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Exploring the biological basis of residual feed intake in beef cattle using multi-Omics analysis.

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Exploring the biological basis of residual feed intake in beef cattle using multi-Omics analysis.

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Abstract

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Efficient feed utilization is critical for both economic sustainability and environmental responsibility in modern livestock production. While there has been extensive research, the multifaceted nature of feed efficiency remains complex, with many biological factors still unexplored. This dissertation examines the physiological foundations of feed efficiency by elucidating some of the complex biological mechanisms associated with residual feed intake (RFI) phenotype in beef cattle, using a range of Omics approaches. We hypothesized that metabolites related to amino acids, carbohydrates, and fatty acids could act as potential biomarkers for RFI. Through a chemical group-based metabolomics method, we identified enriched pathways in feed-efficient steers, notably in arginine biosynthesis and histidine metabolism. This led to the identification of five potential metabolite biomarkers mainly linked to amino acid metabolism, emphasizing a relationship between blood amino acid profiles and RFI. This led us to investigate the expression of genes and associated pathways related to nutrient and energy metabolism, especially in liver tissue, where hepatic metabolism is driven by transcriptional regulation. Low-RFI steers showed upregulation of genes involved in fatty acid transport, β-oxidation, and mitochondrial ATP production. In contrast, a crucial gene in amino acid metabolism responsible for aminoadipate aminotransferase activity exhibited a significant decrease in expression in low-RFI steers. These results indicate that alteration in expression of hepatic genes regulating lipid and amino acid metabolism, and mitochondrial ATP generation is associated with RFI phenotype. We also investigated potential differences in the rumen microbiome and immune gene expression of beef steers with low or high RFI. We observed increased mRNA expression of immune-related genes in both blood and liver tissues of low-RFI beef steers, especially those linked to pathogen detection and phagocytosis. Low-RFI steers also displayed variation in the relative abundance of microbial taxa compared to high-RFI. Lastly, detailed statistical analysis indicated that plasma amino acids such as tyrosine, glycine, and dimethyl sulfone may be promising economic prospects as cost-efficient predictors of RFI in beef cattle. In conclusion, this dissertation provides invaluable insights into some of the intricate biological processes associated with RFI in crossbred beef cattle, enhancing our grasp of the

involved biological mechanisms and laying the groundwork for refining feed utilization in the beef cattle sector of livestock production.

To my wife, Rhoda, and my mum, Olusade

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2D-PAGE	2-Dimensional Polyacrylamide Gel Electrophoresis
ADG	Average daily gain
ATP	adenosine triphosphate
AUC	Area under the curve
BW	Body weight
CIL	Chemical isotope labeling
CP	Crude protein
CV	Coefficient of variation
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
FC	Fold change
FCR	Feed conversion ratio
GHG	Greenhouse gas
GO	Gene ontology
GSEA	Gene set enrichment analysis
GWAS	Genome-wide association studies
HFE	High feed efficient
HIF	Heat increment of feeding
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS	Liquid chromatography–mass spectrometry
LDA	Linear discriminant analysis
LFE	Low feed efficient
MMTW	Mid-test metabolic body weight.
NDF	Neutral detergent fibre
NE	Net energy
NMR	Nuclear magnetic resonance
OTU	Operational taxonomic units
PCoA	principal coordinates analysis
PLS-DA	Partial least square discriminant analysis
RADG	Residual average daily gain
RFI	Residual feed intake
RFID	Radio frequency identification
RIG	Residual intake gain
RNA	Ribonucleic acid
ROC	receiver operating characteristic
RT	Retention time
SEM	Standard error of the mean

TMR	Total mixed ration
USA	United States America
VFA	Volatile fatty acids
WVU	West Virginia University

Chapter 1. Literature Review

Feed efficiency as an economic trait in beef production

Efficiency is a broad economic concept, typically defined as the ratio of outputs to inputs within a given production system (Drummond et al., 2005). Assessing efficiency comprehensively across the entire integrated beef system is challenging due to its multifaceted nature, which encompasses breed differences, diverse cattle classes (growing, finishing, breeding), and variations inherent in biological systems such as diet, lactation, reproduction, and basal metabolism (Lamb et al., 2013). One tool for enhancing beef production is feed efficiency. Identification of individual animal feed efficiency holds the potential to optimize productivity, elevate producer profitability, and reduce environmental footprints (Berry and Crowley, 2012; Kenny et al., 2018). Thus, selecting feed-efficient cattle, ensuring optimal growth performance, identifying cost-effective input combinations, and adopting superior feed efficiency measures have taken center stage in the beef industry (Seabury et al., 2017; Silva et al., 2023). This focus arises, in part, because feed costs constitute approximately 75-80% of the production expenses (Arthur et al., 2001; Nielsen et al., 2013;). Feed efficiency is, therefore, a cornerstone economic trait in beef production, marrying economic growth with environmental conservation (Goldstein et al., 2017). Efficient feed-to-weight gain conversion has been shown to not only trims production costs but also aid in conserving natural resources (Nath et al., 2023). Emphasizing feed efficiency by grasping its biological drivers, adopting advanced management strategies, and leveraging new technologies is pivotal for satisfying the escalating beef demand while ensuring the industry's economic and environmental health (Cantalapiedra-Hijar et al., 2018; Broom, 2021; Ismail and Al-Ansari, et al., 2023).

The significance of feed efficiency in beef production

In our journey to feed an expanding global population, feed efficiency stands as a central component in crafting a thriving and sustainable beef sector (EPA, 2022; Wunderlich and Martinez, 2018). Its relevance in animal studies has grown, especially with the onset of trends favoring livestock with higher productivity, decreased environmental footprints, and reduced grain competition for human consumption (Hayes et al., 2013; Gerber et al., 2013; Alexandra et al., 2015). Enhanced feed efficiency is linked to diminished greenhouse gas emissions (Knapp et al., 2014). For example, Capper et al. (2009) found that US dairy farms have seen a 60% reduction in greenhouse gas emissions per milk unit over the past six decades, primarily due to increased feed efficiency. Boosting feed efficiency in ruminants means less land and fewer resources are needed for feed production (von Keyserlingk et al., 2013). Notably, earlier research using ADG as a feed efficiency metric revealed significant cost savings for livestock producers. Feedlot studies indicated that a 10% rise in ADG bolstered profitability by 18%. In contrast, a 10% hike in feed efficiency led to a 43% profit surge (Fox et al., 2001). Similarly, Basarab et al. (2002) reported that enhancing feed efficiency by 5% could yield an economic benefit four times that of a 5% ADG increase. Through collaborative research, technological innovations, and informed practices, the beef industry can stride towards a future marked by heightened efficiency, resilience, and sustainability.

Measures of feed efficiency

Relative growth rate

Historically, animal performance assessment mainly focused on measuring outputs such as body weight, milk yield, rate of gain, or carcass weight. Yet, it's clear that a variety of factors

influence these outcomes. These include feed energy density and quality (McCarthy et al., 1985; Helferich et al., 1986), management practices like handling techniques and pen density (Voisinet et al., 1997; Mader and Colgan, 2007), and the animals' environmental conditions. Such conditions can involve wind speed, temperature, precipitation, and pen state, each potentially affecting animal performance (Mitlöhner et al., 2001).

Genetics is also crucial in determining animal performance. Numerous studies have estimated genotypic and phenotypic variances in beef cattle performance traits (Mohiuddin, 1993; Koots et al., 1994). Significant correlations have been observed in the variances of average daily gain (ADG) and other weight measures. Given these intricate factors, ADG serves as an essential selection criterion, influencing the time to reach target weights and overall feed requirements. Understanding how animals utilize feed energy after consumption, including energy partitioning, is crucial for an accurate portrayal of biological efficiency. Thus, it's essential to holistically assess various aspects, ranging from genetics and management to environmental conditions and metabolic processes, in understanding feed efficiency and the various measures associated with it.

Feed conversion ratio

Historically, feed efficiency metrics focused on comparing feed intake with growth. The Feed Conversion Ratio (FCR) stands out as a primary index reflecting beef production efficiency (Lamb and Maddock, 2009; Shike, 2013). It represents the relationship between average daily feed consumption and ADG. Animals with low FCRs need less feed per unit of weight gain compared to those with higher FCRs. Even though FCR has heritable traits, its value in genetically enhancing feed efficiency remains limited (Crews, 2005). Selecting for improved FCR will lead to increased cow maintenance requirements and higher feed costs due to the negative correlation between FCR and growth rate and the consequent rise in mature cow size (Koots et al., 1994). This could mean

that efficiency gains during growth might be offset by mature animals' increased feed requirements, leaving overall system feed efficiency unchanged (Archer et al., 1999).

A significant drawback of using FCR as a feed efficiency measure is the challenge in accurately measuring individual animal intake (Herd and Arthur, 2009). The conventional approach uses pen averages to estimate individual consumption. Still, this lacks accuracy, overlooking the vital individual variations essential for comparing animals and selecting superior breeding stock. Thus, selecting based on ratio traits like FCR can lead to inconsistent outcomes due to unpredictable responses in component traits, especially the feed intake trait (Zetouni et al., 2018; Cantalapiedra-Hijar et al., 2018).

Assessment of individual animal feed intake

The early 1970s marked significant advancements in the quest to automate individual animal intake tracking, with the introduction of the Calan Feeding System. Pioneered by Broadbent et al. (1970), this system employed electronically unlocked head gates activated by keys worn by cattle. Through this mechanism, animals could access their feed by opening these gates, and their intake data were systematically recorded (Matamoros et al., 2022). An alternative system was the Pinpointer feeding system (Gonyou and Stricklin, 1981), which utilized stalls equipped with a microprocessor. This system recorded feeding specifics by identifying animals via transponders attached to their necks.

Despite their innovative designs, both systems had their share of challenges. They encountered electronic malfunctions, especially in terms of incorrect animal identification and weather-related issues, necessitating continuous supervision (Cole, 1994). Typically, the adaptation period for these systems ranged from 7 to 21 days, and they were best suited for limited group sizes. A

subsequent study highlighted that the American Calan systems, in particular, necessitated modifications—either by integrating change of state sensors or resorting to labor-intensive visual observation techniques—to effectively observe feeding behavior (Krawczel et al., 2012).

A notable advancement came with the development of the GrowSafe® Feeding System. This system utilized radio frequency identification (RFID) ear tags, eliminating the need for visual monitoring, and was capable of accommodating larger cattle groups (Schwartzkopf-Genswein et al., 1999). While it did come with a higher price tag, the value was evident in the automated behavioral metrics assessment it provided. Other contemporary automated monitoring systems, such as the Insentec system, use load cell feed bunks. This particular system has been especially effective for indoor cattle housing (Tolkamp et al., 2000; Chapinal et al., 2007).

As these systems gained traction, research in the realm of feed efficiency adapted and expanded, the new measures of efficiency emerged. Measures such as residual feed intake (RFI), residual gain (RG), and the integrated approach of residual intake and gain (RIG) have been introduced. These methods aim to offer in-depth information on an individual animal basis, illuminating the nuanced factors influencing feed efficiency (Koch, 1963; Berry and Crowley, 2012, 2005; Cantalapiedra-Hijar et al., 2018).

Residual feed intake (RFI)

The groundbreaking idea of evaluating biological efficiency by measuring its outliers from the energy of an adjusted population beyond the needs for maintenance and production was first introduced by Titus in 1928, with a primary focus on chickens. Building upon this concept, the principle of Residual Feed Intake (RFI) was first applied to beef cattle by Koch et al. in 1963. This metric served as a tool for gauging feed efficiency and predicting anticipated feed consumption.

Under the RFI framework, it's posited that feed intake can be bifurcated into two segments: one segment pertains directly to basal energy necessities and production results, such as growth or milk production. The second segment is the residual component (Koch et al., 1963). RFI is delineated as the divergence between an animal's actual dry matter intake (DMI) and its expected DMI, needed for both maintenance and growth. This expected intake is determined through a regression equation factoring in metabolic body weight (BW) and ADG (Elolimy et al., 2018). Residual feed intake, which showcases moderate heritability, emerges as a potent tool to enhance beef herds' efficiency (Arthur et al., 2001). This is particularly due to its phenotypic correlation with intake, though not with gain. Notably, RFI is not influenced by growth parameters, including BW and ADG (Kennedy et al., 1993; Crews et al., 2005).

Differentiating itself from the FCR, RFI scrutinizes feed intake by breaking it down into two parts: the first corresponds to a specific production threshold, and the second encompasses the remaining feed intake (Montanholi et al., 2010; Santiago et al., 2021; Ewaoluwagbemiga et al., 2023).

Residual average daily gain or residual gain (RADG)

Residual average daily gain (RADG) is a measure of the difference between an animal's actual weight gain and its predicted gain based on its DMI, body weight maintenance, and fat cover (Northcutt 2010). Hence, RADG represents the amount of body weight gain not accounted for by differences in feed intake and mid metabolic test body weight (MMW) (Willems et al., 2013). Although the concept of RADG appears to be similar to RFI as they both contain similar components, the two concepts work in very different ways. Although selection for this trait is likely to produce cattle that grow faster, it is also likely to increase the mature animal size and

therefore increase maintenance requirements, in which case they will require more feed (Crowley et al., 2010).

Residual intake and body weight gain (RIG)

One other evolving measure of feed efficiency is residual intake and growth (RIG). RIG, pioneered for beef cattle by Berry and Crowley (2012), represents a linear combination of Residual Feed Intake (RFI) and residual gain (RG). This linear composition suggests that RIG holds the potential advantages associated with both aspects of feed efficiency. One of the main advantages of RFI and RG is their independence from BW, which was also noted for RIG because this trait had no phenotypic correlation with final BW and MMW (do Nascimento et al., 2020). A similar relationship has been verified in other studies with growing cattle (Berry and Crowley, 2012; Fernandes, 2014; Grion et al., 2014) and lambs (Lima et al., 2017). The improvement in the efficiency of RIG leads to a reduced DMI and increased ADG (Berry and Crowley, 2012; do Nascimento et al., 2016).

RFI traits and limitations

Feed intake, variation, and repeatability

Accurate assessment of feed efficiency requires precise measurements of feed intake and energy utilization. This encompasses parameters such as body weight, growth, and body composition in young cattle as indicated by Arthur et al. (2001) and Basarab et al. (2007, 2011), fat mobilization, milk components (Rius et al., 2012). The variability in feed efficiency can sometimes mask diverse biological realities (Cantalapiedra-Hijar et al., 2018; Martin et al., 2021). A crucial factor regarding feed efficiency is the variation both within and between animals,

particularly in dry matter intake and feed efficiency measures. These rely heavily on average daily feed intakes and coefficients of variation in animals. This variability can arise from differences in digestive or metabolic efficiency. Under specific feeding conditions, different combinations of these efficiencies can result in similar overall efficiency. Although these variations may be insignificant in one environment, they become critical in others. For instance, an organism prioritizing energy for milk production over maintaining reserves might flourish in nutrient-rich environments but falter in nutrient-deficient ones where reserves are vital (Martin et al., 2021).

Basarab (2012) noted that the coefficient of variation (CV) for daily feed intake fluctuated between 11% and 22%, representing daily feed intake differences among animals. Several factors can explain the evolving variation and repeatability of RFI. These include measurement errors in body weight and feed intake, variability in animal responses to compensatory gain, differences in efficiency as animals mature, and changes in diet digestibility due to variations in feeding behavior, rate of passage, and rumen microbial populations (Archer et al., 2002; Carstens & Tedeschi, 2006; Kelly et al., 2010; Durunna et al., 2011). Using a ratio-of-variance approach and assessments over 10-day intervals, researchers found that between-animal repeatability varied widely based on the animal type. They concluded that these levels of repeatability are weak to moderate, with declining estimates when the feeding interval is extended (Kelly et al., 2010; Basarab, 2012). Furthermore, Wang et al. (2006) reported that phenotypic differences in DMI decreased notably from the 7th to the 35th day of a feeding trial. Extending data collection beyond 35 days provided minimal improvement in accuracy.

A commonly observed limitation in RFI models is the use of fixed, short time intervals for RFI calculation. This might not capture the long-term effects on animals throughout their entire productive lifespan. While short-term RFI assessments can indicate improved gain efficiency,

potential drawbacks like adverse impacts on fertility and productive life duration exist (Vallimont et al., 2013; Puillet et al., 2016). As a result, there's a growing emphasis on longer-term assessments for sustainable efficiency (Martin et al., 2021).

Moreover, to accurately measure feed efficiency, animals should be in a nutritional equilibrium state (feed of maintenance). They should receive the same rations ad libitum throughout the feeding or testing period (Thompson, 2016). Maintaining good health is essential to avoid variations in feed consumption that could distort daily intake averages and relative growth rates. Young and growing animals, when unaffected by nutritional or health challenges, should exhibit a linear growth curve (Lui et al., 2011). This curve should remain unaffected by nutritional or health setbacks (Basarab, 2012). Determining each animal's growth curve requires performing a linear regression of weight over time, with measurements recorded periodically (Wang et al., 2006).

RFI calculation

Archer et al. (1997) and Basarab et al. (2003) succinctly described metrics for calculating RFI, considering diverse interwoven factors affecting feed efficiency. Key feed efficiency parameters including; average daily gain, initial body weight, mid-test weight (MWT), and final body weight are established using regression coefficients from the individual animal's growth curve (David et al., 2021). Monitoring feed intake is facilitated by automated feeding systems like the GrowSafe® system. This allows for calculating daily averages for each animal during the testing periods. In each period, DMI is usually calculated as the average daily DMI. ADG and initial BW are obtained from linear regression of BW on the day of the study period. The period mid-BW (mid-test $BW^{0.75}$; MMTW) was calculated as the average of the initial BW and final BW.

Therefore, average daily gain (ADG) and metabolic mid-test BW (mid-test $BW^{0.75}$; MMTW) were regressed against daily DM intake. The RFI equation is generally constructed from the following base: which is the difference between the predicted value from the regression and the actual measured value:

$$\mathbf{DMI\ or\ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \varepsilon,}$$

where Y is the expected DMI (kg/d), β_0 is the regression intercept, β_1 and β_2 are the partial regression coefficients, X_1 is the MMTW (kg), X_2 is the ADG (kg/d), and ε indicates the RFI (kg/d) or residual (Durunna et al., 2011). This residual usually contains the biological variability in intake not explained by the model and any errors in measurement or in the model structure. On the basis of this calculation, animals are classified as either the lowest (negative RFI; feed efficient) or highest (positive RFI; feed inefficient).

Effects of environmental stressors on feed intake and efficiency

Improving feed efficiency enables producers to enhance their net output while reducing both feed costs and environmental impacts (Reynolds et al., 2011; Hill and Wall, 2017). Stress is characterized as a state in an animal that arises from exposure to one or more stressors, whether of external or internal origin (Idowu et al., 2022). Environmental stressors can profoundly affect feed intake and efficiency in livestock, leading to potential challenges in animal production and performance. For example, factors such as high temperatures, humidity, and heat stress have been proven to decrease feed intake and hinder nutrient utilization across various species (Renaudeau et al., 2012; Cheng et al., 2022). Cold stress and exposure to severe weather conditions, on the other hand, can elevate energy demands, further compromising feed efficiency (NRC, 2007). Research also indicates that susceptibility to stress is a significant factor in the biological

differences observed after divergent selection for residual feed intake in beef cattle (Foroutan et al., 2021; Richardson and Herd, 2004). Beef cattle that efficiently convert feed to growth exhibit lower rectal temperatures and produce less metabolic heat (Basarab et al., 2003; Nkrumah et al., 2006; Martello et al., 2016) compared to their less efficient counterparts, suggesting that efficient animals are able to effectively utilize the metabolic heat for ATP generation and other production needs. Furthermore, factors like social stress, transportation, and shifts in housing conditions can adversely affect feeding behavior and nutrient utilization (Lynch et al., 2019; Idowu et al., 2022).

Additionally, environmental stressors can trigger physiological changes, such as altered hormone secretion patterns and reduced rumen functionality, which subsequently lead to diminished appetite and feed intake (Lara and Rostagno, 2013; Bova et al., 2014). Maintaining homeostasis is vital for cattle to achieve and sustain optimal health, indirectly influencing food production. This equilibrium hinges on hormones—powerful compounds secreted by various organs that initiate distinct cellular reactions (Bova et al., 2014). To counteract the detrimental effects of environmental stress on feed intake and animal efficiency, interventions such as ensuring adequate shade, proper ventilation, and effective management strategies become essential (Wheelock et al., 2010). Nutritional strategies, like incorporating feed additives, antioxidants, or prebiotics, can further help counteract the stress-induced decline in feed efficiency (Dikmen et al., 2009; Adeyemi et al., 2019). Recognizing the impact of environmental stressors on feed intake and efficiency is paramount for devising effective management strategies. These strategies aim to enhance livestock performance and welfare, ensuring sustainable animal production systems remain in place (Basarab et al., 2013; Varijakshapanicker et al., 2019; Orihuela et al., 2021).

Influence of diet composition on residual feed intake

A significant factor affecting RFI is the diet composition provided to animals. It's vital to acknowledge that impact of diet composition on RFI can differ depending on an animal's genetic background. Some research points to genotype-by-diet interactions, indicating certain genetic lines might react differently to particular diet types (Herd et al., 2017). The less pronounced variation seen with forage-based diets is expected given their inferior intake properties and their slower transition through the rumen (Forbes, 2005; Kenny et al., 2018). High-forage diets, when compared to concentrate-based ones, may restrict voluntary feed intake, thus diminishing the display of inherent DMI potential and its passage rate through the rumen (Forbes, 2005). The generation of volatile fatty acids (VFA) from microbial fermentation accounts for roughly 75% of a ruminant's energy needs (Bergman, 1990). In terms of total VFA concentration, efficient cattle have recorded higher VFA molar concentrations (Guan et al., 2008). Research has demonstrated that energy-dense diets can influence RFI in cattle (Parson et al., 2021). Diets with high energy density, usually rich in grains and concentrates, have been linked with improved feed efficiency and a lower RFI (Arthur et al., 2001; Nkrumah et al., 2006; Kelly et al., 2010). Individual cattle exhibit variations in their DMI, sometimes deviating from what's expected based on their growth rate or size (Herd and Arthur, 2009). Animals vary in the amounts of manure, methane, and carbon dioxide they produce per DMI unit, and in their ability to generate and retain heat energy (DiGiacomo et al., 2014; Arndt et al., 2015). High-density diets offer more digestible energy, promoting efficient weight gain and less energy waste.

