Wahl, H. Grallert, J. Adamski, T. Illig, C. Prehn, Z. Yu, R. Wang-Sattler, and B. Koletzko. 2014. Changes in the serum metabolite profile in obese children with weight loss. Eur. J. Nutr. 54:173-181. https://doi.org/10.1007/s00394-014-0698-8.

244. Roberts, J. N., K. J. May, and A. Veiga-Lopez. 2017. Time-dependent changes in pregnancy-associated glycoproteins and progesterone in commercial crossbred sheep. Therio. 89:271-279. https://doi.org/10.1016/j.theriogenology.2016.10.029.

245. Robinson, T. P., G. R. W. Wint, G. Conchedda, T. P. Van Boeckel, V. Ercoli, E. Palamara, G. Cinardi, L. D'Aietti, S. I. Hay, and M. Gilbert. 2014. Mapping the global distribution of livestock. PLoS One. 9:e96084. https://doi.org/10.1371/journal.pone.0096084.

246. Römpp, A., J. Both, A. Brunelle, R. M. A. Heeren, O. Laprévote, B. Prideaux, A. Seyer, B. Spengler, M. Stoeckli, and D. F. Smith. 2015. Mass spectrometry imaging of biological tissue: an approach for multicenter studies. Anal. Bioanal. Chem. 407:2329. https://doi.org/10.1007/s00216-014-8410-7.

247. Sajed, T., A. Marcu, M. Ramirez, A. Pon, A. Guo, C. Knox, M. Wilson, J. R. Grant, Y. Djoumbou, and D. S. Wishart. 2016. ECMDB 2.0: A richer resource for understanding the biochemistry of E. coli. Nucleic Acids Res. 44:D495-501. https://doi.org/10.1093/nar/gkv1060.

248. Saia, S., M. Fragasso, P. De Vita, and R. Beleggia. 2019. Metabolomics Provides Valuable Insight for the Study of Durum Wheat: A Review. J. Agr. Food Chem. 67:3069-3085. DOI: 10.1021/acs.jafc.8b07097.

249. Saleem, F., B. N. Ametaj, S. Bouatra, R. Mandal, Q. Zebeli, S. M. Dunn, and D. S. Wishart. 2012. A metabolomics approach to uncover the effect of grain diets on rumen health in dairy cows. J. Dairy Sci. 95:6606-6623. https://doi.org/10.3168/jds.2012-5403.

250. Saleem, F., S. Bouatra, A. C. Guo, N. Psychogios, R. Mandal, S. M. Dunn, B. N. Ametaj, and D. S. Wishart. 2013. The bovine ruminal fluid metabolome. Metabolomics. 9:360-378. https://doi.org/10.1007/s11306-012-0458-9.

251. Salleh, M. S., G. Mazzoni, M. O. Nielsen, P. Løvendahl, and H. N. Kadarmideen. 2018. Identification of expression QTLs targeting candidate genes for residual feed intake in dairy

cattle using systems genomics. J. Genet. Genome Res. 5:035. DOI: 10.23937/2378-3648/1410035.

252. Satterfield, M. C., K. A. Dunlap, D. H. Keisler, F. W. Bazer, and G. Wu. 2013. Arginine nutrition and fetal brown adipose tissue development in nutrient-restricted sheep. Amin. Aci. 45:489–499. https://doi.org/10.1007/s00726-011-1168-8.

253. Satterfield, M. C., K. A. Dunlap, D. H. Keisler, F. W. Bazer, and G. Wu. 2013. Arginine nutrition and fetal brown adipose tissue development in nutrient-restricted sheep. Amin. Aci. 45:489–499. https://doi.org/10.1007/s00726-011-1168-8.

254. Saude, E. J., C. M. Slupksy, and B. D. Sykes. 2006. Optimization of NMR analysis of biological fluids for quantitative accuracy. Metabolomics. 2:113-123. https://doi.org/10.1007/s11306-006-0023-5.

255. Schenkel, F. S., S. P. Miller, and J. W. Wilton. 2004. Genetic parameters and breed differences for feed efficiency, growth, and body composition traits of young beef bulls. Can. J. Anim. Sci. 84:177–185. https://doi.org/10.4141/A03-085.