The presence of dietary fiber, from sources like forages and roughage, also affects RFI. In ruminants, fiber-rich diets enhance rumen fermentation, thereby boosting microbial protein synthesis, leading to more effective feed component use and reduced RFI (Hales et al., 2015).

Moreover, diets high in fiber support gut health and sustain a balanced rumen environment, factors crucial for overall feed efficiency (Belanche et al., 2021; Li et al., 2022).

Gut microbiota, and host metabolism and their influence on feed efficiency

Research also indicates that most microbial profiles are consistent among efficient cattle (Hernandez-Sanabria et al., 2012), with specific bacterial species linked to efficient steers (Guan et al., 2008). Further studies have explored how the rumen epithelial structure adapts to varying physiological states (Kern et al., 2016). The rumen microbiome is instrumental in the digestion and fermentation of feed in ruminants, significantly affecting their overall feed efficiency (Matthews et al., 2019; Liu et al., 2021; Sanjorjo et al., 2023). This microbiome consists of bacteria, archaea, fungi, protozoa, and viruses, collaboratively converting feed components into simpler molecules (Huws et al., 2018; Gilbert et al., 2020; Xu et al., 2021). Bacteria, being the most prevalent and varied group within the rumen, predominantly ferment carbohydrates, yielding VFAs – the primary energy source for the host ruminant. Meanwhile, archaea, especially methanogens, produce methane, representing both a loss of energy and a contributor to greenhouse gas emissions (Hook et al., 2010). Fungi assist in decomposing intricate plant constituents like lignocellulose, augmenting the availability of nutrients for microbial fermentation.

Volatile fatty acids are produced through microbial fermentation, serving as pivotal energy sources once absorbed into the bloodstream (Weimer, 2022). The rumen microbiome's efficiency in nutrient metabolism is directly linked to the ruminant's health and productivity (Liu et al., 2021). Effective microbial fermentation in the rumen correlates with enhanced VFA production and improved feed efficiency (Nathani et al., 2015). However, disturbances in the microbial community or dietary alterations can undermine VFA production, leading to diminished feed

efficiency. Notably, a diverse rumen microbiome often signifies better feed efficiency, given its adaptability to dietary changes and optimization of nutrient breakdown. Research indicates that animals with improved feed efficiency typically possess a more diverse and stable rumen microbiome than those less efficient (Welch et al., 2020). Further studies, such as those by Paz et al. (2018), deduced that the rumen microbial composition accounts for around 20% of efficiency of feed utilization in beef steers.

Moreover, high-efficiency beef cattle, both bulls and heifers, display superior digestibility levels across various metrics such as DM, organic matter (OM), and NDF. This emphasizes the paramount importance of ruminal microbes and epithelial tissue in determining divergence in RFI. Phenotypic differences in residual feed intake in dairy cows have also been linked to specific ruminal microbes and metabolic pathways (Shabat et al., 2016). Additionally, variations in steers' feed efficiency measurements correspond to differences in their rumen microbiomes (Myer et al., 2015). Alterations in animal metabolism play a role in feed efficiency differences (Ferrell and Jenkins 1984). Distinct metabolites involved in this process hint at effective nutrient use, delineating more or less feed-efficient animals. Around 55–60% of rumen fluid metabolites have a correlation with the rumen microbiota, as reported by Saleem et al. (2013). The bovine ruminal fluid composition, characterized by an array of compounds from phospholipids to VFAs, predominantly results from microbial fermentation under the anaerobic conditions within the rumen (Clemmons et al., 2020). These metabolites, integral to understanding feed efficiency variations, are influenced by factors that mold rumen microbial diversity (Clemmons et al., 2020).

Association of methane mitigation with RFI

Globally livestock emissions account for 14.5% of human-caused greenhouse gas emissions, with methane (CH4) contributing 44% of these livestock-related emissions (Gerber, 2013). Various options exist to decrease CH4 emissions, including enhancing feed quality, employing CH4 inhibitors, and selectively breeding for reduced CH4 output (Dini et al., 2018). Arthur and Herd (2005) characterized RFI as a moderately heritable trait linked to CH4 emissions as a result, these animals produce fewer emissions than their high-RFI counterparts (Basarab et al., 2013). Notably, low-RFI animals, with an average RFI of -0.78 kg DMI/d, emitted up to 27% less CH4 than high-RFI animals, which had an average RFI of 0.83 kg DMI/d (Dini et al., 2018). This difference in emission could be attributed to the shorter meal durations observed in more efficient animals or reduced consumption of feed.

Interestingly, studies have indicated that animals with higher intake rates often experience quicker passage rates of rumen particles. This phenomenon isn't necessarily connected to reduced digestibility, especially for high-quality diets (Pérez-Ruchel et al., 2013). Recent research has raised doubts about the viability of using RFI as a strategy to curb enteric CH4 emissions (Jones et al., 2011; Dini et al., 2018). Nevertheless, it's vital to note that these studies worked with populations that lacked significant RFI variation. This limited variation could have influenced the results concerning CH4 emissions.

Physiological mechanisms associated with feed efficiency in beef cattle

The physiological mechanisms that contribute to variations in feed efficiency have been associated with complex entities, with no single mechanism potentially able to assume sole responsibility for the associated changes observed in the phenotype of animals (Oddy et al., 1999; Herd and Arthur, 2009; NRC 2000). Richardson and Herd elucidated numerous physiological

mechanisms responsible for the variability seen in RFI among Angus steers that had been distinctly chosen for low and high RFI.

Digestion of feed and nutrient metabolism

A significant portion of the RFI variation, specifically 73%, could be attributed to metabolic heat production, body composition, and physical activity. To some extent, the biological constituents largely controlling RFI traits (inter-animal variations) have been linked to differences in feed intake, digestion, body composition, metabolism, activity, and thermoregulation (Nkrumah et al., 2006; Herd and Arthur, 2009; Bottje and Carstens, 2009). Increasing consumption of feed usually decreases diet digestibility, mainly because of a reduction in ruminal residency time (SCA, 1990; Kenny et al., 2018). Variations in residual feed intake have been reported to be due to 10% differences in digestion in beef cattle (Richardson et al., 1996; Richardson and Herd, 2004). In fact, a recent study in beef cattle has reported that selection for production components (growth) is accompanied by improvement in feed digestion and absorption of dietary nutrients , suggesting that differences in the processes of digestion and substrate availability normally occur at the portal blood, thereby providing a possible mechanism to explain the variation in the efficiency of feed utilization without the need to invoke variation in nutrient utilization *per se* (Cantalapiedra-Hijar et al., 2018).

Also, various factors can influence nutrient metabolism and feed efficiency in ruminants (Liu et al., 2021), including dietary composition, feed quality, age, breed, physiological state, and environmental conditions (Terry et al., 2020). Proper feed management, with attention to dietary formulation and provision, can enhance nutrient metabolism and feed efficiency. Additionally, optimizing rumen health and microbial balance through appropriate feeding practices and management strategies can contribute to improved nutrient utilization and feed efficiency (Diao et

al., 2019; Matthews et al., 2019). Efficient nutrient utilization is critical for maximizing feed efficiency in ruminants (VandeHaar, 2011; McGrath et al., 2018). The efficiency of energy utilization can be influenced by factors such as the rate of passage of digesta through the gastrointestinal tract, nutrient absorption, and energy expenditure on maintenance and production processes. Optimizing nutrient supply through balanced diets that meet the specific requirements of different production stages can enhance feed efficiency while minimizing wastage and environmental pollution. Carbohydrates are the primary energy source in ruminant diets, and their metabolism is pivotal for feed efficiency. Ruminants have the ability to ferment complex carbohydrates, such as cellulose and hemicellulose, through symbiotic microorganisms in the rumen.

Dietary proteins are broken down into amino acids through microbial fermentation and proteolytic enzymes in the rumen. The resulting amino acids are utilized by the rumen microorganisms for their growth and protein synthesis. Some amino acids escape rumen degradation and are absorbed in the small intestine, contributing to the animal's protein needs. Balancing dietary protein sources and providing essential amino acids are critical for maximizing protein utilization and feed efficiency in ruminants. Differences in RFI have been suggested to be partially due to variations in the cell-level extent of protein turnover of animals (Nkrumah et al., 2006), and the capacity to generate optimal proton gradients across mitochondrial membranes to maximize the efficiency of ATP production (Blaxter, 1989; Bottje and Carstens, 2009). Lipid metabolism is crucial for ruminants as lipids serve as a concentrated source of energy and essential fatty acids. Rumen microorganisms can efficiently hydrogenate unsaturated fatty acids, impacting the fatty acid composition of the rumen and, subsequently, the animal's tissues and milk. Dietary lipid supplementation can influence rumen fermentation and nutrient absorption, affecting feed

efficiency in ruminants. However, excessive lipid supplementation can also disrupt rumen function and impair feed efficiency.

Heat increment of feeding (HIF)

Variation in feed intake is also associated with the differences in the maintenance requirements of ruminants (Herd et al., 2000). Much of these metabolic events in ruminants are largely related to energy transactions, exclusively describing how feed with various compositions may deliver diverse amounts of energy relative to differences in digestion, nutrient absorption, and metabolism (Herd et al., 2004). The amount of energy expended to digest the feed increases concurrently as the feed intake increases owing to changes in the size of digestive organs (Celi et al., 2017). Tissue energy expenditure also increases per unit of weight of the animals (Johnson et al., 1990; Caton et al., 2000) and these are associated with heat increment of feeding (HIF). Forty percent (40%) of the total HIF is associated with gut tissue metabolism, and the remainder are due to elevated metabolism in peripheral tissues (Webster et al., 1975).

Body composition and hormonal influence

An animal's body composition plays a significant role in determining their energy requirements. The energy costs of accumulating protein and lipid tissue differ, with 1.24 kcal g-1 for protein and 9.39 kcal g-1 for lipid tissue (Carstens and Kerley, 2009). Consequently, the variation in fat and lean gain in beef cattle can influence nutrient utilization efficiency. Leaner animals are more likely to exhibit lower RFI (Lancaster et al., 2009). In the context of RFI, the genetic correlation between body composition (chemical composition) and RFI is noteworthy. Richardson et al. (2001) demonstrated that beef steers selected for divergent RFI showed a genetic connection to their body composition.

Also, differences in body chemical composition (metabolites) have been linked to hormonal regulation and skeletal development in steers divergently selected for RFI (Richardson et al., 2004; Lawrence et al., 2011). Nutritional status and body energy reserves are important to the hypothalamic-hypophysis-gonadal axis integrity in cattle (Schilloz et al., 1992; Bova et al., 2014). Hormones including insulin, leptin, glucagon-like peptide-1 (GLP-1), cholecystokinin, and peptide produced via interactions between the gut-hypothalamic axis aid in regulating feed intake (Air et al., 2002; Harada and Inagaki, 2022; Chaudhri et al., 2008). Broadly, leptin has been implicated in mediating multiple physiological functions in the bovine (Chelikani et al., 2003), alongside its control of feed intake by regulating the synthesis and release of orexigenic (neuropeptide Y) and anorexigenic (corticotrophin-releasing hormone) neuropeptides in the hypothalamus (Houseknecht et al., 1998; Ingvartsen and Andersen, 2000). Specifically, leptin concentration was observed to be associated with greater fatness in less efficient beef steers (Minton et al., 1998).

Creatinine which is a predictor of muscle development was reported as negatively associated with inefficient beef steers and possibly suggesting greater muscle mass of the efficient steers (Richardson et al., 2004). Cortisol initiates a variety of bodily reactions, including the release of energy through glycogen, muscle, and adipose tissue breakdown; synthesis of acute-phase proteins to address inflammation; heightened levels of catecholamines; and immune system suppression to prevent autoimmune responses (Cooke, 2017; Gouvêa et al., 2022). Richardson et al. (2004) and Gomes et al. (2017) have documented reduced blood cortisol levels in low- in comparison to high-RFI beef cattle. A similar outcome was noted in crossbred rams after an ACTH challenge (Knott et al., 2008), suggesting that efficient steers have better-coping mechanisms that

shut down the activation of the HPA axis and manage the stressors to the optimal condition for improved growth, health, and performance.

Oxygen transport and blood flow

Tissue oxygenation is a key activity of any organism (Brahimi-Horn and Pouysségur, 2007; Pittman, 2013). Maximization of the efficiency of oxygen utilization has become a subject of relevance. Earlier studies exploring the biochemical variation in feed efficiency traits have reported that around 27% of the difference in residual feed intake was attributed to variations in other processes, such as ion transport (Richardson and Herd, 2004; Kerley et al., 2010). In fact, some studies have demonstrated that feed-efficient cattle show lower blood hemoglobin and hematocrit (Hudson, 2009; Chaves et al., 2015). In line with this finding, a recent study using transcriptomics approaches has reported that the biological functions of proteins evolving heme/iron binding, oxygen binding, and oxygen transporter also contribute to the variations observed in the phenotypic efficiency of beef cattle

Mitochondrial function

The synthesis of adenosine triphosphate by the mitochondria was shown to be correlated to the efficiency of beef cattle (Blaxter, 1989). Mitochondrial conversion of energy as NADH and FADH to ATP is an important contributor to energy supply accounting for approximately 20-30% of resting energy requirements (Zurlo et al., 1990). Therefore, changes in mitochondrial efficiency will have large impacts on energetic and therefore feed efficiency (Bottje and Carstens, 2009). Similarly, other researchers have reported that differences in the energetic efficiency of

mitochondria are associated with phenotypic differences in feed efficiency (Kolath et al., 2006; Herd and Arthur, 2009; Lancaster et al., 2014). Most of these studies focused principally on assessing the mitochondrial function via differences in the mitochondrial number, citrate synthase activity, mitochondrial respiratory control ratio (an indication of the efficiency of electron transfer), and measurement of specific activity of the complexes of the electron transport system (Acetoze et al., 2015; Casal et al., 2018; Zhao et al., 2019). In fact, a study conducted by Kolath et al., 2006 demonstrated that the degree of efficiency of electron transfer in longissimus muscle tissue of low- is greater relative to high-RFI steers, implying a higher degree of coupling between respiration and oxidative phosphorylation but no increase in production of ROS when expressed as a function of respiration rate. Protons pumping across the inner mitochondrial membrane are used to drive ATP synthesis. Mitochondria, because of their role in oxidative metabolism, are particularly susceptible to ROS damage, which can induce proton leak (Brookes, 2005). Lancaster et al. (2014) studied the bovine hepatic mitochondrial function of beef cattle phenotypically divergent for RFI and reported that RFI status affected indices of mitochondrial proton leakage rates and acceptor control ratio in the mitochondria. Similarly, Nitric oxide has been implicated to be involved in the regulation of mitochondrial respiration especially, when nitric oxide synthase is produced in close proximity to the electron transport chain. This action leads to the disruption of the cellular structure of complexes I and II (Hill et al 2012). Also, the mitochondrial DNA has a tendency for ROS oxidation which subsequently results in mitochondrial dysfunction. The large number of proteins that are nuclear-encoded and imported to mitochondria imply the need for regulatory steps that ensure the coordination of the process of mitochondrial biogenesis (Yambire et al., 2019). In fact, the damage to mitochondrial DNA (lacks protective histones), usually arises from the disruption in the importation of nuclear-encoded proteins into mitochondria. This

disruptive event has been documented to have a limited inclination to encode the subunits of proteins, leading to compromised respiratory capacity due to the malfunctioning of crucial respiratory chain complexes (Hill et al., 2012).

Immunity and stress response

The intricate relationship between feed efficiency and health underscores the concept of immune competence in beef production system. Diseases and inflammation, often triggered by stress, can disrupt the metabolic processes involved in efficient feed utilization, emphasizing the need for a comprehensive approach. Beef cattle, naturally, undergo management-driven situations that induce stress responses, resulting in decreased feed consumption (Marques et al., 2019). These stressors encompass events like weaning, transportation, withholding water and feed, and comingling (Filho et al., 2014; Cooke, 2017). Infections can reduce appetite by means of immune system cytokines (Johnson, 1997), resulting in inadequate energy intake, reduced growth or lactation, and decreased feed efficiency. According to Gautron and Laýe (2010), anorexia linked to inflammation diminishes food intake during both acute and chronic inflammatory states. Interestingly, inflammatory stimulations, when in optimal production, benefit the host animals, however, an emergence of immunological imbalance disrupts the intestinal integrity thereby causing tissue damage with consequent impairment in the overall health and performance of cattle (Gu et al., 2012; Zhou et al., 2017; Wu et al., 2022). Most of these earlier studies are centered on cattle experiencing stress-induced inflammation (Ingvartsen and Andersen, 2000). There is a consensus that during any event that results in inflammatory responses, dietary nutrients are partitioned towards the immune-related processes rather than being used for growth and thus reduces animal feed efficiency (Johnson, 1997; Spurlock, 1997; Patience et al., 2015). Recent studies reported that high-feed efficient (low-RFI) animals have a more efficient systemic and

hepatic immune response to fight off inflammation promptly, leading to less energy consumption for combating the inflammatory insults and therefore more energy is available for growth and protein accretion in beef cattle (Alexandre et al., 2015; Paradis et al., 2015).

Activity

Feeding behavior and activities are governed by both physical and biological mechanisms (Allen, 2014; Fitzsimons et al., 2017; Parsons et al., 2021). Previous research found that variation in RFI was associated with distinctive differences in feeding behavior (Allen, 2014; Scanes and Hill, 2017). Kenny et al. (2018) reported that divergent RFI status observed in cattle might partly be due to the diet type offered to the animals. Most earlier studies in beef cattle consuming high concentrate-based diets revealed that feeding behavioral entities including meal frequency, daily feeding duration, feeding rate, meal size, and meal duration were associated with divergent RFI phenotypes (Lancaster et al., 2009; Montanholi et al., 2010). In a recent study, evidence indicates that day-to-day variation in feeding behavior patterns of beef steers consuming a concentrate diet could be useful biomarkers for the prediction of feed efficiency (Parsons et al., 2021). Richardson et al. (2000) reported a phenotypic correlation ($r = 0.32$) between RFI and the daily pedometer count. Similarly, Arthur et al. (2001) conducted a comparable study that revealed that high-RFI steers, on average, took 6 percent more steps than their low-RFI counterparts. It was also noted that high-RFI steers spent approximately 13 percent more time in the feeding stall and engaged in more rumination event. The increased walking distance and the additional time spent standing and ruminating accounted for about 5 percent of the higher feed energy intake observed in the high RFI (low feed efficiency) group when compared to the low RFI group.

Despite these research findings, limited information exists on rations differing in energy content especially a high-forage diet relative to the feeding behavior of cattle kept in confinement

as a predictor of feed efficiency. For instance, some studies reported no relationship between bunk visit duration and residual feed intake phenotype when fed a high-forage diet (Kelly et al., 2010; Basarab et al., 2011; Olson et al., 2020), revealing how the type of diet offered may or may not possibly initiate variations in bunk visit events as associated with residual feed intake phenotypes. Also, studies in sheep and swine with divergent RFI phenotypes, have reported a lack of consistency in feeding behavior metrics such as bunk visit events (frequency and duration) (Cammack et al., 2005; Young et al., 2011). Therefore, to select animals with superior RFI phenotypes, understanding the feeding behavior metrics within feed efficiency phenotypes becomes important.

Omics approach to unravel complex interactions associated with feed efficiency

In recent years, omics technologies have emerged as powerful tools for uncovering molecular patterns associated with feed efficiency in animals. As an increasing number of omics-based intermediate traits become available, integrating multi-omics data offers the potential for deeper insights into the genetic bases of complex traits (Weber et al., 2016; Fonseca et al., 2019).

Genomic Approaches

Genetic regulation significantly influences feed efficiency in ruminants by affecting various physiological and metabolic processes tied to nutrient utilization. By deciphering the genetic foundation of feed efficiency, researchers and livestock producers can craft targeted breeding strategies, bolstering feed efficiency and advocating for sustainable livestock production. Recognizing genetic determinants of feed efficiency can pave the way for more effective livestock production systems. Estimating feed efficiency's heritability is essential for gauging the degree of genetic influence on the trait. Defined as a statistical metric, heritability quantifies the fraction of

phenotypic variation in a trait resulting from genetic variation. A host of studies have recorded moderate to high heritability values for feed efficiency in ruminants, implying a significant genetic influence on this variation (Madilindi et al., 2022; Cavani et al., 2022). These estimates lay the groundwork for considering feed efficiency as a selection criterion in breeding programs, enhancing the prospects for future generations (Xu et al., 2017).

In the same vein, pinpointing specific candidate genes and metabolic pathways crucial to feed efficiency can shed light on this trait's genetic regulation. Several candidate genes associated with feed efficiency in ruminants have been identified primarily through candidate gene association studies and functional analyses. These genes typically correspond to nutrient metabolism, hormonal regulation, and growth-centric processes (Han et al., 2021; Casado et al., 2023). For instance, genes tied to glucose and fatty acid metabolism, as well as those linked with leptin and growth hormone pathways, have been implicated in phenotypic expression of feed efficiency traits (Vijayakumar et al., 2011; Kim et al., 2021). Nonetheless, our grasp on candidate genes remains incomplete, necessitating more research to fully appreciate their roles in feed efficiency. Recent strides in genomics and high-throughput sequencing have revolutionized our study of intricate traits like feed efficiency. Genome-wide association studies (GWAS) and genomic selection stand out as robust methodologies for pinpointing genetic variations correlated with feed efficiency in ruminants. Employing GWAS, the genome of a population is scanned to spot regions or distinct genetic markers associated with a specific trait. These techniques have unveiled novel genetic variants and genomic regions associated with feed efficiency, which are invaluable for targeted breeding initiatives. Gene expression and epigenetic regulation add another layer of complexity to the factors influencing feed efficiency in ruminants. Epigenetic alterations, including DNA methylation and histone modifications, can modify gene expression and thus

influence traits related to feed efficiency. Epigenetics revolves around controlling transcription through assorted chemicals added to DNA or histone proteins. This leads to diverse 'epigenomic marks' that alter chromatin's spatial conformation (Tiffon 2018; Loor, 2022). It's crucial to understand the interplay between genetic variation, gene expression, and epigenetic regulation to unpack the intricate genetic architecture underlying feed efficiency (Capp et al., 2021).

Furthermore, genomics studies have unveiled genetic markers and candidate genes linked to feed efficiency across various livestock species (Lam et al., 2021; Li et al., 2021). By identifying more candidate genes and causal DNA variants through GWAS, the precision of genomic selection for intricate traits, such as carcass merit traits, can be improved. Deepening our understanding of the biological interconnections between the genome and phenome might also hone the accuracy of genomic selection (Meuwissen et al., 2013; Zhang et al., 2019). For instance, research on beef cattle has demonstrated associations between particular genetic variants and RFI (Saatchi et al., 2014). These markers can be employed to select animals with superior feed efficiency, allowing breeding programs to optimize herd productivity.

Transcriptomics, Proteomics, and Metabolomics Approaches.

Recent innovations in molecular profiling techniques enable the efficient and cost-effective collection of extensive omics datasets, encompassing transcriptomics, proteomics, and metabolomics (Serin et al., 2016). Transcriptomics entails analyzing gene expression patterns against various physiological conditions, including feed efficiency. Such studies have spotlighted differentially expressed genes related to metabolism, energy consumption, and immune responses in animals displaying distinct feed efficiency phenotypes (Xiang et al., 2020). Gleaning these molecular changes can offer invaluable insights into the biological pathways underpinning feed

efficiency, guiding nutritional strategies to maximize animal performance. Proteomic investigations facilitate the identification and quantification of proteins in animals with different feed efficiency levels (Baldassini et al., 2018). They have unveiled variations in the abundance and function of proteins linked to nutrient absorption, energy metabolism, and stress response in animals with high and low feed efficiency (Huang et al., 2019). Understanding these proteomic patterns can help in formulating targeted nutritional strategies that bolster feed efficiency and minimize waste. Interestingly, such omics techniques have been employed to study protein expression shifts in the liver and muscles, shedding light on the related regulatory mechanisms in animals (Kuhla et al., 2013). Some investigations have combined two-dimensional gel electrophoresis (2D-PAGE) and electrospray ionization mass spectrometry (ESI-MS) to achieve this (Shevchenko et al., 2006; Bandow et al., 2008).

Metabolomics, meanwhile, focuses on the comprehensive profiling of small-molecule metabolites in biological samples (Lin et al., 2016). By analyzing metabolomic variations, researchers can grasp metabolic shifts between animals with different feed efficiency levels (Beckonert et al., 2007). Notably, studies have uncovered distinct metabolite profiles in high and low feed efficiency animals, highlighting differences in energy metabolism, amino acid catabolism, and lipid biosynthesis pathways (Staerfl et al., 2012; Ramayo-Caldas et al., 2016). Such findings can help tailor nutritional interventions to boost feed efficiency.

Harnessing the synergy of multi-omics datasets can provide a panoramic view of underlying molecular intricacies of feed efficiency in ruminants. As our understanding of these biological systems deepens, livestock producers will be better equipped to enhance feed efficiency in their herds, fostering more sustainable animal production systems. Intensive research and

technological advancements in omics technologies promise to usher in a new era of precision livestock farming, positioning it at the forefront of sustainability and productivity.

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Chapter 2

Chapter 2. Chemical group-based metabolome analysis identifies candidate plasma biomarkers associated with residual feed intake in beef steers

Abstract

Objective: We applied chemical group-based metabolomics to identify blood metabolic signatures associated with residual feed intake in beef cattle.

Methods: A group of 56 crossbred growing beef steers (average BW = 261.3 ± 18.5 kg) were adapted to a high-forage total mixed ration in a confinement dry lot equipped with GrowSafe intake nodes for period of 49 d to determine their residual feed intake classification (RFI). After RFI determination, weekly blood samples were collected three times from beef steers with the lowest RFI (most efficient (HFE); n = 8) and highest RFI and least efficient (least efficient (LFE); n = 8). Plasma was prepared by centrifugation and composited for each steer. Metabolome analysis was conducted using a chemical isotope labeling (CIL)/ liquid chromatography–mass spectrometry,

which permitted the analysis of metabolites containing amine/phenol, carboxylic acid-, and carbonyl-chemical groups, which are metabolites associated with metabolisms of amino acids, fatty acids, and carbohydrates, respectively.

Results: A total number of 495 amine/phenol-containing metabolites were detected and identified; pathway analysis of all these metabolites showed that arginine biosynthesis and histidine metabolism were enriched ($P < 0.10$) in HFE, relative to LFE steers. Biomarker analyses of the amine/phenol-metabolites identified methionine, 5-aminopentanoic acid, 2-aminohexanedioic acid, and 4-chlorolysine as candidate biomarkers of RFI (false discovery rate ≤ 0.05; Area Under the Curve (AUC) > 0.90). A total of 118 and 330 metabolites containing carbonyl- and carboxylic acid-chemical groups, respectively, were detected and identified; no metabolic pathways associated with these metabolites were altered and only one candidate biomarker (methionine sulfoxide) was identified.

Conclusions: These results identified four candidate metabolite biomarkers of RFI in beef cattle which are mostly associated with amino acid metabolism. Further validation using a larger cohort of beef cattle of different genetic pedigrees is required to confirm these findings.

Introduction

Due to rising feed costs, the efficiency of feed nutrient use for better growth performance continues to be of significant interest (Holmgren and Feuz, 2015). Residual feed intake (RFI), a measure of feed efficiency in beef cattle, is known to be moderately heritable (Koch et al., 1963; Herd et al., 2004) and has been improved over the years via genetic selection (Arthur et al. 2001). However, factors other than genetic factors, including differences in host metabolism and gut microbiome contribute to variation in RFI (Herd and Arthur, 2009; Myer et al. 2017).

Consequently, several studies have focused on understanding the physiological mechanisms that cause differences in RFI, the difference between an animal's actual intake versus its predicted feed intake based on average daily gain (ADG) and metabolic body weight (Koch et al., 1963; Herd and Arthur, 2009). Animals with low (negative) RFI values consume less feed than expected and are feed efficient whereas animals with high (positive) RFI values consume more feed than expected and are feed inefficient (Koch et al., 1963).

In recent years, the advent of metabolomics has provided an opportunity to comprehensively analyze multiple metabolites in biological samples. Indeed, several studies have applied metabolomics to provide insight into the metabolic status of animals with varying RFI with a twin goal of identifying blood metabolic signatures that could be used as predictive biomarkers due to the high cost associated with the direct measurement of RFI in animals (Goldansaz et al., 2020). Studies that have attempted to identify candidate biomarkers of RFI mostly applied analytical tools, such as nuclear magnetic resonance and liquid chromatography–mass spectrometry (LC-MS) (Connolly et al., 2019; Goldansaz et al., 2020). However, due to the limited sensitivity and/or quantitative accuracy of these methods, only a small number of high-abundance metabolites can be analyzed (Pan and Raftery, 2007; Imperlini et al., 2016). The chemical isotope labeling (CIL) LC-MS is a metabolomics technique that provides a new opportunity to perform chemical-group-based metabolome profiling (Zhao and Li, 2020). This method can detect thousands of metabolites based on their chemical groups (such as amine/phenol, carbonyl, and carboxylic acid chemical groups) in biological samples thereby allowing a holistic view of the metabolome with highly accurate metabolite quantification (Zhao et al., 2019).

Metabolites containing amine/phenol, carbonyl, and carboxylic acid chemical groups are common intermediates and/or end products of metabolisms of amino acid, carbohydrate (such as

glucose), and fatty acid, respectively. Due to the functional roles of amino acids, carbohydrate, and fatty acids and their associated metabolic pathways on animal health and productivity, we hypothesized that metabolites related to their metabolisms could serve as candidate biomarkers of RFI. Therefore, the objective of this study was to analyze the plasma amine/phenol-, carbonyl- and carboxylic acid-metabolome of crossbred beef steers divergent for high and low RFI to identify blood metabolic signatures that could serve as candidate biomarkers for divergent RFI in beef cattle.

Materials and Methods

The research procedures were approved by the Institutional Animal Care and Use Committees of West Virginia University (protocol number 1608003693). A group of 56 crossbred growing beef steers (average BW = 261.3 ± 18.5 kg) were adapted to a high-forage total mixed ration (TMR; primarily consisting of corn silage; ground hay; and a ration balancing supplement; CP = 13.2%, NDF = 45.9% NDF, and NE_g = 0.93 Mcal/kg) in a confinement dry lot equipped with GrowSafe intake nodes. The dry lot was comprised of 5 pens of 1500 m^2 (with 312 m^2 under roof), each served by 6 GrowSafe 8000 (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) feeding nodes. Steers were assigned to the pens at random and identified with a passive, half-duplex, transponder ear tag (Allflex USA Inc., Dallas–Fort Worth, TX) before entry into the test facility. Specifically, the steers were allowed to adjust to the feeding facilities for 15 days before the start of the trial. After the adjustment period, individual feed intake was measured over 49 days. Daily BW for each animal were regressed on time using simple linear regression to calculate beginning BW, mid-test BW, and average daily gain (ADG). Animal ADG and metabolic mid-test BW (mid-test $BW^{0.75}$) were regressed against individual average daily intake, and RFI was calculated as the residual or the difference between the predicted value of the regression and the

actual measured value. After the RFI values were available, all animals were ranked by RFI coefficients. Based on the RFI coefficients, the most-efficient with the lowest RFI (HFE; n = 8) and the least-efficient with the highest RFI (LFE; n = 8) beef steers were selected and kept on the same diet for additional 21 days (designated in this study as d 50 – 70).

Blood samples from HFE and LFE steers were collected from the coccygeal vessels before the morning feeding on d 56, 63, and 70 into 10-mL vacutainer tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Immediately after collection, the blood samples were placed on ice, and thereafter centrifuged at 1,500 × g for 20 min at 4°C to harvest the plasma. The plasma samples were then frozen at −80°C until later analysis.

Sample preparation for metabolome analysis

The plasma samples collected on d 56, 63, and 70 were composited for each steer. Metabolites from the composited samples were first extracted using methanol-protein precipitation method as previously described by Zhao et al. (2019). The extracts were then re-dissolved in 200 μL water and stored at -80°C until metabolome analysis was performed.

CIL/LC-MS-based metabolomics analysis

In-depth untargeted metabolome profiling of the extracted plasma was done using a CIL/LC-MS-based technique. The technique uses a differential ^{12}C-/^{13}C-isotope labeling to derivatize metabolites based on their chemical groups (amines/phenols, carboxylic acids, and carbonyls) (Zhao et al., 2019). Detailed description of the sample analysis including metabolite labelling, sample normalization using LC–ultraviolet quantification of the labeled metabolites, and LC-MS operating conditions and set-up have been described in a previous study (Zhao et al., 2019). Relative quantification of the ^{12}C-/^{13}C-labeled metabolites based on peak ratio values was analyzed using a Bruker Compact quadrupole time-of-flight MS (Bruker, Billerica, MA) linked to

an UltiMate 3000 ultra-high-performance LC system (Thermo Scientific, MA). For each plasma sample, a total number of 16 LC-MS data files were generated (8 HFE samples and 8 LFE samples).

Metabolite data processing and identification

All 16 raw LC-MS data files were processed using IsoMS Pro 1.0 using the procedures described by Mung and Li (2017). Briefly, the ^{12}C-/^{13}C-peak pairs were extracted from each run by the IsoMS software. In this step, the redundant pairs (those of adduct ions and dimers) and noise signal (having a singlet peak) were filtered out, only retaining a protonated ion of a peak pair for one true metabolite and then, the peak intensity ratio was calculated for each peak pair. The IsoMS-Quant program was then used to determine the chromatographic peak ratio of each peak pair and to generate the final metabolite-intensity table (Huan and Li, 2015). Metabolite identification was done using a two-tier identification approach. In tier 1, peak pairs from metabolite-intensity tables were searched against a chemical isotope-labeled (CIL) metabolite library based on accurate mass and retention time (RT) (Huan and Li, 2015). This CIL library contains 1060 unique endogenous metabolites including 711 amines/phenols, 187 carboxylic acids, 85 hydroxyls, and 77 carbonyls. In tier 2, linked identity (LI) library was used for the identification of the remaining peak pairs based on accurate mass and predicted RT information. The LI Library contains over 2000 metabolic-pathway-related metabolites extracted from the KEGG database (Li et al., 2013)

Statistical analysis

Metabolite intensity values of each chemical group (amine/phenol, carbonyl, hydroxyl, and carboxylic acid) were separately imported MetaboAnalyst 5.0 software (https:

//www.metaboanalyst.ca/) for statistical analysis (Chong et al., 2019). Prior to statistical testing, log-transformation, normalization by median, and autoscaling of the data were performed. Median normalization was performed with the aim of eliminating undesirable inter-sample variations, and to ensure individual samples were truly comparable to one another. Auto-scaling was applied to make metabolites more comparable to each other in magnitude. Partial least squares discriminant analysis (PLS-DA) scores plot was generated to visualize the metabolome difference between treatments. Volcano plot analysis was performed to identify those metabolites that differed (false discovery rate (FDR) ≤ 0.05) between LFE and HFE steers. The utility of the metabolites with FDR ≤ 0.05 to serve as potential biomarkers of RFI was further tested using a receiver operating characteristic (ROC) curves as calculated by the ROCCET web server (Xia et al., 2013). Area under the curve (AUC), a value that combines sensitivity and specificity for a diagnostic test was used (Xia et al., 2013). Metabolites having AUC > 0.90 were chosen as potential biomarkers associated with RFI (Xia et al., 2013). Pathway analysis of all metabolites was also performed with a *Bos taurus* KEGG pathway library using global test for enrichment method and relative-betweeness centrality for topology analysis, to determine altered nutrient pathways between the two groups of animals.

Results and Discussion

Amine/phenol-metabolome associated with divergent RFI

A total number of 495 amine/phenol-containing metabolites were detected and identified (https://www.frontiersin.org/articles/10.3389/fanim.2021.783314/full). The PLS-DA plot showed clear separation between the two groups of steers (Figure 2.1) indicating that plasma amine/phenol-metabolome of the beef steers is associated with selection for RFI. A total of 42

differentially abundant (FDR ≤ 0.05) metabolites were detected between HFE and LFE steers (Figure 2.2). Plasma concentrations of 21 metabolites, including isomers of 4-chlorolysine, citrulline, ornithine, arginine, histamine, taurine, carnosine were greater (FDR ≤ 0.05) in HFE steers while 21 metabolites, including 3 isomers of 5-aminopentanoic acid, methionine, prolyl-methionine, and 2-aminohexanedioic acid were greater (FDR ≤ 0.05) in LFE steers (Table 2.2). The results of the ROC analysis revealed that four metabolites (methionine, 5-aminopentanoic acid, 2-aminohexanedioic acid, and 4-chlorolysine) with respective AUC values of 0.969, 0.906, 0.953, and 0.938 had sufficient specificity and sensitivity to qualify as candidate biomarkers of divergent high and low RFI values (Figure 2.3). The box plots showing the distributions of these candidate biomarkers in LFE and HFE steers are shown in Figure 2.4. Results of the pathway analysis of all metabolites showed that arginine biosynthesis and histidine metabolism were enriched ($P < 0.10$) in HFE, relative to LFE steers (Figure 2. 5).

Carbonyl-metabolome associated with divergent RFI

A total of 118 carbonyl-containing metabolites were detected and identified (https://www.frontiersin.org/articles/10.3389/fanim.2021.783314/full). The PLSDA score plot showed a slight overlap, indicating little or no alterations in the carbonyl-metabolome of both groups (Figure 2.6a). A total of 5 differentially abundant (FDR ≤ 0.05) metabolites were detected (Figure 2.6b). Plasma concentrations of two metabolites (ethyl acetoacetic acid and 7-oxoheptanoic acid) were greater (FDR ≤ 0.05) in HFE whereas three metabolites (2-hydroxymethyl-4-oxobutanoic acid, glycolaldehyde, and koeniginequinone B were greater (FDR ≤ 0.05) in LFE steers (data not shown). All the differentially abundant metabolites had AUC values less than 0.9 indicating that none of them had sufficient specificity and sensitivity to qualify as candidate biomarkers of divergent high and low RFI values. Pathway analysis of all the carbonyl-

metabolites revealed no altered ($P > 0.10$) metabolic pathway (https://www.frontiersin.org/articles/10.3389/fanim.2021.783314/full).

Carboxylic acid-metabolome associated with divergent RFI

A total of 330 carboxyl-containing metabolites were detected and identified (https://www.frontiersin.org/articles/10.3389/fanim.2021.783314/full). The PLSDA score plot showed a slight overlap, indicating slight alterations in the carboxyl-metabolome between HFE and LFE steers (Figure 2.7a). A total of 5 differentially abundant (FDR ≤ 0.05) metabolites were detected. (Figure 2.7b). Plasma concentrations of four metabolites (5-carboxy-alpha-chromanol, ureidoacrylic acid, 6-hydroxynicotinic acid, N-acetyl-L-proline) were greater (FDR ≤ 0.05) in HFE whereas only one metabolite (methionine sulfoxide) was greater (FDR ≤ 0.05) in LFE steers. The results of the ROC analysis revealed that only methionine sulfoxide with AUC value of 0.938 had sufficient specificity and sensitivity to qualify as candidate biomarker of divergent high and low RFI values (Figure 2.8). Pathway analysis of all the carboxylic acid-metabolites revealed no altered ($P > 0.10$) metabolic pathway (See link above).

Metabolites containing amine/phenol chemical group are common intermediate and/or end products of amino acid metabolism (Zhao et al., 2019). Amino acid metabolism contributes largely to the productivity of farm animals due to its functional roles in various biochemical and metabolic processes in the cells of animals including growth, production, and reproduction. Altered plasma amine/phenol-metabolome of the beef steers is a testament to the significance of amino acid metabolism to productivity and feed efficiency of beef steers. In this study, four amine/phenol-containing metabolites were identified as candidate biomarkers to classify beef steers into high and low-RFI groups: methionine, 5-aminopentanoic acid, 6-aminohexanoic acid, 4-chlorolysine,

and 7-cyano-7-carbaguanine. Relative concentration of plasma methionine was lower in HFE, relative to LFE steers. Methionine is known to be the first limiting amino acid in growing beef cattle when microbial protein is the only source of amino acids (Richardson and Hatfield, 1978), and its deficiency in diet has been reported to be associated with poor growth performance in growing beef cattle (Ragland-Gray et al., 1997). In addition to the role of methionine in tissue protein synthesis, methionine can serve as a precursor for synthesis of other amino acids such as taurine, cysteine, apolipoprotein, and can donate its methyl groups, via S-adenosyl methionine, for synthesis of choline, carnitine, creatine, and phospholipids which are all essential for improved skeletal muscle and hepatic lipid metabolism for energy supply especially during reduced supply of glucose. In this study, lower plasma level of methionine in HFE steers was accompanied with higher plasma levels of taurine and creatine, which are known to regulate lipid metabolism (da Silva et al., 2014; Ibrahim et al., 2019). In a similar study, Karisa et al. 2014 reported that plasma concentration of creatine was associated with RFI. In the same study, creatine was reported to interact with AMP activated protein kinase which is known to stimulate hepatic and skeletal muscle fatty acid oxidation. When there is an insufficient glucose supply due to low DMI, as observed in HFE steers, to meet energy demands for growth and other physiological processes, there is normally an increased hepatic lipid catabolism to generate acetyl-CoA, which can enter the citric acid cycle to generate energy in the form of ATP and/or be converted to oxidative fuels including ketones (Rui, 2014). In a previous study, Mukiibi et al., 2018 reported upregulation of hepatic genes responsible for lipid secretion, transport and efux in more-efficient beef cattle compared to less-efficient ones. In dairy cattle, Salleh et al., 2018 reported that RFI status is associated with regulation of energy via hepatic lipid metabolism. Considering these facts, we speculate that reduced plasma concentration of methionine in HFE steers was probably due to its

increased uptake by the hepatic cells to synthesize other metabolites that aid lipid beta-oxidation to compensate for reduced energy supply due to low DMI.

4-chlorolysine, a derivative of lysine, was identified as a candidate biomarker of RFI in this study. Lysine plays a significant role in tissue protein synthesis and energy metabolism (Tomé, and Bos, 2007). Plasma lysine concentration has been reported to be associated with RFI in two previous studies in beef heifer and steer (Karisa et al., 2014; Jorge-Smeding et al., 2019), although no AUC values were provided in both studies. In our study, lower level of 4-chlorolysine in HFE steers also corresponded to higher levels of 5-aminopentanoic acid and 2-aminohexanedioic acid, which were also identified as candidate biomarkers in this study. 5-aminopentanoic acid and 2-aminohexanedioic acid are intermediate products of lysine degradation (Guidetti and Schwarcz, 2003). In fact, in a recent study, 2-aminohexanedioic acid, also known as aminoadipic acid, has been previously identified as a candidate serum metabolite biomarker of RFI in sheep (Goldansaz et al., 2020). A study in rat revealed induced cell death and reduced tissue protein synthesis with *in vitro* supplementation of aminoadipic acid (Nishimura et al., 2000). Thus, a high level of 4-chlorolysine and low levels of 2-aminohexanedioic acid and 5-aminopentanoic acid would be expected to result in increased tissue protein synthesis in HFE steers.