256. Scherf, B. D. 2000. World watch list for domestic animal diversity. Ed. 3. Food and Agriculture Organization of the United Nations. Rome. https://www.cabdirect.org/cabdirect/abstract/20013016397.

257. Scholz, A., L. Bünger, J. Kongsro, U. Baulain, and A. Mitchell. 2015. Non-invasive methods for the determination of body and carcass composition in livestock: Dual-energy X-ray absorptiometry, computed tomography, magnetic resonance imaging and ultrasound: Invited review. Animal. 9:1250-1264. doi:10.1017/S1751731115000336.

258. See, K., A. J. Bailer, E. J. Behnke, R. S. Baker, K. E. Clark, and P. T. Russell. 2007. Predicting pregnancy status and fetal number in time-dated pregnant ewes using serum

progesterone and ultrasound. Res. J. Anim. Sci. 1:65-71. https://medwelljournals.com/abstract/?doi=rjnasci.2007.65.71.

259. Senchuk, I. 2019. Evaluation of metabolic status of ewes infected with ketosis. Agri. Sci. Euro-North-East. 20:265-272. doi:10.30766/2072-9081.2019.20.3.265-272.

260. Shajahan-Haq, A. N., M. S. Cheema, and R. Clarke. 2015. Application of metabolomics in drug resistant breast cancer research. Metabolites. 5:100-118. https://doi.org/10.3390/metabo5010100.

261. Shah, S. H., W. E. Kraus, and C. B. Newgard. 2012. Metabolomic profiling for the identification of novel biomarkers and mechanisms related to common cardiovascular diseases. Circulation. 169:1110-1120. https://doi.org/10.1161/CIRCULATIONAHA.111.060368.

262. Shrestha, J. N. B., and D. P. Heaney. 2003. Review of Canadian, Outaouais and Rideau Arcott breeds of sheep: 1. Development and characterization. Sm. Rumin. Res. 49:79-96. https://doi.org/10.1016/S0921-4488(03)00070-1.

263. Silva, S. R. 2017. Use of ultrasonographic examination for in vivo evaluation of body composition and for prediction of carcass quality of sheep. Sm. Rumin. Res. 152:144-157. https://doi.org/10.1016/j.smallrumres.2016.12.020.

264. Sinclair, K. D., C. Allegrucci, R. Singh, D. S. Gardner, S. Sebastian, J. Bispham, A. Thurston, J. F. Huntley, W. D. Rees, C. A. Maloney, and R. G. Lea. 2007. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. PNAS. 104:19351-19356. https://doi.org/10.1073/pnas.0707258104.

265. Singh, A. K. 2016. Experimental methodologies for the characterization of nanoparticles. Eng. Nano. pp.125-170. DOI: 10.1016/B978-0-12-801406-6.00004-2.

266. Singh, R., N. Gautam, A. Mishra, and R. Gupta. 2011. Heavy metals and living systems: An overview. Indian J. Pharmacol. 43:246-53. doi: 10.4103/0253-7613.81505.

267. Simo, C., C. Ibanez, A. Valdes, A. Cifuentes, and V. Garcia-Canas. 2014. Metabolomics of genetically modified crops. Int. J. Mol. Sci. 15:18941-18966. https://doi.org/10.3390/ijms151018941.

268. Skelton, D. M., D. R. Ekman, D. Martinović-Weigelt, G. T. Ankley, D. L. Villeneuve, Q. Teng, and T. W. Collette. 2014. Metabolomics for in situ environmental monitoring of surface waters impacted by contaminants from both point and nonpoint sources. Environ. Sci. Technol. 48:2395–2403. https://doi.org/10.1021/es404021f.

269. Skene, D., B. Middleton, C. Fraser, J. L. A. Pennings, T. R. Kuchel, S. R. Rudiger, C. S. Bawden, and A. J. Morton. 2017. Metabolic profiling of presymptomatic Huntington's disease sheep reveals novel biomarkers. Sci. Rep. 7:43030. https://doi.org/10.1038/srep43030.