Two amino acid metabolism-related pathways, histidine metabolism and arginine biosynthesis, were enriched in HFE steers, relative to LFE. Histidine metabolism results in production of several metabolites including glutamate, histamine, and carnosine, all of which were increased in HFE steers. Glutamate promotes neural functioning, cell proliferation, and the production of other amino acids (Wunschiers 2012; Holecek 2020). Histamine can function as a homeostatic neurotransmitter while carnosine exhibits anti-inflammatory and cytoprotective effects by scavenging free radicals and reducing protein glycation mostly in skeletal muscle

(Mendelson 2008; Wunschiers 2012; Holecek 2020). Arginine biosynthesis pathway synthesizes arginine and several intermediate products including citrulline and ornithine, all of which were increased in HFE steers. Arginine helps in the control of normal cell division, wound healing, and removal of ammonia via the urea cycle (Rhoads and Wu, 2009; Wunschiers 2012). Enrichment of these aforementioned pathways and their associated metabolites in HFE steers is evidence of better health and immune status, relative to LFE steers. Our results concur with prior investigations that have attempted to associate blood metabolites with RFI in cattle; several of these studies have reported an association of a good number of amino acid metabolism-related metabolites including creatine, tyrosine, glycine, glutamine, ornithine, aspartate, lysine, and valine with RFI in beef cattle, although no AUC values for these metabolites were reported (Karisa et al., 2014; Clemmons et al., 2017, Jorge-Smeding et al., 2019).

Several studies that analyzed the hepatic transcriptome of beef cattle with divergent RFI reported altered expressions of genes related to lipid and carbohydrate metabolisms (Mukiibi et al., 2018; Higgins et al., 2019), indicating possibility of differences in their hepatic metabolisms which would be expected to lead to alterations in blood concentrations of their metabolites. In our study, none of the plasma metabolites related to carbohydrate and fatty acid metabolisms (carbonyl- and carboxylic acid-metabolome), except methionine sulfoxide, which is an oxidized form of methionine, qualified as candidate biomarkers in this study. In fact, very few related metabolites were differentially abundant, and no associated metabolic pathways were different between HFE and LFE steers. These results were unexpected given the significant roles of carbohydrate and fatty acid metabolisms to the health and productivity of ruminants. In fact, blood glucose, an important carbonyl-containing metabolite, and non-esterified fatty acids (NEFA), which are carboxylic acid-containing metabolites, are often used as markers of health and energy

status in ruminants (Adewuyi et al. 2005; Gleghorn et al. 2004). A possible explanation for the lack of difference may be because glucose is in continuous supply via gluconeogenesis in ruminants (Young, 1977). Increased concentrations of blood NEFA reflect extensive fat mobilization from body reserves due to negative energy balance and fatty acid release from adipocytes most especially during lactation period in high-yielding dairy cows (Bowden 1971). Unlike in dairy cows, there is a little need for an extensive body fat mobilization which is not expected to cause a significant change in blood NEFA (Clemmons et al. 2017). In agreement with our results, two previous studies observed no differences in blood glucose and NEFA in beef cattle divergent for low and high RFI (Bourgon et al., 2017; Clemmons et al. 2017).

Conclusions

The findings of the present study demonstrate differences in the plasma amine/phenol-metabolome of beef steers with divergent high and low RFI values indicating an association between blood amino acid metabolic signatures and RFI divergence in beef steers. Two amino acid metabolic pathways, histidine metabolism and arginine biosynthesis, were found to be associated with RFI. Five candidate metabolite biomarkers of divergent RFI related to the amino acid metabolism pathway (methionine, methionine sulfoxide, 5-aminopentanoic acid, 2-aminohexanedioic acid, and 4-chlorolysine) were identified in this study. Validation studies using a larger cohort of beef cattle of different genetic pedigree are needed to confirm the robustness of the candidate plasma biomarkers identified in this study.

Tables and Figures

Table 2. 1 Performance of beef steers with divergent residual feed intake

	HFE	LFE	SE	*P*-value
RFI	-1.93	2.01	0.32	0.01
Initial BW (kg)	258	258	11.2	0.95
Final BW (kg)	276	278	4.07	0.57
ADG	0.76	0.86	0.17	0.58
DMI	11.9	16.0	0.66	0.01

HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake.

Table 2. 2 Differentially abundant amine/phenol-metabolites in beef steers with divergent residual feed intake

Metabolite	FC (HFE/LFE)	FDR
4-Chloro-L-lysine	1.58	0.01
Threoninyl-hydroxyproline	1.43	0.01
Mesalazine	1.39	0.01
Isomer 1 of 4-Chloro-L-lysine	1.27	0.02
Isomer 2 of 4-chloro-L-lysine	1.24	0.03
Asparaginyl-alanine	1.23	0.02
Carnosine	1.23	0.01
Creatinine	1.21	0.03
Histamine	1.20	0.01
Benzyl salicylic acid	1.19	0.01
Taurine	1.17	0.01
Glutamate	1.16	0.01
Creatine	1.16	0.01
L-alpha-aspartyl-L-hydroxyproline	1.13	0.01
Arginyl-cysteine	1.12	0.04
Imidazoleacetic acid	1.12	0.05
Valyl-glutamate	1.12	0.01
Isomer of N-formimino-L-glutamic acid	1.11	0.05
Citrulline	1.11	0.03
Methylguanidine	1.10	0.04
Ornithine	1.09	0.02
5-Aminopentanoic acid	0.90	0.01
Isomer 1 of 5-aminopentanoic acid	0.93	0.00
Isomer 2 of 5-aminopentanoic acid	0.90	0.01
Methionine	0.89	0.01
Isomer of methionine	0.88	0.02
5-Hydroxylysine	0.88	0.02
4-Guanidinobutanal	0.87	0.01
2-Hydroxy-4-methylbenzaldehyde	0.86	0.05
L-Cysteinylglycine disulfide	0.86	0.02
Azetidinecarboxylic acid	0.86	0.03
Isoleucyl-alanine	0.85	0.01
Glutamyl-glutamine	0.85	0.03
Prolyl-methionine	0.84	0.03
Cystathionine sulfoxide	0.84	0.03
2-Aminohexanedioic acid	0.83	0.00
Glutaminyl-glutamic acid	0.83	0.01
Hydroxyprolyl-cysteine	0.83	0.03

Salsoline-1-carboxylic acid	0.81	0.02
Glutaminyl-methionine	0.80	0.02
Butylparaben	0.79	0.03
Methionyl-glutamic acid	0.79	0.01

HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake.
FC: fold change relative to LFE
Only metabolites with false discovery rate (FDR) ≤ 0.05 are shown.

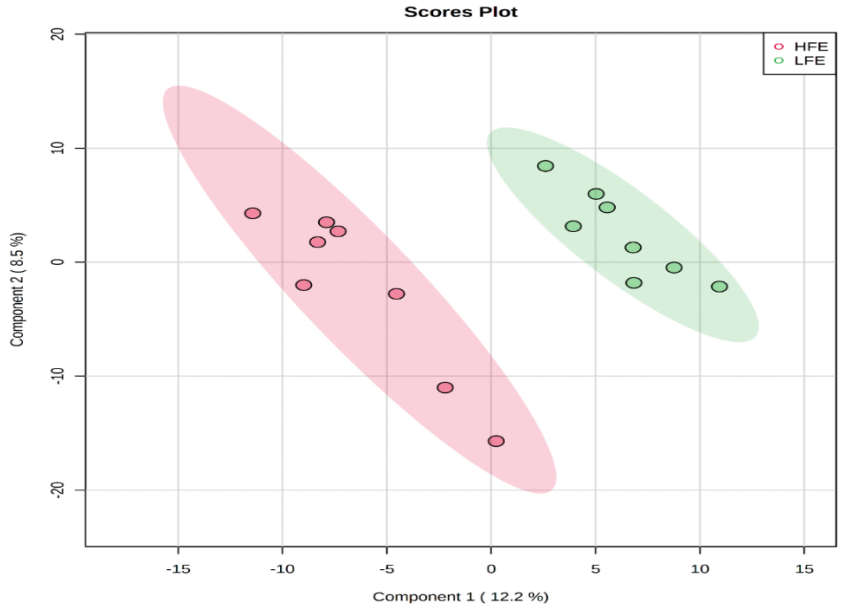


Figure 2. 1 PLS-DA scores plot of amine/phenol-metabolome of LFE and HFE steers.
HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake.

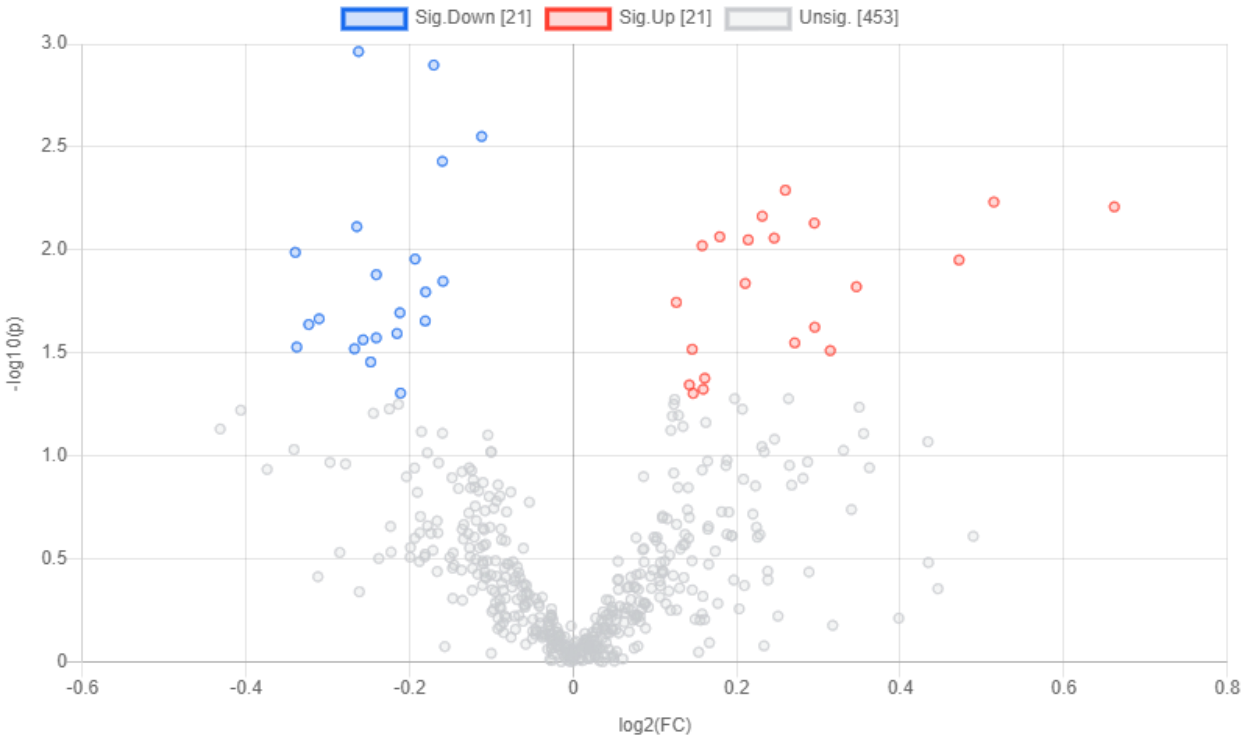


Figure 2. 2 Volcano plot showing the differentially abundant amine/phenol-containing metabolites. Metabolites with false discovery ratio ≤ 0.05 (red or blue) are differentially increased or reduced in HFE, relative to LFE. HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake.

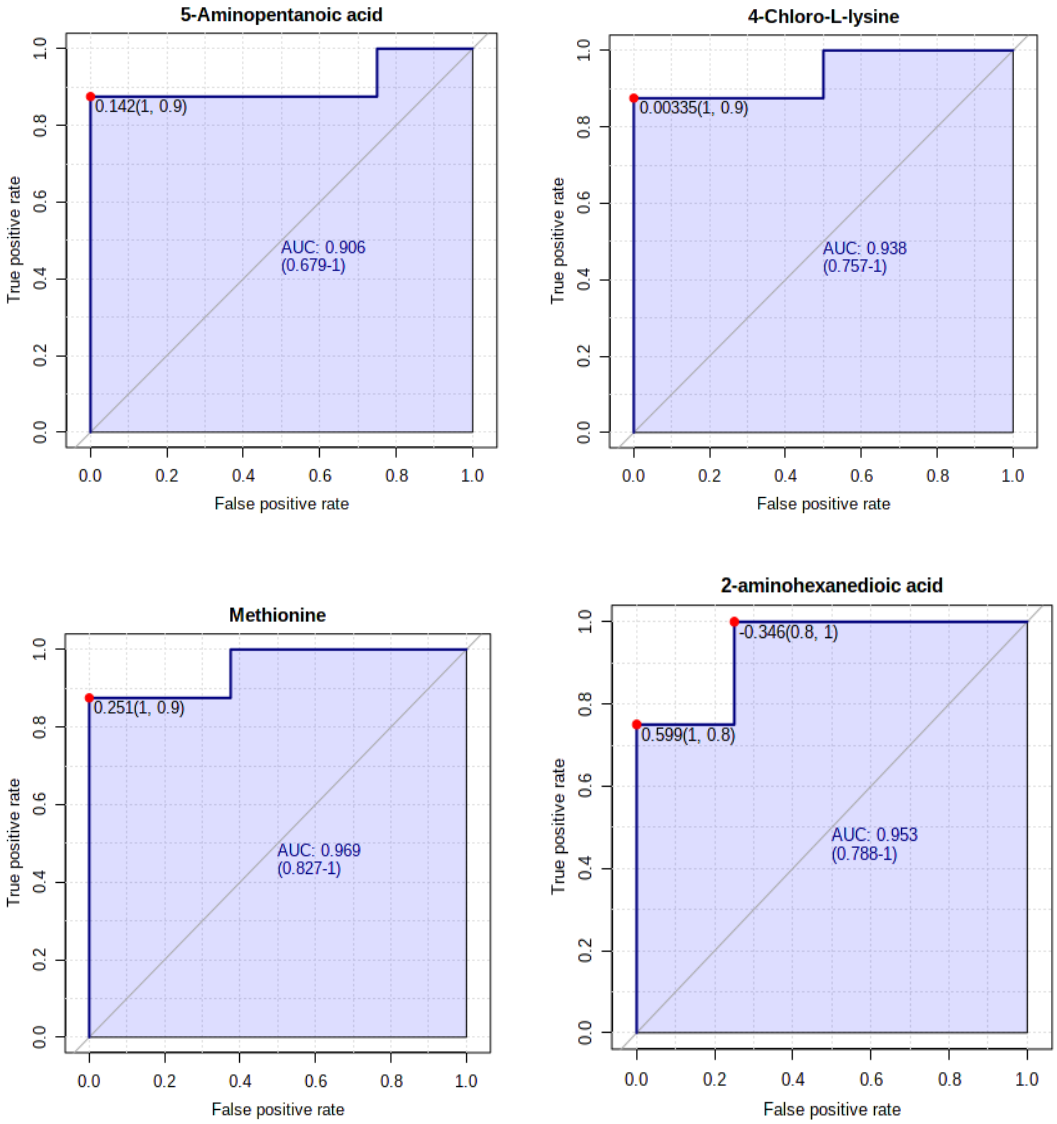


Figure 2. 3 Biomarker analysis of plasma amine/phenol metabolome. ROC curve analysis of candidate plasma amine/phenol biomarkers (methionine, 5-aminopentanoic acid, 2-aminohexanedioic acid, and 4-chlorolysine) of beef steer with divergent RFI values.

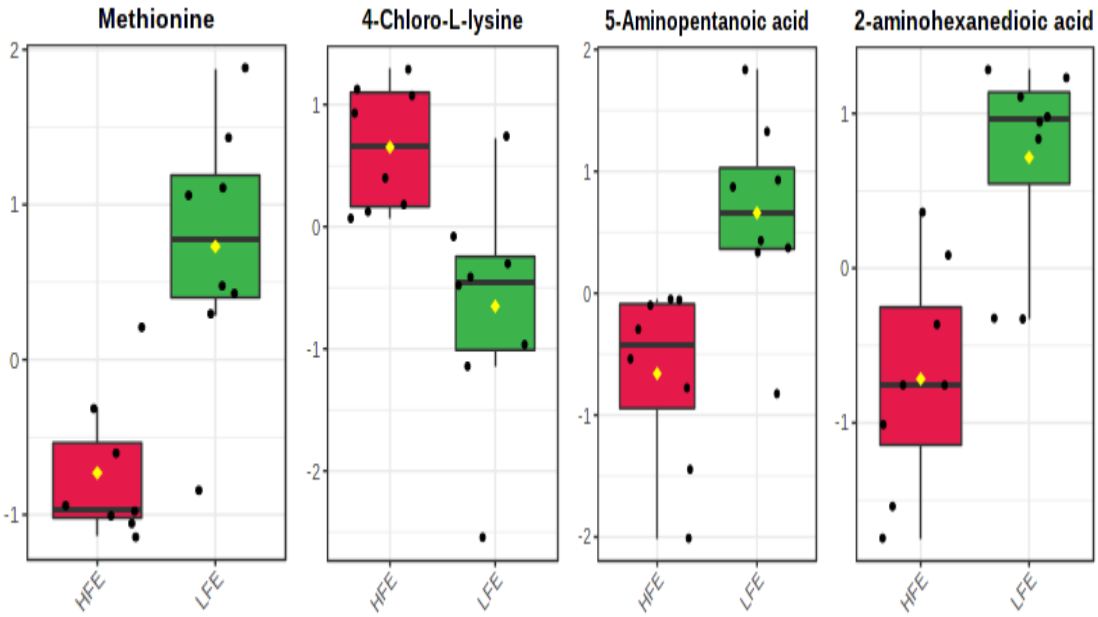


Figure 2. 4 Relative distributions of the candidate plasma amine/phenol biomarkers of beef steer with divergent RFI values. HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake

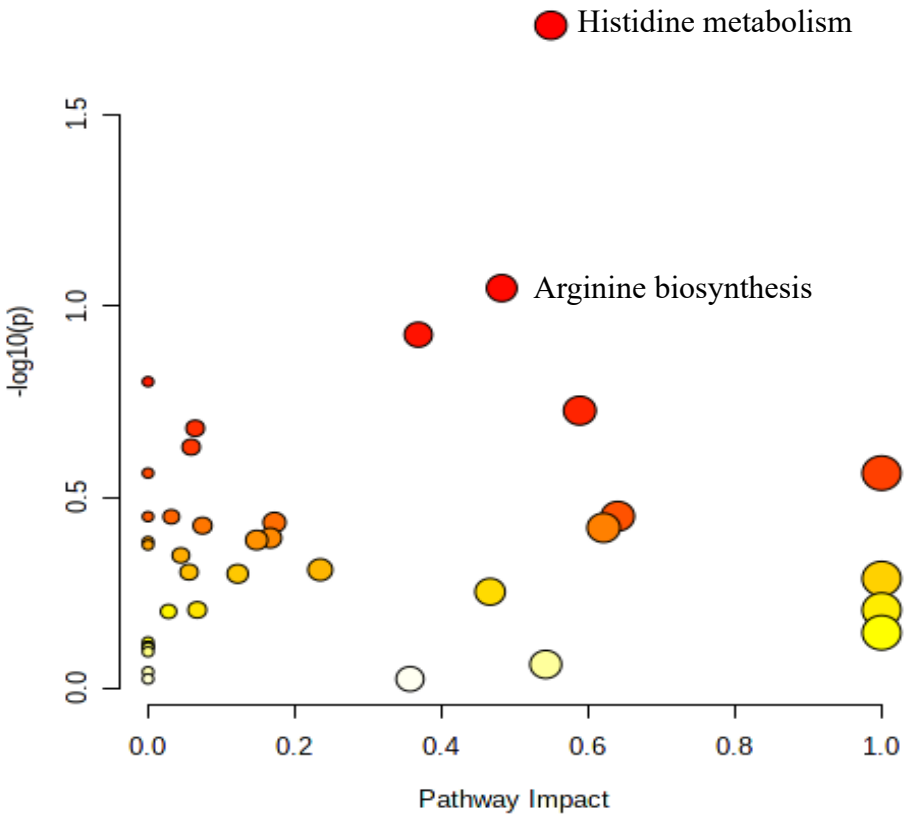
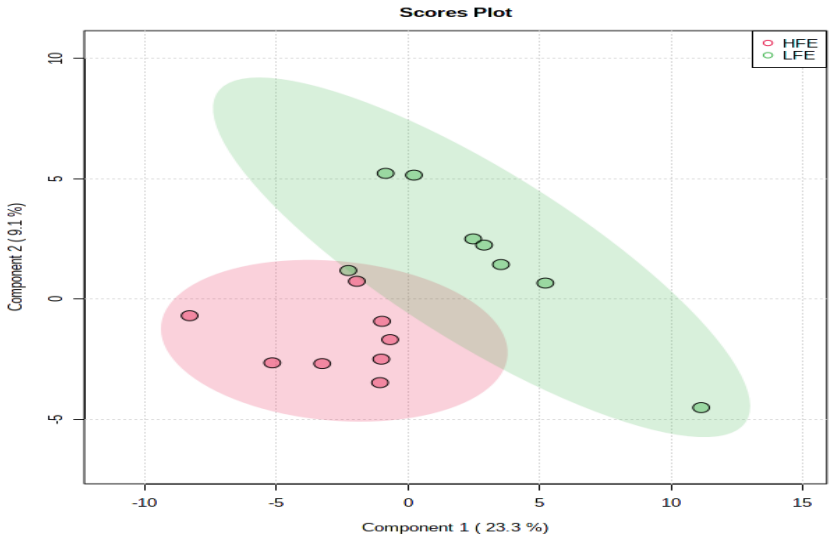


Figure 2. 5 Pathway analysis of the amine/phenol-metabolites of beef steers with divergent RFI values. Metabolic pathways with -log10(P) ≥ 1.0 (equivalent to P ≤ 0.10) are enriched in HFE steers, relative to LFE. HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake.

A.



B

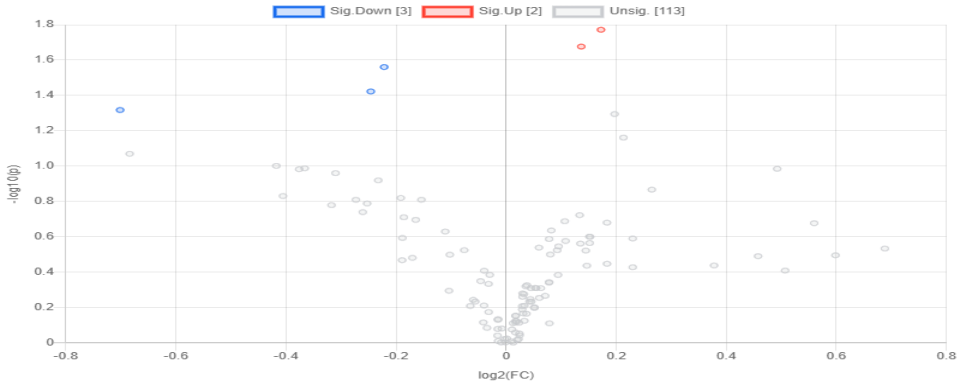
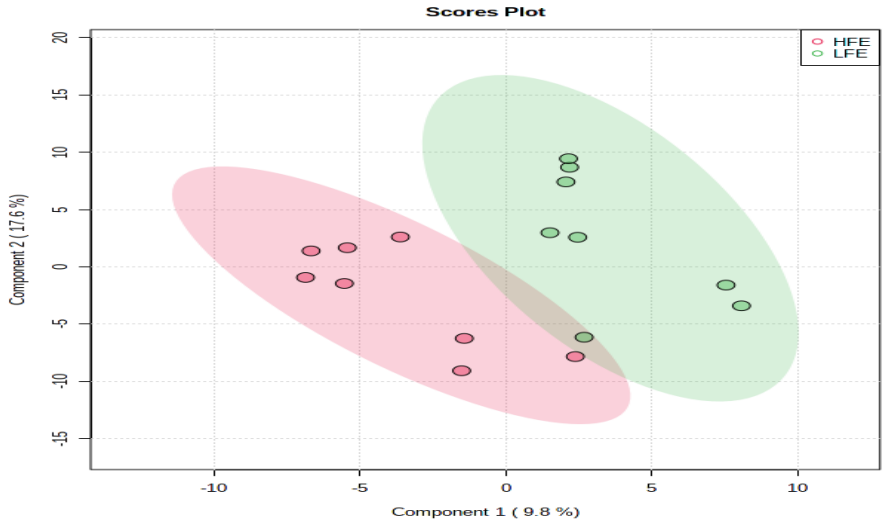


Figure 2. 6 A. PLS-DA scores plot of carbonyl-metabolome of LFE and HFE steers; B. Volcano plot showing the differentially abundant carbonyl-containing metabolites. Metabolites with false discovery ratio ≤ 0.05 (red or blue) are differentially increased or reduced in HFE, relative to LFE. HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake.

A.



B.

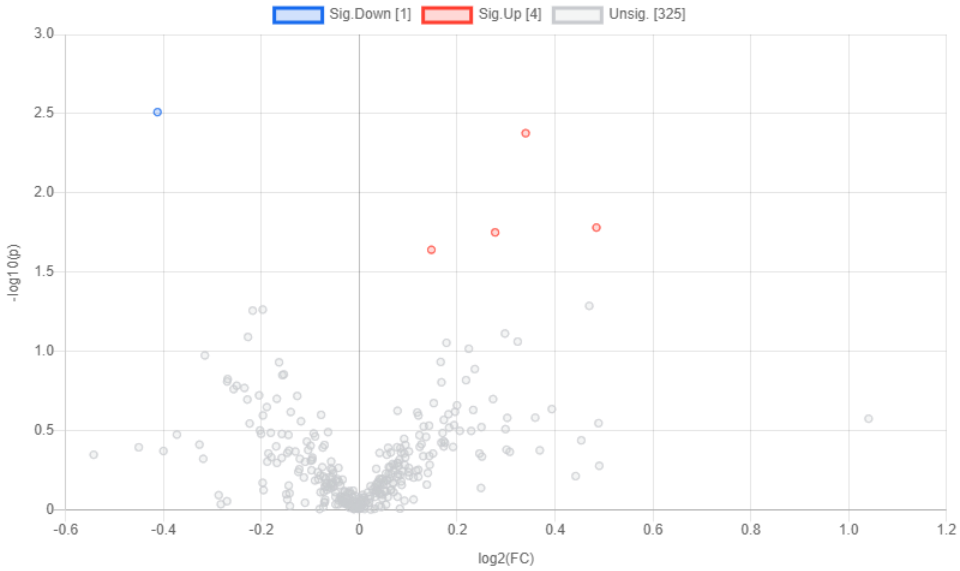


Figure 2. 7 A. PLS-DA scores plot of carboxyl-acid-metabolome of LFE and HFE steers; B. Volcano plot showing the differentially abundant carboxylic acid-containing metabolites. Metabolites with false discovery ratio ≤ 0.05 (red or blue) are differentially increased or reduced in HFE, relative to LFE. HFE = beef steers with negative residual feed intake; LFE = beef steers with positive residual feed intake.

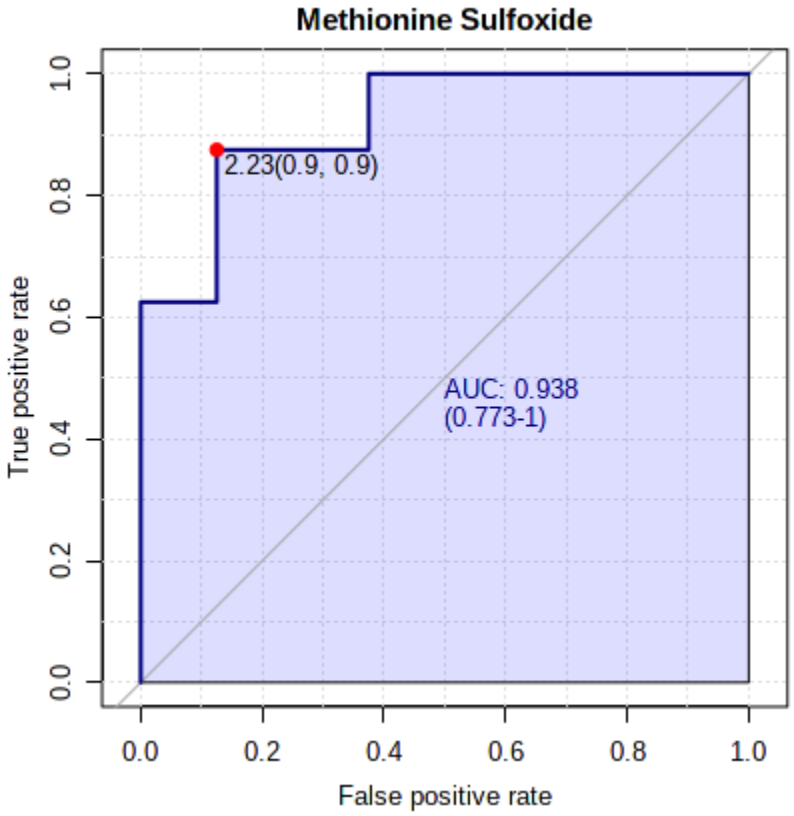


Figure 8. Biomarker analysis of plasma carboxylic acid-metabolome. ROC curve analysis of methionine sulfoxide.

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Chapter 3

Chapter 3. Residual feed intake in beef cattle is associated with differences in hepatic mRNA expression of fatty acid, amino acid, and mitochondrial energy metabolism genes

Abstract

Objective: To analyze the mRNA expression of genes involved in hepatic fatty acid, amino acid, and mitochondrial energy metabolism in crossbred beef steers with divergent low and high residual feed intake (RFI).

Methods: Low-RFI beef steers (n = 8; RFI = - 1.93 kg/d) and high-RFI beef steers (n = 8; RFI = + 2.01kg/d) were selected from a group of 56 growing crossbred beef steers (average BW = 261 ± 18.5 kg) fed a high-forage total mixed ration after a 49-d performance testing period. At the end of the 49-d performance testing period, liver biopsies were collected from the low-RFI and high-RFI beef steers for RNA extraction and cDNA synthesis. The mRNA expression of 84 genes each related to fatty acid metabolism, amino acid metabolism, and mitochondrial energy metabolism were analyzed using pathway-focused PCR-based arrays.

Results: The mRNA expression of 8 genes (*CRAT, SLC27A5*, *SLC27A2*, *ACSBG2*, *ACADL*, *ACADSB*, *ACAA1*, and *ACAA2*) involved fatty acid transport and β-oxidation were upregulated (FC > 2.0, $P < 0.05$) in low-RFI, compared to high-RFI steers. Among those involved in amino acid metabolism, hepatic mRNA expression of a gene encoding for aminoadipate aminotransferase, an enzyme related to lysine degradation, was downregulated (FC = -5.45, $P = 0.01$) in low-RFI steers, whereas those of methionine adenosyltransferase I and aspartate aminotransferase 2, which both link amino acid and lipid metabolism, were upregulated (FC > 2, $P < 0.05$). Two mitochondrial energy metabolism genes (*UQCRC1* and *ATP5G1*) involved in ATP

synthesis via oxidative phosphorylation were upregulated (FC > 2; $P < 0.05$) in low- compared to high-RFI beef steers.

Conclusions: The results of this study demonstrated that low-RFI beef steers exhibit upregulation of molecular mechanisms related to fatty acid transport, fatty acid β-oxidation, and mitochondrial ATP synthesis, which suggest that low-RFI beef steers have enhanced metabolic capacity to maximize capture of energy and nutrients from feeds consumed.

Introduction

Due to high feed costs associated with animal production, residual feed intake (RFI), calculated as the difference between actual and expected dry matter intake required for maintenance and growth in animals (Koch et al., 1963), is of great economic importance. Compared to beef cattle with high (or positive) RFI, those with low (or negative) RFI are more feed efficient because they consume less feed than expected while maintaining similar growth performance. Residual feed intake is phenotypically independent of the level of production, suggesting that RFI variation in animals reflects differences in metabolic processes (Koch et al., 1963; Nkrumah et al., 2006; Elolimy et al., 2019).

Despite the great economic importance of RFI, the underlying biological mechanisms controlling this trait in beef cattle are still not well understood. Recent studies have suggested that low-RFI beef cattle possess several mechanisms that enable them to maximize capture of energy and nutrients from feed consumed (Elolimy et al., 2019). One of the most important organs for metabolic process is the liver, which regulates whole-body energy metabolism and acts as the main site of nutrient and energy metabolism that are essential for growth and productivity of animals (Bauchart et al., 1996; Baldwin et al., 2004). Studies have shown that fatty acids (including acetate

and long-chain fatty acids) and acetyl-CoA produced from catabolism of fatty acids and amino acid are the primary carbon sources oxidized in the liver to provide energy for the body (Drackley et al., 2001). Indeed, work from our laboratory and other researchers using either liver transcriptomics or plasma metabolomics (Alexandre et al., 2015; Mukiibi et al., 2018) have demonstrated that several biologically relevant pathways, such as lipid and amino acid metabolism, are associated with RFI in beef cattle, given their roles in energy production and tissue protein synthesis.

Furthermore, the major energy-generating organelle in body tissues, including the liver, is the mitochondria which is considered a metabolic hub for the regulation of hepatic metabolism of nutrients such as lipids and proteins, and it is known to produce approximately 90% of cellular energy (Saraste, 1999). Due to the role of hepatic mitochondria in energy metabolism, several studies have demonstrated that variation in RFI could be associated with differences in liver mitochondrial energy metabolism (Rolfe and Brand, 1997; Kolath et al., 2006; Lancaster et al., 2014). For instance, Lancaster et al. (2014) revealed that low-RFI steers had greater hepatic mitochondrial rate than high-RFI beef steers. In a similar study, Ramos and Kerley (2013) observed increased activities of some proteins of respiratory complexes in muscle of low-RFI beef steers when compared with low-efficiency steers. Since hepatic metabolism is controlled in large part by transcriptional and/or post-transcriptional regulation of several genes/enzymes which catalyze key nutrient and energy metabolic reactions, it was necessary to determine how changes in expression of nutrient and energy metabolism pathway-focused genes are associated with selection for low or high RFI. Therefore, the objective of the present study was to analyze the mRNA expression of genes involved in hepatic fatty acid, amino acid, and mitochondrial energy metabolism in crossbred beef steers with low or high RFI to give more insight into the biological

mechanisms associated with RFI divergence. We hypothesized that differences in the expression of some nutrient or/and energy metabolism-related genes would be associated with divergence in RFI in beef steers.

Materials and Methods

Animals, feeding, RFI determination

The Institutional Animal Care and Use Committees of West Virginia University (protocol number 1608003693) approved the research procedures used in this study. A group of 56 crossbred growing beef steers (average BW = 261 ± 18.5 kg) were fed a high-forage total mixed ration formulated to achieve gains of about 0.9 kg per day (Table 3.1), which is a typical backgrounding diet fed in West Virginia. The beef steers were kept in a dry lot equipped with GrowSafe intake nodes (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) to measure individual feed intake and In-Pen Weighing Positions (IPW, Vytelle LLC) to measure daily BW of individual animals (Wells et al., 2021). The use of IPW to measure BW has enabled the measurement of feed efficiency with sufficient accuracy with a test period of 49 d (Wells et al., 2021; MacNeil et al., 2021). Steers were identified with a passive, half-duplex, transponder ear tag (Allflex USA Inc., Dallas–Fort Worth, TX) before entry into the test facility. The steers were first allowed to adjust to the facilities and diet for 15 days before the start of the trial. After the adjustment period, individual feed intake was measured over 49 days. Daily BW for each animal was regressed on time using simple linear regression to calculate beginning BW, mid-test BW, and average daily gain (ADG). Values of steer's ADG and mid-test metabolic BW (mid-test $BW^{0.75}$) were regressed against individual average daily intake (in dry matter basis) and RFI was calculated as the residual or the difference between the predicted value of the regression and the actual measured value based on the following

equation: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$, where Y is the observed DMI (kg/d), β_0 is the regression intercept, β_1 and β_2 are the partial regression coefficients, X_1 is the mid-test metabolic BW (kg), X_2 is the ADG (kg/d), and ε indicates the RFI (kg/d; Durunna et al., 2011). At the end of the RFI testing period, all animals were ranked by RFI coefficients. Based on the RFI coefficients, the most-efficient with the lowest RFI (low-RFI; n = 8) and the least-efficient with the highest RFI (high-RFI; n = 8) beef steers were selected.

Liver biopsy collection, RNA extraction, and gene expression

At the end of the 49-d RFI testing, liver biopsies were collected by needle biopsy under local anesthesia. After cutting the skin, liver tissue was extracted using a 14-gauge biopsy needle (Tru-Core-II Automatic Biopsy Instrument: Angiotech, Lausanne, Switzerland). Approximately 1000 mg of liver tissue samples obtained by one puncture were immediately stored in RNAprotect tissue tubes (Cat No: 76163; Qiagen, Germantown, MD), which contain RNAprotect tissue reagent that immediately stabilizes RNA in tissue samples to preserve the gene expression profile, and immediately stored at -80°C until analyzed. Total RNA was isolated with RNeasy Micro Kit (Cat No: 74004; Qiagen) following the manufacturer's protocol. Total RNA concentration (>100 ng/ul) was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was verified by formaldehyde gel. The RNA samples were then used to synthesize complementary DNA (cDNA) using RT^2 First Strand Kit (Cat. No. 330401; Qiagen) following the manufacturer's instructions.

The mRNA expressions of 84 genes each related to fatty acid metabolism, amino acid metabolism, and mitochondrial energy metabolism were analyzed using the RT^2 Profiler PCR Array Cow Fatty Acid Metabolism (PABT-007ZA; Qiagen), RT^2 Profiler PCR Array Human Amino Acid Metabolism I (PAHS-129ZA; Qiagen), and RT^2 Profiler PCR Array Cow

Mitochondrial Energy Metabolism (PABT-008ZA; Qiagen), respectively following the manufacturer's instructions. Each array consisted 84 metabolism-related genes, five housekeeping genes (actin, Glyceraldehyde-3-phosphate dehydrogenase, Hypoxanthine phosphoribosyltransferase 1, TATA box binding protein, and Tyrosine 3-monooxygenase), one genomic DNA control to detect gDNA contamination, three reverse transcription controls, and three positive PCR controls. Real-time PCR was performed using a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR cycling conditions were as follows: 95 °C for 10 min, 40 cycles of denaturation at 95°C for 15 s and 60°C for 1 min.

Statistical analysis

Differences in average RFI values, ADG and DMI between low- and high-RFI groups were determined by student's t-test. All mRNA expression data were analyzed using the Qiagen web-based platform, GeneGlobe (https://geneglobe.qiagen.com). The comparative cycle threshold (Ct) method was used for relative quantification of the gene expression (Pfaffl, 2001). Delta-delta-Ct ($^{\Delta\Delta}$Ct) method with normalization of the raw data using the geometric mean of the 5 housekeeping genes was used to calculate the differences in mRNA expression of the genes between low- and high-RFI beef steers (Pfaffl, 2001). The PCR Arrays used have an average amplification efficiency of 99% with a 95% CI from 90 – 110%, which enable them to accurately analyze multiple genes simultaneously utilizing the $^{\Delta\Delta}C_T$ method. The mRNA expression of genes with absolute fold change (FC) ≥ 2.0 having false discovery rate-adjusted *P*-values (FDR; Benjamini and Hochberg, 1995)) ≤ 0.05 were considered to be differentially expressed.

Results and discussion

Table 3.2 shows the results of the growth performance of the low- and high-RFI beef steers. The average RFI values of low- and high-RFI steers were -1.93 and 2.01 kg/d, respectively (P = 0.01). The initial BW, final BW, ADG, and gain:feed ratio were similar between the two groups ($P > 0.05$); however, high-RFI steers had greater ($P = 0.01$) DMI (16.0 kg/d) than low-RFI steers (11.9 kg/d).

The mRNA expression of the 84 genes involved in hepatic fatty acid metabolism are shown in this link (https://www.frontiersin.org/articles/10.3389/fanim.2022.828591/full). The genes having FDR ≤ 0.10 are shown on Table 3.3. Out of the 84 genes analyzed, the mRNA expression of 8 genes encoding several enzymes such as acetyl-CoA transferases, acyl-CoA dehydrogenases, acyl-CoA synthetases, and fatty acid transport were upregulated (FC ≥ 2.0, FDR ≤ 0.05) in low-RFI, compared to high-RFI steers.

The mRNA expression of the 84 genes involved in hepatic amino acid metabolism are shown in this link (https://www.frontiersin.org/articles/10.3389/fanim.2022.828591/full). The genes having FDR ≤ 0.10 are shown on Table 3.4. The mRNA expression of only 3 genes encoding aminoadipate aminotransferase (FC = -5.45, FDR = 0.01), methionine adenosyltransferase I (FC = 2.07, FDR = 0.03), and aspartate aminotransferase 2 (FC = 3.96, FDR = 0.05) were differentially expressed.

The mRNA expression of the 84 genes involved in mitochondrial energy metabolism are shown in (https://www.frontiersin.org/articles/10.3389/fanim.2022.828591/full). The genes having FDR ≤ 0.10 are shown on Table 3.5. The mRNA expression of 2 genes (*UQCRC1* and *ATP5G1*) encoding ubiquinol-cytochrome C reductase core protein 1 and ATP synthase, respectively were upregulated (FC > 2; FDR < 0.05) in low-RFI beef steers, compared to high-

RFI beef steers (Table 3.4). No other mitochondrial energy metabolism genes were differentially regulated.

Hepatic mRNA expressions of fatty acid metabolism genes

The mRNA expression of genes involved in fatty acid transport (*CRAT*, *SLC27A5*, and *SLC27A2*) were upregulated in low-RFI beef steers. Since fatty acid oxidation occurs mostly in the mitochondrial matrix, fatty acids (in the form of fatty acyl-CoA) must be transported across the mitochondrial outer and inner membranes for β-oxidation to occur (Kerner and Hoppel, 2000). Carnitine O-acetyltransferase is a member of the carnitine acyltransferase family which promotes the translocation of long-chain fatty acids across the mitochondrial membrane (Kerner and Hoppel, 2000). Both *SLC27A2* and *SLC27A4* genes are members of the solute carrier family 27 that encode fatty acid transport protein 2 and 4, respectively (Schaffer and Lodish, 1994; Krammer et al., 2011). Fatty acid transport proteins have been proposed to function as both direct transporters of long chain fatty acids (LCFA) as well as enzymes that activate LCFA via conjugation with Co-enzyme A, a reaction catalyzed by Acyl-CoA synthetases (Anderson and Stahl, 2013). Thus, upregulation of *SLC27A2* and *SLC27A4* in low-RFI steers partly explained the greater mRNA expression of gene *ACSBG2* encoding acyl-CoA synthetase in low-RFI beef steers, compared to high-RFI beef steers. Acyl-CoA synthetase catalyzes the activation of free fatty acids to their CoA thioesters before they can participate in any cellular metabolic pathways such as oxidation, elongation or unsaturation, protein acylation, and conversion into phospholipids (Steinberg et al., 2000). *ACSBG2* belongs to a family of genes encoding enzymes capable of activating long chain fatty acids which are known to originate primarily from the diet (Steinberg et al., 2000).