270. Song, W., M. Puttabyatappa, L. Zeng, D. Vazquez, S. Pennathur, and V. Padmanabhan. 2020. Developmental programming: Prenatal bisphenol a treatment disrupts mediators of placental function in sheep. Chemosphere. 243:125301. https://doi.org/10.1016/j.chemosphere.2019.125301.

271. Spring, P. 2013. The challenge of cost effective poultry and animal nutrition: optimizing existing and applying novel concepts. Lohm. Info. 48:38-47. https://www.lohmann-information.com/content/l_i_48_artikel5.pdf.

272. Srivastava, A., G. M. Kowalski, D. L. Callahan, P. J. Meikle, and D. J. Creek. 2016. Strategies for extending metabolomics studies with stable isotope labelling and fluxomics. Metabolites. 6:E32. https://doi.org/10.3390/metabo6040032.

273. Sreekumar, A., L. M. Poisson, T. M. Rajendiran, A. P. Khan, Q. Cao, J. Yu, B. Laxman, R. Mehra, R. J. Lonigro, Y. Li, M. K. Nyati, A. Ahsan, S. Kalyana-Sundaram, B. Han, X. Cao, J. Byun, G. S. Omenn, D. Ghosh, S. Pennathur, D. C. Alexander, A. Berger, J. R. Shuster, J. T. Wei, S. Varambally, C. Beecher, and A. M. Chinnaiyan. 2009. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature. 457:910–914.

274. Statistics Canada. Table 32-10-0425-01. Sheep and lambs on census day. https://doi.org/10.25318/3210042501-eng.

275. Statistics Canada. Table 32-10-0129-01. Number of sheep and lambs on farms (x 1,000). https://doi.org/10.25318/3210012901-eng.

276. Steckeler, P., F. Weber, H. Zerbe, A. Rieger, K. Voigt. 2019. Evaluation of a bovine visual pregnancy test for the detection of pregnancy-associated glycoproteins in sheep. Repro. Dom. Anim. 54:280–288. https://doi.org/10.1111/rda.13356.

277. Steinshamn, H., R. A. Inglingstadb, D. Ekebergb, J. Mølmanna, and M. Jørgensen. 2014. Effect of forage type and season on Norwegian dairy goat milk production and quality. Small Rum. Res. 122:18–30. https://doi.org/10.1016/j.smallrumres.2014.07.013.

278. Strahl, B. D., and C. D. Allis. 2000. The language of covalent histone modifications. Nature. 403:41-45. https://doi.org/10.1038/47412.

279. Sumaryadi, M., and W. Manalu. 1999. Prediction of litter size based on hormones and blood metabolites concentrations during pregnancy in Javanese thin-tail ewes. Asian-Aus. J. Anim. Sci. 12:682-688. https://doi.org/10.5713/ajas.1999.682.

280. Summer, L. W., Z. Lei, B. J. Nikolau, and K. Saito. 2015. Modern plant metabolomics: advanced natural product gene discoveries, improved technologies, and future prospects. Nat. Prod. Rep. 32:212-229. https://doi.org/10.1039/C4NP00072B.

281. Sun, L., Y. Guo, Y. Fan, H. Nie, R. Wang, and F. Wang. 2017. Metabolic profiling of stages of healthy pregnancy in Hu sheep using nuclear magnetic resonance (NMR). Therio. 92:121-128. https://doi.org/10.1016/j.theriogenology.2017.01.025.

282. Sun, H., D. Wang, B. Wang, J. Wang, H. Liu, L. L. Guan, and J. X. Liu. 2015. Metabolomics of four biofluids from dairy cows: potential biomarkers for milk production and quality. J. Proteome Res. 14:1287−1298. https://doi.org/10.1021/pr501305g.

283. Sun, L., Yixuan Guo, Y. Fan, H. Nie, R. Wang, and F. Wang. 2017. Metabolic profiling of stages of healthy pregnancy in Hu sheep using nuclear magnetic resonance (NMR). Therio. 92:121-128. https://doi.org/10.1016/j.theriogenology.2017.01.025.