The first and rate-limiting step in fatty acid β-oxidation in mitochondria is catalyzed by acyl-CoA dehydrogenases (ACADs; Ghisla and Thorpe, 2004). The mRNA expression of two

genes, *ACADL* and *ACADSB*, encoding acyl-CoA dehydrogenases were upregulated in low-RFI steers. *ACADL* and *ACADSB* catalyze the first step in mitochondrial β-oxidation of long and short-chain fatty acids, respectively (Ghisla and Thorpe, 2004). Acetyl-CoA transferases are a group of enzymes involved in β-oxidation pathway of fatty acid degradation and various fatty acid biosynthetic pathways (Wanders et al., 2001). Acetyl-CoA acyltransferase 1 and 2 (*ACAA1* and *ACAA2*), both of which were upregulated in low-RFI steers, are key regulators of fatty acid β-oxidation in peroxisomes and mitochondria, respectively (Wanders et al., 2001; Li, 2008). Acetyl-CoA acyltransferase 1 catalyzes the splitting of 3-ketoacyl-CoA to acetyl-CoA and acyl-CoA, which are both involved in fatty acid elongation and degradation in peroxisomes, while *ACAA2* catalyzes the last step of mitochondrial β-oxidation of fatty acids (Wanders et al., 2001; Li, 2008). Collectively, upregulated expression of these aforementioned genes suggests enhanced mitochondrial and peroxisomal fatty acid β-oxidation in low-RFI steers.

The major function of hepatic β-oxidation of fatty acids is energy production (Kunau et al. 1995). Fatty acid oxidation can generate 2.5 times more energy than metabolism of carbohydrate via oxidative phosphorylation. Metabolism of fatty acids is a major source of energy for the skeletal muscle and β-oxidation of fatty acids in the liver produces ketone bodies that serve as essential energy source for extra-hepatic organs. These results agree with several hepatic transcriptomic studies that demonstrated lipid oxidation and transport as the most significant pathway associated with RFI divergence in beef cattle (Alexandre et al., 2015; Mukiibi et al., 2018). For instance, Mukiibi et al., 2018 analyzed the hepatic transcriptome of three breeds of beef cattle and revealed that expression of genes related to lipid synthesis and accumulation were observed to be downregulated in the liver tissues of low-RFI animals, which was consistent across the three beef breeds. In a similar study, the gene, *ACACB*, encoding fatty acid synthase, an enzyme

that promotes lipid synthesis, was observed to be downregulated in low-RFI beef steers (Alexandre et al., 2015). In dairy cattle, Salleh et al., 2018 reported upregulation of co-expressed genes involved in lipid and cholesterol biosynthesis in liver of high-RFI cows, compared to low-RFI cows. Previous studies in pigs have also demonstrated that altered hepatic lipid metabolism is closely related to feed efficiency (Lkhagvadorj et al., 2010; Zhao et al., 2016). Taken together, increased hepatic expression of these fatty acid metabolism-related genes in low-RFI beef steers, relative to high-RFI beef steers, suggests an improved efficiency of energy utilization, thus allowing for a similar level of growth performance despite lower DMI.

Hepatic mRNA expression of amino acid metabolism genes

Hepatic mRNA expression of a gene, *AADAT*, encoding for aminoadipate aminotransferase was downregulated in low-RFI steers. Aminoadipate aminotransferase is a protein involved in a metabolic pathway that synthesizes kynurenine and glutaric acid, products of tryptophan and lysine metabolism, respectively (Sauer et al., 2011). Although most previous studies have focused on its role in kynurenine biosynthesis, the function of *AADAT* in hepatic lysine degradation to aminoadipate has been well described in several studies (Higashino et al., 1971; Goh et al., 2002). In fact, the enzyme has been reported to have a higher catalytic efficiency for biosynthesis of aminoadipate than for kynurenine (Han et al., 2008). Downregulation of the activity of aminoadipate aminotransferase in the liver tissue of low-RFI steers suggests increased availability of lysine for tissue protein synthesis in low-RFI steers. This result probably explains the reduced plasma concentrations of 5-aminopentanoic acid and 2-aminohexanedioic acid (aminoadipic acid), and increased plasma concentration of chloro-lysine in low-RFI beef steers observed in our companion paper as shown in chapter 2. In agreement with our results, a recent study in sheep demonstrated reduced serum concentration of aminoadipic acid and increased serum concentration of lysine in low-RFI sheep, relative to high-RFI sheep (Goldansaz et al., 2020).

The mRNA expression of a gene, *GOT2*, encoding for mitochondrial aspartate aminotransferase isoenzyme 2 was upregulated in the liver tissue of low-RFI steers. Mitochondrial aspartate aminotransferase is involved in several metabolic processes; the enzyme links amino acid metabolism to carbohydrate metabolism by catalyzing the reaction of L-aspartate and α-ketoglutarate to form oxaloacetate and L-glutamate, which both fuel the tricarboxylic acid cycle for ATP synthesis (Jiang et al., 2016). Mitochondrial aspartate aminotransferase also has high affinity for LCFA and is known to facilitate cellular transport of both saturated and unsaturated LCFA, a key step in energy-generating mitochondrial beta-oxidation of fatty acids (Roepstorff et al., 2004; Bradburry et al., 2011), which is in line with upregulation of fatty acid metabolism genes observed in this study.

Methionine adenosyltransferase is an enzyme specific to the liver and is primarily involved in the conversion of methionine to S-adenosyl-methionine, a biological methyl donor (Avila et al., 2002; Mato et al., 2002). S-adenosylmethionine is the key methyl donor for the synthesis of several compounds (Perez-Mato et al., 1999), including phosphatidylcholine that is required for export of very-low-density lipoproteins from the liver to adipose and muscle tissues where they can either be hydrolyzed to provide fatty acids as substrates for ATP-generating fatty acid oxidation or stored as fat when energy is not needed (Avila et al., 2002; Alves-Bezerra and Cohen, 2017). Methionine is also a precursor for synthesis of succinyl-CoA, homocysteine, cysteine, choline, creatine, methylarginine, and carnitine which are directly or indirectly involved in lipid metabolism; carnitine, in particular, is essential for the transfer of LCFA across the inner mitochondrial membrane (Roe and Ding, 2001). Increased mRNA expression of *MAT1A*, a gene encoding methionine adenosyltransferase in low-RFI steers suggests increased conversion of methionine to s-adenosyl-methionine in the liver. As shown in chapter 2, we applied a chemical group-based

metabolomics technique to identify blood metabolic signatures associated with RFI and observed lower plasma concentration of methionine in low-RFI beef steers when compared with high-RFI beef steers, which is in line with the result of the current study. It is important to note that the PCR array panel used was designed for human mRNA and its cross-reaction with the specific bovine mRNA was not validated in this study. Therefore, these results should be interpreted with caution.

Hepatic mRNA expressions of mitochondrial energy metabolism genes

Ubiquinol-cytochrome c reductase core protein 1 is a sub-unit of mitochondrial respiratory complex III and is known to play an essential role in regulating mitochondrial function, although its exact function is not yet determined (Yi et al., 2020). Mitochondrial respiratory complex III is one of the series of multi-subunit protein complexes of the electron transport chain (ETC), a key player in mitochondrial energy production (Lehninger et al., 1993). The ETC generates a potential difference across the mitochondrial membrane, by pumping protons from the mitochondrial matrix to the intermembrane space, which is used to power ATP synthesis (Osellame et al., 2012). Similar to our results, Casal et al., 2018 determined the mRNA expression of some select genes involved in mitochondrial respiratory chain in the liver of Hereford steers with divergent RFI phenotypes and reported upregulation of some genes, including *UQCRC1* in low-RFI steers. Lancaster et al., 2014 observed greater ADP-stimulated respiration rates (state 3 respiration rates) in liver of low-RFI beef steers than their high-RFI counterparts. Similar result was observed by Kolath et al. (2006) who reported greater ADP-stimulated respiration rates in muscle of low-RFI beef steers compared to steers with high-RFI. In another study, Ramos and Kerley, 2013 reported greater quantity of mitochondrial complex proteins in beef cattle with low-RFI, relative to high-RFI cattle. In contrast to our results, other studies that utilized total RNA sequencing or microarray, reported no differentially expressed genes related to electron transport chains between beef cattle with

divergent RFI (Alexandre et al., 2015; Tizioto et al., 2015; Zarek et al., 2017), which may be due to several reasons, including difference in methodology because transcriptomics analysis is not as specific and targeted as qPCR-based analysis.

The gene, *ATP5G1*, encodes a subunit of the mitochondrial ATP synthase and it is an important component of complex V of the oxidative phosphorylation chain (He et al., 2017). Mitochondrial ATP synthase catalyzes ATP synthesis during oxidative phosphorylation using the energy provided by the proton electrochemical gradient to meet cellular energy needs (Keogh et al., 2015; He et al., 2017). In rats, low feed intake has been demonstrated to result in increased expression of muscle and hepatic gene transcripts involved in ATP production to compensate for the lower caloric intake per unit weight (Gredilla et al., 2001; Sreekumar et al., 2002). This suggests that increased expression of *ATP5G1* is probably an adaptive response of low-RFI steers to low DMI that may result in increased ATP production. Taken together, since mitochondrial oxidative phosphorylation provides over 90% of the energy needed for mammalian metabolism (Bermejo-Nogales et al., 2015), upregulation of *UQCRC1* and *ATP5G1* supports the notion that low-RFI beef steers can synthesize ATP more efficiently than high-RFI beef steers. It is important to note that the potential impact of these genes on liver function was not fully evaluated in this study as it was not determined if these mRNA changes are translated into proteins or how they affect important metabolic changes that could influence feed efficiency. In addition, the validation of the use of the reference genes and amplification efficiency of the PCR array was based on the information provided by the manufacturer. However, the results of this study provide evidence to support the concept that divergence in RFI is associated with changes in mRNA expression of genes involved in lipid metabolism, amino acid metabolism, and mitochondrial ATP production in the liver of crossbred beef steers.

Conclusions

This study revealed differential hepatic mRNA expression of multiple genes involved in amino acid, fatty acid, and mitochondrial energy metabolism in beef steers with divergently low- or high-RFI. Future studies are needed to determine how these mRNA changes are translated into proteins or how they affect important metabolic changes that could influence measures of feed efficiency in beef cattle.

Tables and Figures

Table 3. 1 Ingredient and nutrient composition of the basal diet[1]

Item	**Value**[2]
Ingredient composition	
Triticale silage	49.5
Ryegrass silage	47.5
Concentrate supplement[3]	3.0
Nutrient composition[4]	
DM, %	44.5
CP	13.7
aNDF	59.7
ADF	31.5
EE	3.14
Ca	0.66
P	0.37
NE_m, Mcal/kg	1.37
NE_g, Mcal/kg	0.91

[1]Composition of basal diet calculated from analysis and concentration of individual ingredients.
[2]Values are presented on a % DM basis unless indicated otherwise.
[3]Traditions 50% beef supplement (Southern States Cooperative, Richmond, VA) contained processed grain by-products, plant protein products, ground limestone, urea, salt, cane molasses, potassium sulfate, magnesium sulfate, sodium selenite, vitamin A supplement, calcium carbonate, vegetable oil, manganous oxide, vitamin D3 supplement, vitamin E supplement, zinc oxide, lecithin, phosphoric acid, basic copper chloride, magnesium chloride, propylene glycol, natural and artificial flavors, ferrous sulfate, calcium iodate, and cobalt carbonate; Guaranteed analysis: 50% CP; 5% Ca; 0.55% P; 2% Na; 3.9% salt; 1% K, and 66,000 IU/kg vitamin A.
[4]DM = dry matter; CP = crude protein; aNDF = neutral detergent fiber (amylase treated); ADF = acid detergent fiber; EE = ether extract; NE_m = net energy of maintenance; NE_g = net energy of gain.

Table 3. 2 Growth performance of low and high-RFI beef steers[1]

Item[2]	**Low-RFI**	**High-RFI**	**SEM**	***P*-value**
RFI, kg/d	-1.93	2.01	0.32	0.01
Initial BW, kg	258	258	11.2	0.95
Final BW, kg	276	278	4.07	0.57
ADG, kg/d	0.76	0.86	0.17	0.58
DMI, kg/d	11.9	16.0	0.66	0.01
Gain: feed	0.06	0.05	0.004	0.29

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers

[2]ADG = average daily gain; DMI = dry matter intake; BW = body weight; SEM = standard error of mean.

Table 3. 3 Fold change in hepatic fatty acid metabolism gene expression in low compared with high-RFI beef steers[1]

Gene symbol	Gene name	FC2	FDR
ACAA2	Acetyl-CoA acyltransferase 2	5.47	0.01
ACADSB	Acyl-CoA dehydrogenase, short/branched chain	4.9	0.01
ACSBG2	Acyl-CoA synthetase bubblegum family member 2	4.34	0.01
CRAT	Carnitine O-acetyltransferase	2.37	0.01
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	9.37	0.01
SLC27A5	Solute carrier family 27 (fatty acid transporter), member 5	3.35	0.01
ACAA1	Acetyl-CoA acyltransferase 1	5.17	0.02
ACADL	Acyl-CoA dehydrogenase, long chain	4.34	0.02
ECHS1	Enoyl CoA hydratase, short chain, 1, mitochondrial	5.01	0.06
BDH1	3-hydroxybutyrate dehydrogenase, type 1	-1.15	0.08
FABP5	Fatty acid binding protein 5 (psoriasis-associated)	-1.29	0.09
SLC27A1	Solute carrier family 27 (fatty acid transporter), member 1	-2.73	0.09

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers.
[2]Fold change (FC; relative to high-RFI).

Table 3. 4 Fold change in hepatic amino acid metabolism gene expression in low compared with high-RFI beef steers

Gene symbol	**Gene name**	**FC[1]**	**FDR**
AADAT	Aminoadipate aminotransferase	-5.45	0.01
MAT1A	Methionine adenosyltransferase I	2.07	0.03
GOT2	Aspartate aminotransferase 2	3.96	0.05
NIT2	Nitrilase family, member 2	2.85	0.07
GFPT1	Glutamine--fructose-6-phosphate transaminase 1	2.35	0.08
MCCC2	Methylcrotonoyl-CoA carboxylase 2 (beta)	2.65	0.10

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers.
[2]Fold change (FC; relative to high-RFI).

Table 3. 5 Fold change in hepatic mitochondrial energy metabolism gene expression in low compared with high-RFI beef steers[1]

Gene symbol	Gene name	FC[1]	FDR
UQCRC1	Ubiquinol-Cytochrome C Reductase Core Protein 1	2.07	0.01
ATP5G1	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C1	2.38	0.01
NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa	-1.31	0.01
NDUFC2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa	-1.76	0.01
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	1.84	0.01
COX6B1	Cytochrome c oxidase subunit VIb polypeptide 1	-1.56	0.01
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	-1.49	0.01
UQCRC2	Ubiquinol-cytochrome c reductase core protein II	-1.50	0.01
ATP5G3	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C3	-1.65	0.01
NDUFB2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	-1.50	0.01
NDUFV3	NADH dehydrogenase (ubiquinone) flavoprotein 3, 10kDa	1.52	0.01
ATP5J2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F2	-1.70	0.01
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	-1.50	0.02
NDUFS4	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa	-1.33	0.02
OXA1L	Oxidase (cytochrome c) assembly 1-like	-1.68	0.02
SLC25A22	Solute carrier family 25 (mitochondrial carrier: glutamate), member 22	1.79	0.04
NDUFAB1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa	-1.64	0.04
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	1.65	0.04
CYC1	Cytochrome c-1	-1.42	0.05
NDUFA3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9kDa	-1.25	0.05
TBP	TATA box binding protein	1.81	0.05
NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	-1.37	0.05
NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa	1.60	0.06

ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	1.42	0.07
HPRT1	Hypoxanthine phosphoribosyltransferase 1	-1.48	0.08
NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	1.24	0.08
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	1.28	0.08
UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	1.76	0.08
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	1.26	0.08
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa	2.05	0.09

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers.
[2]Fold change (FC; relative to high-RFI).

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Chapter 4

Chapter 4. Identification of key pathways associated with residual feed intake of beef cattle based whole blood transcriptome data analyzed using gene set enrichment analysis

Abstract

Objective: We applied whole blood transcriptome analysis and gene set enrichment analysis to identify key pathways associated with divergent selection for low or high RFI in beef cattle.

Methods: A group of 56 crossbred beef steers (average BW = 261.3 ± 18.5 kg) were adapted to a high-forage total mixed ration in a confinement dry lot equipped with GrowSafe intake nodes for period of 49 d to determine their residual feed intake (RFI). After RFI determination, weekly whole blood samples were collected three times from beef steers with the lowest RFI (most efficient; low-RFI; n = 8) and highest RFI (least efficient; high-RFI; n = 8). Prior to RNA extraction, whole blood samples collected were composited for each steer. Sequencing was performed on an Illumina NextSeq2000 equipped with a P3 flow. Gene set enrichment analysis (GSEA) was used to analyze differentially expressed gene sets and pathways between the two groups of steers.

Results: Results of GSEA revealed pathways associated with metabolism of proteins, cellular responses to external stimuli, stress, and heat stress were differentially inhibited (false discovery rate (FDR) < 0.05) in high-RFI compared to low-RFI beef cattle, while pathways associated with binding and uptake of ligands by scavenger receptors, scavenging of heme from plasma, and erythrocytes release/take up oxygen were differentially enriched (FDR < 0.05) in high-RFI, relative to low-RFI beef cattle.

Conclusions: Taken together, our results revealed that beef steers divergently selected for low or high RFI revealed differential expressions of genes related to protein metabolism and stress responsiveness.

Introduction

Residual feed intake (RFI), a measure of feed efficiency, continues to be of great economic importance due to increasing cost of animal feeds (Koch et al., 1963). Residual Feed Intake is the difference between an animal's actual feed intake and its predicted feed intake for a given level of maintenance and body weight gain (Koch et al., 1963). Feed efficient animals consume less than expected and have a low (negative) RFI, while inefficient animals consume more than expected and have a high (positive) RFI. Thus, beef cattle selected for low RFI have decreased feed costs because they consume less dry matter when compared with high-RFI beef cattle while maintaining similar growth performance.

Due to the great economic importance of RFI, the biological mechanisms underlying variation in this trait have always been of great interest; however, these mechanisms have not been fully understood. Difference in RFI has been suggested to be an indication of differences in metabolism rather than differences in growth performance because the trait is phenotypically independent of growth performance (Koch et al., 1963). Several studies have applied whole transcriptome analysis of several tissues such as liver and ruminal epithelium to further understand the biological mechanisms regulating feed efficiency traits including RFI in beef cattle (Alexandre et al., 2015; Kong et al., 2016; Mukiibi et al., 2018). For instance, Kong et al., 2016 analyzed the rumen epithelial transcriptome from low-RFI and high-RFI beef steers and observed increased tissue morphogenesis and greater expression of mitochondrial genes in low-RFI compared to high

RFI steers. Mukiibi et al. (2018) analyzed liver tissue transcriptome profile and observed differential expressions of genes involved in nutrient metabolisms and cellular development in beef steers divergent for low and high RFI. However, these studies involve invasive sample collection procedures. Despite the convenience of collection and relatively non-invasive accessibility of blood in ruminants, very few attempts have been made to apply whole-blood transcriptome to understand the biological mechanisms associated with RFI in animals. Genes expressed in peripheral blood cells have been demonstrated to reflect physiological changes in different body tissues and can highlight biological processes related to overall metabolism. Therefore, the objective of this study was to analyze the whole-blood transcriptome data of beef steers via gene-set enrichment analysis to identify key pathways associated with divergent selection for low or high RFI in beef cattle.

Materials and Methods

Animals and sample collection

A total of 56 crossbred growing beef steers with average BW of 261.3 ± 18.5 kg were fed a high-forage total mixed ration (TMR; primarily consisting of triticale silage; rye grass silage; and a ration balancing supplement; (https://www.frontiersin.org/articles/10.3389/fvets.2022.848027/full) in a confinement dry lot equipped with GrowSafe intake nodes (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) to measure individual feed intake and In-Pen Weighing Positions (Vytelle LLC) to measure daily BW for a total of 49 d after 15-d adjustment period to the feeding facilities. The use of In-Pen Weighing Positions has enabled the measurement of feed efficiency with sufficient accuracy with a test period of 49 d (Wells et al., 2021). Daily BW for each animal were regressed on time to

calculate beginning BW, mid-test BW, and average daily gain (ADG). Animal ADG and metabolic mid-test BW (mid-test $BW^{0.75}$) were regressed against individual average daily intake, and RFI was calculated as the residual or the difference between the predicted value of the regression and the actual measured value based on the following equation: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$, where Y is the observed DMI (kg/d), β_0 is the regression intercept, β_1 and β_2 are the partial regression coefficients, X_1 is the mid-test metabolic BW (kg), X_2 is the ADG (kg/d), and ε indicates the RFI (kg/d; Durunna et al., 2011). After the determination of RFI values for all animals, the most-efficient with the lowest RFI (low-RFI; n = 8) and the least-efficient with the highest RFI (high-RFI; n = 8) beef steers were selected, kept separate from others, and kept on the same diet for additional 21 d (designated in this study as d 50 – 70). On d 56, 63, and 70, 10 mL of blood samples were collected before morning feeding into tubes containing sodium heparin. Immediately after collection, subsamples (500 µL each) were transferred into RNA-protect tubes (Cat. No. 76554; Qiagen) containing a reagent that lyses blood cells and stabilizes intracellular RNA and stored at −80°C until later analysis.

RNA extraction, library preparation, and sequencing

Prior to RNA extraction, whole blood samples collected on d 56, 63, and 70 were composited for each steer. Total RNA was extracted from the composited samples using RNeasy Protect Animal Blood kit (Cat. No. 73224; Qiagen) following the manufacturer's instructions. RNA concentration was measured using a NanoDrop One C spectrophotometer with an A260:A280 ratio from 1.8 to 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). All RNA samples had RNA integrity numbers > 8.0. Dual indexed RNA Libraries were prepared from 100 – 250 ng of total RNA per sample using the KAPA RNA HyperPrep Kit with RiboErase (Human, Mouse, Rat) Globin Reduction method in the WVU Genomics Core according to the kit

manufacturer's instructions. Library quality was assessed by electrophoretic analysis on the Agilent 4200 TapeStation system with High Sensitivity D1000 screentape. RNA libraries were sequenced in a dual indexed 2 × 50 paired-end run on an Illumina NextSeq2000 equipped with a P3 flow.

Data and statistical analysis

For the RNA-seq data, reads were trimmed using Trimmomatic v 0.39 to remove low-quality base calls and adapter sequences (Bolger et al., 2014), and then aligned to the Bovine reference genome ARS-UCD1.2 (Rosen et al., 2020) using HISAT2 v 2.2.1 (Kim et al., 2015). Resulting files were sorted and indexed, and PCR and optical duplicate reads were marked using SamTools v1.12 (Li et al., 2009). The numbers of reads mapping to each gene for each sample were counted using the R/Bioconductor package GenomicAlignments v 1.26.0 (Lawrence et al., 2013). Log_2 fold change values were computed using DESeq2 version 1.30.1 (Love et al., 2014). We used gene set enrichment analysis (GSEA), a pathway enrichment method that utilizes predefined gene sets from the reactome pathways (Jassal et al., 2020), to analyze differentially expressed gene sets using the R/Bioconductor package fgsea v 1.16.0. The GSEA was performed to determine the key pathways that were enriched or inhibited by considering the expression levels of sets of biologically related genes (Reimand et al., 2019). Genes identified by DESeq2 as expressing over a minimal threshold were ranked by Log_2 fold change and analyzed by the GSEA algorithm (Luo et al., 2009). The altered pathways were filtered based on FDR ≤ 0.05 and were arranged in the order of their normalized enrichment scores.

Results

The average RFI values of low- and high-RFI steers were -1.93 and 2.01, respectively. An average of 36 million reads per sample was generated (https://www.frontiersin.org/articles/10.3389/fvets.2022.848027/full). Results of GSEA revealed gene sets (pathways) associated with metabolism of proteins, cellular responses to external stimuli, stress, heat stress, and regulation of HSF-1-mediated heat shock response were differentially inhibited (FDR = 0.01) in high-RFI compared to low-RFI beef cattle (Table 4.1; https://www.frontiersin.org/articles/10.3389/fvets.2022.848027/full). The gene set associated with metabolism of proteins consists of 248 genes, and 85 of which were leading edge genes (significantly enriched genes). Both cellular response to external stimuli and cellular response to stress shared the same nineteen leading edge genes including *HSPA1A, HSPH1, BAG2, DNAJA1, DNAJB1, H3C13, H2BC7, H4C2, ELOC, JUN,* and *HSPA4.* Five of the leading edge genes (*HSPA1A, HSPH1, BAG2, HSPA4,* and *DNAJB1*) associated with cellular response to external stimuli and stress were also leading edge genes in gene sets associated with response to heat stress and regulation of HSF-1-mediated heat shock response (Table 4.1).

Gene sets associated with binding and uptake of ligands by scavenger receptors, scavenging of heme from plasma, erythrocytes take up/release carbon dioxide and release/take up oxygen share the same leading edge genes (*HBB, HBA1,* and *HBA*) and were all differentially enriched (FDR < 0.05) in high-RFI, relative to low-RFI beef cattle (Table 4.1).

Discussion

Understanding the biological mechanisms regulating feed efficiency using easily accessible and non-invasive sample such as blood is essential to the future of livestock production systems in terms of profitability and animal welfare concern. In this study, protein metabolism is

the most enriched metabolic pathway based on the number of leading-edge genes (such as *LOC101907518, RPL39, LOC101902490, UBE2D1, FUCA2*) in the gene set. In addition to the function of amino acids as the building blocks of proteins, amino acids regulate key metabolism essential for growth, performance, reproduction, and immunity (Wu, 2009). Research studies have shown that protein (amino acids) metabolism is essential for optimizing efficiency of nutrient absorption and metabolism to enhance immunity against diseases and stress, growth performance, and milk production of animals (Wu, 2009). Several published articles have identified protein metabolism as one of the most important metabolic processes associated with RFI in animals (Richardson and Herd, 2004). Elolimy et al., 2019 reported differences in signaling mechanisms controlling protein turnover in ruminal epithelium of beef cattle divergent for low- or high-RFI. In a similar study, Kong et al., 2016 reported increased expression of genes involved in protein and cell turnover in the ruminal epithelium of low-RFI beef cattle, compared with high-RFI beef cattle. Mukiibi et al., 2018 performed RNA-seq analysis of liver tissue in beef cattle divergent for low and high RFI and observed downregulation of genes involved in amino acid degradation and urea synthesis in low-RFI beef cattle. In fact, some studies have reported significant association of blood metabolites involved in urea cycle with RFI in beef cattle (Jorge-Smeding et al., 2014; Goldansaz et al., 2020). Our results and those of others that utilized tissues with relatively more invasive collection methods suggest that amino acid metabolism plays a considerable role in regulating RFI of beef cattle and its enrichment in low-RFI beef steers probably explains their similar growth performance with high-RFI beef steers despite lower DMI.

Amino acids play a functional role in regulating stress response, including oxidative stress, in animals (Coleman et al., 2020). Stress response has significant implication on health and production efficiency of animals (Lyles et al., 2014). In fact, difference in stress responsiveness

has been suggested to contribute to variation in feed efficiency of beef cattle (Richardson and Herd, 2004; Knott et al., 2008). In this study, we observed downregulation of gene set including *HSPA1A, HSPH1, BAG2,* and *DNAJA1* associated with cellular responses to external stimuli, stress, heat stress, and regulation of HSF-1-mediated heat shock response in high-RFI beef steers, which suggests that these steers are more susceptible to stress. When an animal can no longer cope with a stressor, level of blood cortisol increases via activation of hypothalamic–pituitary–adrenal axis (HPA) axis which causes a fight or flight response that increases energy expenditure. Thus, stress response in animals is often determined by blood cortisol level and activity of the HPA axis (Ralph and Tilbrook, 2016). In a study that determined the response of beef heifers to an exogenous adrenocorticotropic hormone (ACTH) challenge, there was a positive association of plasma cortisol level with RFI status and low-RFI had a lower cortisol response than high-RFI heifers indicating that low-RFI heifers coped better with the stress challenge (Kelly et al., 2017). Richardson et al. (2004) and Gomes et al. (2013) reported lower blood levels of cortisol in low-RFI beef cattle when compared to high-RFI beef cattle. A similar result was observed in crossbred rams following ACTH challenge (Knott et al., 2008). Taken together, downregulation of genes associated with cellular responses to external stimuli, stress, heat stress, and regulation of HSF-1-mediated heat shock response in high-RFI beef steers suggests that low-RFI steers have better adaptive mechanisms to cope with environmental stressors, thereby, reducing energy expenditure and increasing energy availability for improved growth performance and better feed efficiency.

In this study, we observed enrichment of gene sets (*HBB, HBA1,* and *HBA*) associated with erythrocytes take up/release carbon dioxide, release/take up oxygen, scavenging of heme from plasma, and binding and uptake of ligands by scavenger receptors in high-RFI, relative to low-RFI beef cattle. Erythrocytes contain hemoglobins which carry oxygen to the body and are

continuously exposed to high oxygen content pre-disposing them to oxidative stress damage (Olsson et al., 2007; Maurya et al., 2015). Heme scavenger proteins, such as hemopexin and alpha-1-microglobulin, scavenge extracellular heme, are synthesized from hemoglobin degradation via the activity of heme-oxygenase. Heme-oxygenase production is an enzyme that is inducible by stressors such as oxygen free radicals (Nielsen et al., 2010; Kalapotharakos et al., 2019). Previous investigations have shown that cellular expression of alpha-1-microglobulin is enriched during increased oxidative stress and heme exposure (Olsson et al., 2007; Kalapotharakos et al., 2019). In ruminants, oxidative stress has been implicated in many pathophysiological conditions that are relevant for growth performance, reproduction, and health (Miller et al 1993).

In fact, several studies have shown that oxidative damage of cell organelles and biomolecules is a source of energy drain and negatively affects several cellular processes including lipid and protein metabolism (Bottje and Carstens, 2009; Radi, 2018). The major source of intracellular reactive oxygen species production is the mitochondria (Boveris and Chance, 1973) and previous studies have reported higher mitochondrial ROS production in less feed-efficient compared to high feed efficient animals (Iqbal et al., 2005; Bottje and Carstens, 2009; Grubbs et al., 2013). In addition, Casal et al. (2020) reported increased hepatic abundance of protein carbonyls and thiobarbituric acid reactive species, products of protein and lipid oxidative damage, and reduced protein expression of antioxidant enzymes, including mitochondrial manganese superoxide dismutase, in high-RFI when compared with low-RFI beef steers. Similarly, Tizioto et al. (2016) observed upregulation of oxidative stress-induced transcription factors in muscle of high-RFI beef steers. Therefore, it is reasonable to speculate that enrichment of genes associated with erythrocytes take up/release carbon dioxide, scavenging of heme from plasma, and binding and uptake of ligands by scavenger receptors in high-RFI compared to low-RFI beef steers

suggests that they may be more prone to oxidative stress, thereby resulting in reduced efficiency of energy use for metabolic processes.

It is important to note that though whole blood transcriptome data might encompass gene activities of several body tissues and organs including liver, kidney, muscles, and rumen, the contribution of each tissue to the whole blood transcriptome is not known and should be determined in future studies. In addition, biological validation of the RNA-Seq data on selected genes by RT-qPCR is also needed to confirm the results of this study.

Conclusion

Results of GSEA of whole blood transcriptome data in beef steers divergently selected for low or high RFI revealed differential expression of genes related to protein metabolism, erythrocytes take up/release carbon dioxide and release/take up oxygen, and stress responsiveness. These results are similar to those of several studies that utilized other tissues including liver, muscle, and ruminal epithelium. Thus, this study demonstrates the suitability of whole blood transcriptome data for understanding the biological mechanisms regulating RFI in animals. Due to the small number of animals used in this study and the effect of different diets and breeds on RFI ranking, further validation using a larger cohort of beef cattle fed different diets is needed to confirm these findings.

Tables and Figures

Table 4. 1 Altered pathways identified by Gene Set Enrichment Analysis in high-RFI compared to low-RFI beef steers

Pathway	FDR	NES	Gene set size (# of leading-edge genes)	Leading edge genes
Binding and uptake of ligands by scavenger receptors	0.01	1.82	4 (3)	*HBB, HBA1, HBA*
Scavenging of heme from plasma	0.01	1.82	4 (3)	*HBB, HBA1, HBA*
Erythrocytes take up carbon dioxide and release oxygen	0.01	1.68	3 (3)	*HBB, HBA1, HBA*
Erythrocytes take up oxygen and release carbon dioxide	0.01	1.68	3 (3)	*HBB, HBA1, HBA*
O_2/CO_2 exchange in erythrocytes	0.01	1.68	3 (3)	*HBB, HBA1, HBA*
Metabolism of proteins	0.01	-1.70	248 (85)	*LOC101907518, RPL39, LOC101902490, UBE2D1, FUCA2, B4GALT6, FBXL3, SOCS3, COMMD8, RPLP2, RPL34*
Cellular responses to external stimuli	0.01	-1.99	69 (19)	*HSPA1A, HSPH1, BAG2, DNAJA1, JUN, HSPA4, UBE2D1, DNAJB1, H3C13, H2BC7, H4C2, ELOC, ELOB, H2AC8, SIRT1, FLCN, ATP6V1G1, HSPA14, H2BU1*
Cellular responses to stress	0.01	-1.99	69 (19)	*HSPA1A, HSPH1, BAG2, DNAJA1, JUN, HSPA4, UBE2D1, DNAJB1, H3C13, H2BC7, H4C2, ELOC, ELOB, H2AC8, SIRT1, FLCN, ATP6V1G1, HSPA14, H2BU1*
Cellular response to heat stress	0.01	-2.05	16 (5)	*HSPA1A, HSPH1, BAG2, HSPA4, DNAJB1*
Regulation of HSF1-mediated heat shock response	0.01	-2.05	16 (5)	*HSPA1A, HSPH1, BAG2, HSPA4, DNAJB1*

High-RFI = feed inefficient beef steers; low-RFI = feed-efficient beef steers,
False discovery rate (FDR) ≤ 0.01; NES, normalized enrichment score (high-RFI vs. low-RFI).
Leading edge genes are those that are enriched within the gene set.
See (https://www.frontiersin.org/articles/10.3389/fvets.2022.848027/full) for the full list of leading edge genes associated with metabolism of proteins.

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Chapter 5

Chapter 5. Characterization of rumen microbiome and immune genes expression of crossbred beef steers with divergent residual feed intake phenotypes

Abstract

Objective: We investigated whole blood and hepatic mRNA expressions of immune genes and rumen microbiome of crossbred beef steers with divergent residual feed intake phenotype to identify relevant biological processes underpinning feed efficiency in beef cattle.

Methods: Low-RFI beef steers (n = 20; RFI = - 1.83 kg/d) and high-RFI beef steers (n = 20; RFI = + 2.12kg/d) were identified from a group of 108 growing crossbred beef steers (average BW = 282 ± 30.4 kg) fed a high-forage total mixed ration after a 70-d performance testing period. At the end of the 70-d testing period, liver biopsies and blood samples were collected for total RNA extraction and cDNA synthesis. Rumen fluid samples were also collected for analysis of the rumen microbial community.

Results: The mRNA expression of 84 genes related to innate and adaptive immunity was analyzed using pathway-focused PCR-based arrays. Differentially expressed genes were determined using *P*-value ≤ 0.05 and fold change (FC) ≥ 1.5 (in whole blood) or ≥ 2.0 (in the liver). Gene ontology analysis of the differentially expressed genes revealed that pathways related to pattern recognition receptor activity, positive regulation of phagocytosis, positive regulation of vitamin metabolic process, vascular endothelial growth factor production, positive regulation of epithelial tube formation and T-helper cell differentiation were significantly enriched (FDR < 0.05) in low-RFI steers. In the rumen, the relative abundance of *PeH15*, *Arthrobacter*, *Moryella*, *Weissella*, *and Muribaculaceae* was enriched in low-RFI steers, while *Methanobrevibacter, Bacteroidales_BS11_gut_group, Bacteroides and Clostridium_sensu_stricto_1* were reduced.

Conclusions: In conclusion, our study found that low-RFI beef steers exhibit increased mRNA expression of genes related to immune cell functions in whole blood and liver tissues, specifically those involved in pathogen recognition and phagocytosis regulation. Additionally, these low-RFI steers showed differences in the relative abundance of some microbial taxa which may partially account for their improved feed efficiency compared to high-RFI steers.

Introduction

A vital role in animal development and health status is a cascade of events between the gut microbiomes and the host organism (Zhou et al. 2017). Studies have shown that the rumen microbiota have a profound impact on the health, performance, and immune system of the host (Jami et al., 2014; Huws et al., 2018). Rumen microbiome has been implicated as one of the major contributors to the variation in host feed efficiency in ruminants (Shabat et al., 2016; Xue et al., 2022; Clemmons et al., 2022), due to their ability to produce the vast majority of energy precursors

(sugar, Acetyl CoA, lactate, H2) needed by the host animal coupled with other required micro-nutrients, such as all water-soluble vitamins (Hungate, 2013; Mizrahi et al., 2018).

Over the past few decades, priority has been given to feed efficiency in the beef production system owing to an ever-increasing demand for animal products coupled with associated economic and environmental significance (Hegarty et al., 2007). The most commonly used measure of feed efficiency in beef cattle is residual feed intake (RFI), which is the difference between observed feed intake and feed intake predicted from the animal's maintenance and needs (Koch et al., 1963; Muir et al., 2018). In comparison to high-RFI cattle, low-RFI cattle consume less feed while maintaining normal growth levels. Several studies have sought to understand the metabolic processes underlying variation in RFI (Alexandre et al., 2015; Olivieri et al., 2016; Fonseca et al., 2019). Some of the metabolic processes associated with RFI include energy metabolism, protein turnover, rumen microbial metabolism, and the immune system (Tizioto et al., 2016; Mukiibi et al., 2018). In fact, rumen microbial activities and fermentation can influence ruminants' performance, nutrient metabolism, and immune system (Jami et al., 2014; Huws et al., 2018).

Innate and adaptive immune responses have high metabolic demands involving the repartitioning of nutrients when exposed to environmental stressors (Hotamisligil and Erbay, 2008). Due to the energy cost associated with immune system activation, immune competence is suggested to be one of the major physiological processes that contribute to variation in RFI in Angus beef cattle (Herd et al., 2009). Despite these findings, differences in the metabolic demands of critical physiological processes in low- and high-RFI cattle such as immune responses and rumen microbiome have not been extensively studied. Furthermore, no studies have evaluated mRNA expression of innate and adaptive immunity-related genes and their associated regulatory pathways in beef steers' blood and liver with divergent RFI phenotypes. Investigating rumen

microbial community composition and diversity can provide insights into the mechanisms that regulate feed efficiency and help develop strategies to improve feed utilization and production efficiency in beef cattle. We hypothesized that selection for low- or high-RFI in beef cattle is associated with differences in hepatic and whole-blood immune gene expression and alteration in the relative abundance of rumen microbial taxa. Therefore, the objective of this study was to characterize the rumen microbiome and immune gene transcriptome of crossbred beef steers with divergent RFI phenotypes in order to gain insights into the mechanisms underlying differences in RFI.

Materials and Methods

Animals and RFI determination

The use of animals in this experiment was approved by the Institutional Animal Care and Use Committees of West Virginia University (protocol number 1608003693). This study involved feeding a high-forage total mixed ration (TMR; primarily consisting of corn silage; ground hay; and a ration balancing supplement; CP = 13.2%, NDF = 45.9% NDF, and NEg = 0.93 Mcal/kg) to 108 crossbred growing beef steers (average body weight of 282 ± 30.4 kg; age = 310 + 17 d) in a confinement dry lot equipped with GrowSafe intake nodes (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) for a total of 70 d. Steers had unrestricted access to the experimental diet and water. Individual steer's feed intake and daily BW were measured with GrowSafe automated feed intake and In-Pen Weighing Positions (IPW Positions, Vytelle LLC), respectively (MacNeil et al., 2021; Wells et al., 2021). Average daily gain (ADG) and metabolic mid-test BW (mid-test $BW^{0.75}$; MMTW) were regressed against daily DM intake. The following equation was used to calculate

RFI, which is the difference between the predicted value from the regression and the actual measured value: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$, where Y is the expected DMI (kg/d), β_0 is the regression intercept, β_1 and β_2 are the partial regression coefficients, X_1 is the MMTW (kg), X_2 is the ADG (kg/d), and ε indicates the RFI (kg/d) (Durunna et al., 2011). After calculating RFI values for all animals, the beef steers with the lowest RFI (n = 20; referred to as low-RFI) and the ones with the highest RFI (n = 20; referred to as high-RFI) were identified as the most and least efficient, respectively.

Blood, rumen fluid and liver biopsy collection

On day 70, 10 mL of blood was collected from each animal prior to morning feeding and placed into tubes containing sodium heparin. Subsequently, subsamples of 500 µL each were promptly transferred into RNA-protect tubes (Cat. No. 76554; Qiagen) that contains a reagent capable of lysing blood cells and stabilizing intracellular RNA. The samples were stored at -80°C until they were later analyzed. Liver biopsy procedure was also carried out on d 70 as described by Swanson et al., 2000. After excising the skin, liver tissue was extracted using a 14-gauge biopsy needle (TruCore-II Automatic Biopsy Instrument: Angiotech, Lausanne, Switzerland) and during a single puncture, approximately 1g of liver samples were obtained from each of the beef steers. Liver biopsy procedure was also carried out on d 70 as described by Swanson et al., 2000. After excising the skin, liver tissue was extracted using a 14-gauge biopsy needle (TruCore-II Automatic Biopsy Instrument: Angiotech, Lausanne, Switzerland) and during a single puncture, approximately 1g of liver samples were obtained from each of the beef steers. Liver samples were immediately stored in RNAprotect tissue tubes (Cat No: 76163; Qiagen, Germantown, MD) containing RNAprotect tissue reagent that immediately stabilizes RNA in tissue samples to

preserve the gene expression profile, and thereafter stored at -80°C until later analysis. Liver samples were immediately stored in RNAprotect tissue tubes (Cat No: 76163; Qiagen, Germantown, MD), and were immediately stored at -80°C until they were analyzed. On the same day (day 70), rumen fluid samples were collected 4 hr after feeding as described by Sidney et al., 2023. Briefly, an orally administered stomach tube connected to a vacuum pump (Ruminator; **profs-products.com**, Wittibreut, Bayern, Germany) was used. To reduce saliva contamination, the first 150 mL of the collected rumen fluid samples were discarded. Subsequently, approximately 200 mL of rumen fluid was collected and promptly stored at -80°C until later analysis.