284. Sundekilde, U. K., N. A. Poulsen, L. B. Larsen, and H. C. Bertram. 2013. Nuclear magnetic resonance metabonomics reveals strong association between milk metabolites and somatic cell count in bovine milk. J. Dairy Sci. 96:290-299. https://doi.org/10.3168/jds.2012-5819.

285. Susmel, P., and E. Piasentier. 1992. Assessment of pregnancy in Bergamasca ewes by analysis of plasma progesterone. Sm. Rumin. Res. 8:325-332. https://doi.org/10.1016/0921-4488(92)90213-N.

286. Thornton, P. K. 2010. Livestock production: recent trends, future prospects. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365:2853-2867. https://doi.org/10.1098/rstb.2010.0134.

287. Thureen, P., K. Baron, P. Fennessey, and W. W. Hay. 2002. Ovine placental and fetal arginine metabolism at normal and increased maternal plasma arginine concentrations. Pediatr. Res. 51:464–471. https://doi.org/10.1203/00006450-200204000-00011.

288. Torii, Y., Y. Kawano, H. Sato, K. Sasaki, T. Fujimori, J. Kawada, O. Takikawa, C. K. Lim, G. J. Guillemin, Y. Ohashi, and Y. Ito. 2016. Quantitative metabolome profiling reveals the involvement of the kynurenine pathway in influenza-associated encephalopathy. Metabolomics. 12:84. https://doi.org/10.1007/s11306-016-1011-z.

289. Torres, S., and C. Fernanda. 2019. Effect of maternal nutrient restriction on fetal skeletal muscle growth and metabolism in sheep. Doctoral dissertation, Texas A&M University. http://hdl.handle.net/1969.1/186572.

290. Trivedi, D. K., K. A. Hollywood, and R. Goodacre. 2017. Metabolomics for the masses: The future of metabolomics in a personalized world. New Hor. Trans. Med. 3:294-305. https://doi.org/10.1016/j.nhtm.2017.06.001.

291. Tsikas, D., R. H. Böger, J. Sandmann, S. M. Bode-Böger, and J. C. Frölich. 2000. Endogenous nitric oxide synthase inhibitors are responsible for the l-arginine paradox. FEBS Lett. 478:1–3. https://doi.org/10.1016/S0014-5793(00)01686-0.

292. Tweeddale, H., L. Notley-Mcrobb, and T. Ferenci. 1998. Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool ("metabolome") analysis. J. Bacteriol. 180:5109–5116. DOI: 10.1128/JB.180.19.5109-5116.1998.

293. Ul-Haq, A., M. Tabassum Ch., F. Ahmad, J. Shafi, M. Ashraf, M. Javed, and S. Ur-Rehman. 2014. Effect of dietary acidification with citric acid on carcass characteristics, haemogram and serum metabolite values of broiler chicken. Pakistan J. Life Soc. Sci. 12:36-41. https://search-ebscohost-com.login.ezproxy.library.ualberta.ca/login.aspx?direct=true&db=a9h&AN=101045347&site=eds-live&scope=site.

294. Umer, S., S. J. Zhao, A. Sammad, B. W. Sahlu, Y. Pang, and H. Zhu. 2019. AMH: Could it be used as a biomarker for fertility and superovulation in domestic animals? Genes. 10:1009. https://doi.org/10.3390/genes10121009.

295. VandeHaar, M. J., L. E. Armentano, K. Weigel, D. M. Spurlock, R. J. Tempelman, and R. Veerkamp. 2016. Harnessing the genetics of the modern dairy cow to continue improvements in feed efficiency. J. Dairy Sci. 99:4941-4954. https://doi.org/10.3168/jds.2015-10352.

296. Verma, M., M. J. Khoury, and J. P. A. Ioannidis. 2013. Opportunities and challenges for selected emerging technologies in cancer epidemiology: mitochondrial, epigenomic, metabolomic, and telomerase profiling. Cancer Epidemiol. Biomar. Prev. 22:189–200. DOI: 10.1158/1055-9965.EPI-12-1263.

297. Vinayavekhin, N., E. A. Homan, and A. Saghatelian. 2010. Exploring disease through metabolomics. ACS Chem. Bio. 5:91-103. DOI: 10.1021/cb900271r.

298. Viswanathan, V. 2008. Rapid web application development: a ruby on rails tutorial. IEEE Software. 25:98-106. DOI: 10.1109/MS.2008.156.

299. Wallace, J. M. 2011. Adaptive maternal, placental and fetal responses to nutritional extremes in the pregnant adolescent: lessons from sheep. in: C.G.N. Mascie-Taylor, L. Rosetta (Eds.) Reproduction and adaptation. University Press. Cambridge. 112–127. https://doi.org/10.1017/CBO9780511973642.007.

300. Wang, X., and H. N. Kadarmideen. 2019. Metabolomics analyses in high-low feed efficient dairy cows reveal novel biochemical mechanisms and predictive biomarkers. Metabolites. 9:151. https://doi.org/10.3390/metabo9070151.

301. Wang, Z., J. D. Nkrumah, C. Li, J. A. Basarab, L. A. Goonewardene, E. K. Okine, D. H. Crews, Jr, and S. S. Moore. 2006. Test duration for growth, feed intake, and feed efficiency in

beef cattle using the GrowSafe system. J. Anim. Sci. 84:2289–2298. doi:10.2527/ jas.2005-715.

302. Wang, J., K. Cui, T. Ma, F. Zhang, S-Q. Wang, Y. Tu, Q-Y. Diao, and N-F. Zhang. 2018. Effects of dietary methionine deficiency followed by replenishment on the growth performance and carcass characteristics of lambs. Anim. Pro. Sci. 59:243-248. https://doi.org/10.1071/AN16643.

303. Wang, J., Z. Xu, H. Zhang, Y. Wang, X. Liu, Q. Wang, J. Xue, Y. Zhao, and S. Yang. 2020. Meat differentiation between pasture-fed and concentrate-fed sheep/goats by liquid chromatography quadrupole time-of-flight mass spectrometry combined with metabolomic and lipidomic profiling. Meat Sci. 108374. https://doi.org/10.1016/j.meatsci.2020.108374.

304. Wang-Sattler, R., Z. Yu, C. Herder, A. C. Messias, A. Floegel, Y. He, K. Heim, M. Campillos, C. Holzapfel, B. Thorand, and H. Grallert. 2012. Novel biomarkers for pre-diabetes identified by metabolomics. Mol. Syst. Biol. 8:615. https://doi.org/10.1038/msb.2012.43.

305. Warth, B., A. Parich, C. Bueschl, D. Schoefbeck, N. K. N. Neumann, B. Kluger, K. Schuster, R. Krska, G. A., M. Lemmens, and R. Schuhmacher. 2015. GC–MS based targeted metabolic profiling identifies changes in the wheat metabolome following deoxynivalenol treatment. Metabolomics. 11:722–738. https://doi.org/10.1007/s11306-014-0731-1.

306. Washburn, S. E., M. A. Caudill, O. Malysheva, A. J. MacFarlane, N. A. Behan, B. Harnett, L. MacMillan, T. Pongnopparat, J. T. Brosnan, and M. E. Brosnan. 2015. Formate metabolism in fetal and neonatal sheep. Am. J. Physiol. Endocrinol. Metab. 308:E921–E927. https://doi.org/10.1152/ajpendo.00046.2015.

307. Watkins, S. M., and J. B. German. 2002. Metabolomics and biochemical profiling in drug discovery and development. Cur. Opin. Mol. Therap. 4:224-228. https://europepmc.org/article/med/12139307.

308. Weikard, R., E. Altmaier, K. Suhre, K. M. Weinberger, H. M. Hammon, E. Albrecht, K. Setoguchi, A. Takasuga, and C. Kühn. 2010. Metabolomic profiles indicate distinct physiological pathways affected by two loci with major divergent effect on Bos taurus growth and lipid deposition. Physiol. Genomics. 42A:79-88. https://doi.org/10.1152/physiolgenomics.00120.2010.

309. Wester, T. J., G. E. Lobley, L. M. Birnie, and M. A. Lomax. 2000. Insulin stimulates phenylalanine uptake across the hind limb in fed lambs. J. Nutr. 130:608–611. https://doi.org/10.1093/jn/130.3.608.

310. Widmann, P., A. Reverter, R. Weikard, K. Suhre, H. M. Hammon, E. Albrecht, and C. Kuehn. 2015. Systems biology analysis merging phenotype, metabolomics and genomic data identifies non-SMC condensing I complex, subunit G (NCAPG) and cellular maintenance processes as major contributors to genetic variability in bovine feed efficiency. PLoS One. 10:e0124574. https://doi.org/10.1371/journal.pone.0124574.

311. Wikoff, W. R., A. T. Anfora, J. Liu, P. G. Schultz, S. A. Lesley, E. C. Peters, G. Siuzdak. 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. PNAS. 106:3698-3703. DOI: 10.1073/pnas.0812874106.

312. Winter, G., and J. O. Krömer. 2013. Fluxomics - connecting 'omics analysis and phenotypes. Environ. Microbiol. 15:1901-16. https://doi.org/10.1111/1462-2920.12064.

313. Wishart, D. S., D. Tzur, C. Knox, R. Eisner, A. C. Guo, et al. 2007. HMDB: the Human Metabolome Database. Nuc. Acids Res. 35:D521–D526. https://doi.org/10.1093/nar/gkl923.

314. Wishart, D. S. 2008. Metabolomics: application to food science and nutrition research. Tren. Food. Sci. Tech. 19:482-493. https://doi.org/10.1016/j.tifs.2008.03.003.

315. Wishart, D. S. 2008b. Quantitative metabolomics using NMR. TrAC. Tre. Analy. Chem. 27:228-237. https://doi.org/10.1016/j.trac.2007.12.001.

316. Wishart, D. S., M. J. Lewis, J. A. Morrissey, M. D. Flegel, K. Jeroncic, Y. Xiong, D. Cheng, R. Eisner, B. Gautam, D. Tzur, and S. Sawhney. 2008. The human cerebrospinal fluid metabolome. J. Chrom. B. 871:164-173. https://doi.org/10.1016/j.jchromb.2008.05.001.

317. Wishart, D. S. 2009. Computational strategies for metabolite identification in metabolomics. Bioanalysis. 1:1579-1596. https://doi.org/10.4155/bio.09.138.

318. Wishart, D. S. 2012. Systems biology resources arising from the human metabolome project. In: Genetics meets metabolomics: from experiment to systems biology. Edited by Suhre K. New York: Springer. 157–175. DOI:10.1007/978-1-4614-1689-0_11.

319. Wishart, D. S. 2016. Emerging applications of metabolomics in drug discovery and precision medicine. Nat. Rev. Drug Discov. 15:473. doi: 10.1038/nrd.2016.32.

320. Wishart, D. S., C. Knox, A. C. Guo, R. Eisner, N. Young, B. Gautam, D. D. Hau, N. Psychogios, E. Dong, S. Bouatra, and R. Mandal. 2009. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 37:D603–610. https://doi.org/10.1093/nar/gkn810.

321. Wishart, D. S., T. Jewison, A. C. Guo, M. Wilson, C. Knox, Y. Liu, Y. Djoumbou, R. Mandal, F. Aziat, E. Dong, and S. Bouatra. 2013. HMDB 3.0—The Human Metabolome Database in 2013. Nucleic Acids Res. 41:D801–807. https://doi.org/10.1093/nar/gks1065.

322. Wishart, D. S., R. Mandal, A. Stanislaus, and M. Ramirez-Gaona. 2016. Cancer metabolomics and the human metabolome database. Metabolites. 2:6. https://doi.org/10.3390/metabo6010010

323. Wooldridge, A. L., R. J. Bischof, H. Liu, G. K. Heinemann, D. S. Hunter, L. C. Giles, R. A. Simmons, Y. C. Lien, W. Lu, J. D. Rabinowitz, and K. L. Kind. 2018. Late-gestation maternal dietary methyl donor and cofactor supplementation in sheep partially reverses protection against allergic sensitization by IUGR. Am. J. Physio. Reg. Integ. Comp. Physio. 314:R22-R33. https://doi.org/10.1152/ajpregu.00549.2016.

324. Wyness, L. 2016. The role of red meat in the diet: nutrition and health benefits. Proc. Nutr. Soc. 75:227-32. doi: 10.1017/S0029665115004267.

325. Yanagihara, M., F. Nakajima, and T. Tobino. 2018. Metabolomic responses of an estuarine benthic amphipod to heavy metals at urban-runoff concentrations. Water Sci. Technol. 78:2349–2354. https://doi.org/10.2166/wst.2018.518.

326. Yang, X., M. Zhang, T. Lu, S. Chen, X. Sun, Y. Guan, Y. Zhang, T. Zhang, R. Sun, B. Hang, X. Wang, M. Chen, Y. Chen, and Y. Xia. 2020. Metabolomics study and meta-analysis on the association between maternal pesticide exposome and birth outcomes. Environ. Res. 182:109087. doi:10.1016/j.envres.2019.109087.

327. Yazdani, M., K. B. P. Elgstøen, H. Rootwelt, A. Shahdadfar, Ø. A. Utheim, and T. P. Utheim. Tear metabolomics in dry eye disease: A review. Int. J. Mol. Sci. 20:3755. doi:10.3390/ijms20153755

328. Xia, J., D. Broadhurst, M. Wilson, and D. Wishart. 2013. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics. 9:280-299. https://doi.org/10.1007/s11306-012-0482-9.

329. Zaher, H. A., S. A. Alawaash, and A. A. Swelum. 2020. Effects of season and breed on the reproductive performance of sheep. J. Anim. Repro. Biotech. 35:149–154. https://doi.org/10.12750/JARB.35.2.149.

330. Zeder, M. A. 2008. Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion, and impact. PNAS. 105:11597-11604. doi: 10.1073/pnas.0801317105.

331. Zygoyiannis, D. 2006. Sheep production in the world and in Greece. Sm. Rum. Res. 62:143-147. https://doi.org/10.1016/j.smallrumres.2005.07.043.

332. Zhang, A., H. Sun, P. Wang, Y. Han, and X. Wang. 2012. Recent and potential developments of biofluid analyses in metabolomics. J. Proteo. 75:1079-1088. https://doi.org/10.1016/j.jprot.2011.10.027.

333. Zhang, A., H. Sun, P. Wang, Y. Han, and X. Wang. 2012. Modern analytical techniques in metabolomics analysis. Analyst. 137:293-300. https://doi.org/10.1039/C1AN15605E.

334. Zhang, X., W. Wang, F. Mo, Y. La, C. Li, and F. Li. 2017. Association of residual feed intake with growth and slaughtering performance, blood metabolism, and body composition in growing lambs. Sci. Rep. 7:12681. doi:10.1038/s41598-017-13042-7.

335. Zhang, L., J. Xue, Q. Wang, W. Lv, H. Mi, Y. Liu, and L. Yang. 2018. Changes in expression of ISG15, progesterone receptor and progesterone-induced blocking factor in ovine thymus during early pregnancy. Therio. 121:153-159. https://doi.org/10.1016/j.theriogenology.2018.08.018.

336. Zhang, J., Y. Gao, H. Guo, Y. Ding, and W. Ren. 2019. Comparative metabolome analysis of serum changes in sheep under overgrazing or light grazing conditions. BMC Vet. Res. 15:469. https://doi.org/10.1186/s12917-019-2218-9.

337. Zhang, X., L. Zhang, W. Sun, X. Lang, J. Wu, C. Zhu, J. Jia, J. Jin, Y. La, and D. P. Casper. 2020. Study on the correlation between BMPR1B protein in sheep blood and reproductive performance. J. Anim. Sci. 98:skaa100. https://doi.org/10.1093/jas/skaa100.

338. Zhang, X., T. Wang, J. Song, J. Deng, and Z. Sun. 2020. Study on follicular fluid metabolomics components at different ages based on lipid metabolism. Repro. Biol. Endocrinol. 18:42. https://doi.org/10.1186/s12958-020-00599-8.