DNA extraction, 16S rRNA sequencing and sequence analysis

The thawed rumen fluid samples were centrifuged at 15,000×g, and the resulting pellets were used for DNA extraction using a PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA). The concentration and purity of the extracted DNA were assessed using a NanoDrop 2000 UV-vis Spectrophotometer (Thermo Scientific, Wilmington, DE, United States). The integrity of DNA was tested using 0.7% agarose gel electrophoresis (Axygen Biosciences, Union City, CA, United States). The DNA samples were prepared for PCR using Qiagen QIAseq phased primers that target the V3/V4 regions of the 16S gene following the manufacturer's instruction (Qiagen; catalog number: 333845). The forward and reverse primer sequences are CCTACGGGNGGCWGCAG and GACTACHVGGGTATCTAATCC, respectively. Following the PCR amplicon cleaning, the samples were sequenced on a v3 MiSeq 600-cycle flowcell to generate 2×276 bp PE reads. After demultiplexing, quality control and adapter trimming of the raw sequence files using Illumina binary base call Convert v4.0. The demultiplexed fastq files generated were imported into Qiime2 (Bolyen et al., 2019) for subsequent analysis. Annotation of

the operational taxonomic units (OTUs) was performed using the greengenes database (v. 13.8) with a similarity threshold of 97% (DeSantis et al., 2006). Analyses of the OTU data were performed using MicrobiomeAnalyst platform (microbiomeanalyst.ca; Chong et al., 2020). First, cumulative-sum scaling and log2 transformation of the OTU abundance data were performed for normalization. Rarefaction curves, alpha diversity (Chao1 index) and beta diversity (Bray-Curtis distance matrix based on principal coordinates analysis (PCoA)) were generated. Differentially abundant taxa at the phylum and genus levels were analyzed and determined using the linear discriminant analysis (LDA) effect size method (LEfSe) based on Kruskal–Wallis test of $\alpha \leq 0.05$ and logarithmic LDA score cut-off of 2.0.

RNA extraction, cDNA synthesis and immune gene expression

Total RNA was isolated from the liver and whole blood samples using RNeasy Micro Kit (Cat No: 74004; Qiagen) and RNeasy Protect Animal Blood kit (Cat. No. 73224; Qiagen). RNA concentration was measured using a NanoDrop One C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All RNA samples with RNA integrity numbers > 8.0 and an A260:A280 ratio of 1.8 to 2.0 were used to synthesize cDNA using RT^2 First Strand Kit (Cat. No. 330401; Qiagen). The expression of 84 genes associated with innate and adaptive immune responses was analyzed using the cow RT2 Profiler PCR Array (PABT-052ZA; Qiagen) according to the manufacturer's instructions. Detailed description of the RT2 Profiler PCR Array has been published in an earlier study from our lab. Briefly, real-time PCR analysis was carried out on a QuantStudio 5 Block Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following cycling conditions: 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s and 60 °C 1 min.

Gene expression, gene ontology and pathway analyses

The Qiagen platform-web GeneGlobe (https://www.qiagen.com) was utilized for analyzing the immune gene expression data. Relative quantification of the gene expression was determined using the comparative cycle threshold (Ct) method (Pfaffl, 2001). To determine the differential mRNA expression between the low- and high-RFI beef steers, the delta-delta-Ct (ΔΔCt) method was employed, with normalization of the raw data using the geometric mean of the five housekeeping genes, as described by Pfaffl (2001). The mRNA expression with *P*-value ≤ 0.05 and fold change (FC) ≥ 1.5 (in blood) and ≥ 2.0 (in liver) were considered to be differentially expressed. Gene ontology (GO) terms and pathways analyses of differentially expressed genes were performed using a web-based geneontology software (http://www.geneontology.org) as described by Ashburner et al. 2000. Significantly enriched pathways among the differentially expressed genes were catalogued using FDR value of 0.05.

Results

Growth performance of the low and high-RFI beef steers

The RFI values of low- and high-RFI steers were –1.83 kg/d and +2.12 kg/d ($P = 0.001$, SE = 0.41), respectively. The initial BW, final BW, and ADG were not different between the two groups ($P > 0.05$); however, low-RFI steers had lower ($P = 0.01$) DMI and feed:gain ratio compared to the high-RFI steers (Table 5.1).

Sequencing results and rumen microbial community

The high-throughput sequencing yielded approximately 166,378 ± 22,215 reads per sample. The rarefaction analysis revealed that the number of sequences utilized for all the samples

was sufficient to ascertain the overall number of sequence types. To identify the differentially abundant taxa mostly affected between the two groups of steers, we compared the rumen microbial population using the metagenomic biomarker discovery approach, LEfSe. This method employs a nonparametric Wilcoxon sum-rank test, followed by linear discriminant analysis, to evaluate the effect size of each differentially abundant taxon. At the phylum level, the microbial community composition of the rumen samples was predominantly composed of Bacteroidota and Firmicutes (Figure 5.1). There was no difference in alpha (Figure 5.2; $P = 0.31$ or beta (Figure 5.3; $P = 0.53$) diversity indices between the two groups of beef steers. Likewise, there was no treatment effects found at the phylum level. At the genus level, the relative abundance of *PeH15, Arthrobacter, Moryella, Weissella* and *Muribaculaceae* were enriched in low-RFI steers, while *Methanobrevibacter, Bacteroidales_BS11_gut_group, Bacteroides* and *Clostridium_sensu_stricto_1* were reduced (Figure 5.4). The relative abundance of *Clostridium sensu stricto 1, Bacteroides, Bacteriodales_BS11_gut_group* were reduced in the low-RFI steers while those of *Weissella, PeH15, Arthrobacter Muribaculaceae and Moryella* were greater compared to the high-RFI steers (Figure 5.4).

Whole-blood and hepatic immune gene expression

To assess the differential expression of both innate and adaptive immune genes between the low-RFI and high-RFI steers, we utilized 84 gene array panel for transcriptome analysis. The genes having P-value ≤ 0.05 and FC ≥ 1.5 or 2.0 in the blood or liver, respectively, were considered differentially expressed as presented in Tables 5.2 and 5.3. Comparing differential gene expression between low-RFI and high-RFI steers, out of the 84 genes analyzed, only eight were significantly upregulated in the blood (Table 2) and twenty in the liver of low-RFI steers (Table 5.3).

Interestingly, five of these differentially expressed genes (IL17A, CXCL10, MPO, IL2 and LY96) had overlapping expression in both the blood and liver (Figure 5.5).

Gene ontology (GO) and functional pathways

Functional analysis, pathway and GO enrichment of the DE genes revealed top 15 or 20 most significant pathways in whole blood or liver respectively (Tables 5.4 and 5.5). From the whole blood transcriptome gene set, the topmost enriched pathways are directly related to pattern recognition receptor signaling, positive regulation of tumor necrosis factor production, macrophage activation and differentiation, and positive regulation of interleukin-10 production among others (Table 5.4). Of interest, LY96, IL2, IL15 and IL17A were most common in several pathways. While in the liver, most significantly enriched pathways include positive regulation of immunoglobulin production, positive regulation of interleukin-13 production, vascular endothelial growth factor production, and regulation of complement-dependent cytotoxicity (Table 5.5). Additionally, we found IL2, CSF2 and IL17A, some of the most upregulated genes in the blood of low-RFI to be connected to the production and regulation of interleukin-17 and interleukin-23 production pathways.

Discussion

This study determined the rumen microbiome and immune gene expression profile of beef steers with divergent RFI using 16S rRNA gene sequencing and targeted transcriptome analyses, respectively. The results of our study revealed a lower relative abundance of *Methanobrevibacter*, a genus of archaea that belongs to the *Methanobacteriaceae* family, in low-RFI compared to high-RFI beef steers. This might imply that the low-RFI steers could partition their methane production

via alternative pathways especially when a lower proportion of H_2 and CO_2 is being produced during the fermentation process by the rest of microbiota. For instance, carbohydrates are fermented to propionic acid with no net loss of CO_2 and thus lower substrate for *Methanobrevibacter* to produce methane (Ungerfeld, 2020; Pereira et al., 2022). Previous studies have shown that *Methanobrevibacter* are predominant methanogens in the rumen of ruminants and their abundance has also been correlated with higher levels of methane emissions (Berchielli et al., 2017; de Jesus et al., 2019) and poorer feed efficiency (Zhou et al., 2010; Lopes et al., 2021). Cattle with negative RFI phenotype have been reported to have reduced daily methane production (Hegarty et al., 2007). In addition, we noted that the relative abundance of *Muribaculaceae and Moryella* were greater in Low-RFI beef cattle. *Muribaculaceae* is a family of bacteria that produces enzymes capable of degrading complex carbohydrates and has been reported to produce short-chain fatty acids (Barouei et al., 2017; Obanda et al., 2018), which play important roles in regulating immune function and energy metabolism. A recent study revealed that the abundance of *Muribaculaceae* in the rumen is positively correlated with feed efficiency and other production traits such as milk components (Jiang et al., 2020) and negatively correlated with methane production in Holstein dairy cows (Cunha et al. 2017).

As seen in our result, the relative abundance of *Moryella* was greater and that *Clostridium_sensu_stricto_1* was lower in low- compared to high-RFI. Previous studies have shown that species of *Moryella* play a key role in the breakdown of complex carbohydrates and the production of volatile fatty acids (VFAs) such as acetate, propionate, and butyrate, which are important energy sources that support improved health and performance of ruminants (Carlier et al., 2007; Hu et al., 2020). A study correlating individual RFI values with bacterial abundances in feces reported that *Clostridium* I is associated with high RFI in chickens (Siegerstetter et al., 2018;

Liu et al., 2021). In fact, an overgrowth of *Clostridium sensu stricto 1* was reported to be associated with necrotic enteritis in human subjects, consequently depicting unhealthy microbiota (Lakshminarayanan et al., 2013; Yang et al., 2019). Therefore, the lower relative abundance of *Clostridium_sensu_stricto_1* in low-RFI beef steers might suggest a robust and healthy microbiome which might translate to better use of nutrients.

Of outmost importance, we provide the first evidence of increased relative abundance of *Weissella, PeH15* and *Arthrobacter* in the low-RFI steers. These genera have been identified as probiotics with immune-boosting potential in humans, fish and chicken (Li et al., 2006; Lee et al., 2012). Probiotics in ruminants influence enzyme production leading to efficient digestion of nutrients, improved growth and performance and robust immunity (Arowolo and He, 2018; Idowu et al., 2022; Kulkarni et al., 2022). In this sense, greater relative abundance of *Weissella, PeH15* and *Arthrobacter* in the rumen of low-RFI group suggest a possible role in activation and initialization of immunomodulatory properties, improved growth, and feed efficiency enhancement. Immune response is related to cascades of metabolic processes and require high metabolic demands. This is also largely connected to the probiotic activities of rumen microbiome.

Due to the energy cost associated with immune system activation, immune competence is suggested to be one of the major physiological processes that contributes to variation in RFI in Angus beef cattle (Schmid-Hempel, 2003; Hine et al., 2021). Our study showed that certain innate immune genes such as LY96, TLR4 and MBL-2 which play a significant role in detection of lipopolysaccharide, pattern recognition receptor, microphage differentiation and positive regulation of phagocytosis were found upregulated in the blood and liver of low-RFI beef steers with divergent RFI phenotypes. This is important in the initial pathogen recognition and subsequent activation of downstream immune signaling pathways that recruit the adaptive immune

response. In addition, toll-like receptors (TLRs), nod-like receptors (NLRs), scavenger receptors, and C-type lectin receptors are pattern-recognition receptors that play a vital role in maintaining pathogen specificity and consequent protection against microbial invasion (Takeuchi et al., 1999; Takeuchi et al., 2002). Therefore, the enrichment of pathways including pattern recognition receptor, lipopolysaccharide-mediated signaling pathway, microphage differentiation and positive regulation of phagocytosis in our study may suggest that low-RFI steers possess a better mechanism for pathogen recognition, reduction of endotoxin and other bacterial products both in the systemic circulation and the hepatocytes. Observed upregulation in expression levels of LY96, TLR4 and MBL-2 and their associated pathways in the liver of low-RFI animals is reasonable because the liver is constantly exposed to gut-derived bacterial products and endotoxins through its main blood supply, the portal vein and is rich in Kupffer cells which helps in detoxification of endotoxins leading to increased concentration of circulating endotoxin with consequent systemic inflammation (Dixon et al., 2013; Nakamoto and Kanai, 2013). This immunological imbalance impairs efficient partitioning of nutrients leaving livestock in poor condition of growth and performance (Sordillo, 2016).

Pathways such as positive regulation of cytokine production involved in inflammatory response and vascular endothelial growth factor production were enriched, and vital pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF-α), CASP1 (interlukine-1 convertase), interlukine-6 (IL-6), C-X-C motif chemokine ligand 10 (CXCL10) and interferon beta and gamma (IFN-β /γ) were found to be differentially upregulated both in the blood and liver of low-RFI beef steers. Interestingly, we also found that interferon (IFN-β /γ), interferon gamma-induced protein 10 (CXCL10 or IP-10) and their associated pathways were enriched in low-RFI compared to high-RFI beef steers. Taken together, our results indicated that the upregulated pro-

inflammatory cytokines coupled with other innate immune genes mediate complex signaling cascade of events in low-RFI beef steers towards recognizing, binding, and marking of pathogens for destruction while maintaining cellular homeostasis. These further suggest a robust innate immune system in low-RFI steers, capable of initiating a prompt response against foreign entities compared to high-RFI steers.

The GO terms associated with positive regulation of immunoglobulin production, T cell differentiation involved in immune response**,** and other vital metabolic process were the most overrepresented pathways for differentially co-expressed genes such as GATA3, IL6, TBX21, IL4 and MEF2C including the enrichment of alpha-beta T cell differentiation in the liver of low-RFI beef steers. These pathways might suggest that the animals possess a robust adaptive immune mechanism for balancing both the catabolic-and anabolic-immune pathways despite their lower dry matter intake. Overall, we showed a significant correlation between the microbial community, immune response and divergent RFI phenotypes. Mostly, dietary nutrients are partitioned towards the immune related processes rather than being used for growth and thus reduces animal's feed efficiency. This is extremely relevant for immune-metabolic axis in livestock. (Johnson, 1997; Spurlock, 1997; Patience et al., 2015). Therefore, the upregulation of our immune genes set and the enriched pathways in both the blood and liver of the low-RFI beef steers suggest that low-RFI beef steers possess a mechanism that allows for a prompt response to pathogen or any other foreign substances and consequently showcase a robust repertoire of both innate- and adaptive-immunity compared to high-RFI beef steers.

Conclusion

In summary, our study demonstrates that low-RFI beef cattle possess a robust and efficient immune response to inflammation, characterized by the upregulation of genes involved in pathogen recognition, intracellular signaling, activation of antimicrobial mechanisms, and phagocytotic killing. These animals exhibit a superior ability to quickly eliminate pathogens and effectively compared to their high-RFI counterparts. Additionally, the relative abundance of *Methanobrevibacter* was lower in low-RFI beef steers, which was probably associated with a reduced methane production. The increased abundance of *Weissella, PeH15,* and *Arthrobacter* in low-RFI steers suggests a potential role of these taxa in the rumen microbiome in initiating immunomodulatory properties, improved growth, and feed efficiency. Future studies utilizing larger cohorts of steers are needed to further investigate the functional characterization of rumen microbes that may be important for the immune system efficiency and nutrient-harvesting in ruminants.

Tables and Figures

Table 5. 1 Growth performance of the low and high-RFI beef steers

Parameters	**[1]Low-RFI**	**[2]High-RFI**	**SE**	***P*-value**
RFI, Kg/d	-1.83	2.12	0.41	0.01
Initial BW, Kg	313	345	10.18	0.14
Final BW, Kg	430	467	12.92	0.19
ADG, Kg/d	1.68	1.74	0.05	0.60
DMI, Kg/d	9.02	11.5	0.33	0.01
F:G	2.38	2.98	0.09	0.01

[1]Low-RFI = feed-efficient beef steers, [2]High-RFI = feed inefficient beef steers. ADG, average daily gain; DMI, dry matter intake; BW, body weight; F: G, feed: gain ratio; SE, standard error of mean.

Table 5. 2 Fold change of whole blood innate and adaptive immune genes expression in low- compared with high-RFI steers[1].

Gene symbol	**Gene name**	**FC[2]**
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	24.22
IL17A	Interleukin 17A	19.13
IL2	Interleukin 2	3.86
MBL2	Mannose-binding lectin (protein C) 2, soluble	2.21
MPO	Myeloperoxidase	1.91
LY96	Lymphocyte antigen 96	1.89
CXCL10	Chemokine (C-X-C motif) ligand 10	1.75
IL15	Interleukin 15	1.60

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers.

[2]Fold change (FC; relative to high-RFI)

Table 5. 3 Fold change of hepatic innate and adaptive immune genes expression in low- compared with high-RFI steers[1].

Gene symbol	Gene name	FC[2]
IL2	Interleukin 2	36.08
IFNB1	Interferon, beta 1, fibroblast	23.09
TNF	Tumor necrosis factor	22.67
CXCL8	Interleukin 8	11.53
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	5.88
IL4	Interleukin 4	5.48
CD40LG	CD40 ligand	5.02
IL17A	Interleukin 17A	4.82
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	4.67
TLR5	Toll-like receptor 5	4.65
MPO	Myeloperoxidase	4.46
IL13	Interleukin 13	4.36
CXCL10	Chemokine (C-X-C motif) ligand 10	4.36
LYZ	Lysozyme	4.10
IFNG	Interferon, gamma	4.00
LY96	Lymphocyte antigen 96	3.51
STAT4	Signal transducer and activator of transcription 4	3.49
TBX21	T-box 21	3.46
IL6	Interleukin 6 (interferon, beta 2)	3.39
GATA	GATA binding protein 3	3.35

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers.

[2]Fold change (FC; relative to high-RFI).

Table 5. 4 Gene ontology showing enriched biological processes, molecular functions, and cellular components of whole blood innate and adaptive immune genes in low- compared with high-RFI steers[1].

	Gene(s)	**Raw *P*-value**	**FDR[2]**
GO biological process			
Detection of lipopolysaccharide	LY96	3.54E-06	1.70E-03
Positive regulation of interleukin-23 production	CSF2, IL17A	4.55E-06	2.12E-03
Positive regulation of interleukin-17 production	IL2, IL15	2.65E-05	7.34E-03
Macrophage differentiation	IL15, CSF2	2.65E-05	7.20E-03
Toll-like receptor 4 signaling pathway	LY96	3.19E-05	8.36E-03
Positive regulation of tyrosine phosphorylation of STAT protein	IL2, IL15, CSF2	6.79E-07	6.52E-04
Receptor signaling pathway via JAK-STAT	IL15, CSF2	7.06E-05	1.43E-02
Defense response to fungus	MPO, IL17A	8.37E-05	1.59E-02
Positive regulation of tumor necrosis factor production	LY96, IL17A	2.01E-06	1.16E-03
Pattern recognition receptor signaling pathway	LY96, MBL2	2.58E-06	1.38E-03
Positive regulation of phagocytosis	IL15, MBL2	2.00E-04	3.31E-02
GO molecular function			
Toll-like receptor 4 binding	LY96	1.90E-06	1.42E-03
Lipopolysaccharide immune receptor activity	LY96	3.54E-06	1.99E-03
Pattern recognition receptor activity	LY96, MBL-2	2.91E-05	1.31E-02
GO cellular component			
Lipopolysaccharide receptor complex	LY96	4.55E-06	2.85E-03

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers.

[2]False discovery rate (FDR; relative to high-RFI)

Table 5. 5 Gene ontology showing enriched biological processes, molecular functions, and cellular components of hepatic innate and adaptive immune genes in low- compared with high-RFI steers[1].

	Gene (s)	**Raw *P*-value**	**FDR[2]**
GO biological process			
Positive regulation of immunoglobulin production	IL13, IL6, TBX21, IL4, IL	1.23E-09	2.42E-07
Positive regulation of interleukin-10 production	IL13, IL6, CD40LG, IL4,	3.75E-08	5.62E-06
T-helper cell differentiation	GATA3, IL6, TBX21, IL4	5.39E-08	7.68E-06
Macrophage activation	IL13, IFNG, IL4, TNF	9.23E-08	1.19E-05
Positive regulation of interleukin-13 production	GATA3, IL4	1.67E-07	1.96E-05
Vascular endothelial growth factor production	TNF, IL6, IFN	2.22E-07	2.46E-05
Microglial cell activation	IFNG, IL4, TNF	5.63E-07	5.55E-05
Positive regulation of cytokine production involved in inflammatory response	IL6, TNF, IL17A	2.30E-06	1.80E-04
Regulation of acute inflammatory response	IL6, IL4, TNF	2.92E-06	2.19E-04
Regulation of complement-dependent cytotoxicity	IL13, IL4	6.31E-06	4.07E-04
Wnt signaling pathway involved in kidney development	GATA3	1.05E-05	6.22E-04
Positive regulation of vitamin metabolic process	IFNG, TNF	1.05E-05	6.36E-04
Detection of lipopolysaccharide	LY96	2.94E-05	1.50E-03
Positive regulation of mast cell activation involved in immune response	IFNG, TNF	6.91E-05	2.96E-03
Positive regulation of interleukin-23 production	GATA3	3.78E-05	1.82E-03
Positive regulation of interleukin-5 production	GATA3	3.78E-05	1.82E-03
Positive regulation of isotype switching to IgG isotypes	TBX21, IL4	4.72E-05	2.17E-03
GO molecular function			
Tumor necrosis factor receptor binding	TNF, CD40LG,	3.51E-10	1.97E-07
CD40 receptor binding	CD40LG	3.54E-08	1.59E-05
CXCR chemokine receptor binding	CXCL8, CXCL10	9.73E-07	3.65E-04
Interleukin-8 receptor binding	CXCL8,	6.31E-06	2.18E-03
Toll-like receptor 4 binding	LY96	1.58E-05	4.73E-03
Interleukin-2 receptor binding	GATA3, IL2	2.20E-05	5.83E-03
Lipopolysaccharide immune receptor activity	LY96	2.94E-05	7.34E-03
Toll-like receptor binding	LY96	9.51E-05	2.14E-02
GO cellular component			
Lipopolysaccharide receptor complex	LY96	3.78E-05	1.77E-02

[1]Low-RFI = feed-efficient beef steers, high-RFI = feed inefficient beef steers.

[2]False discovery rate (FDR; relative to high-RFI)

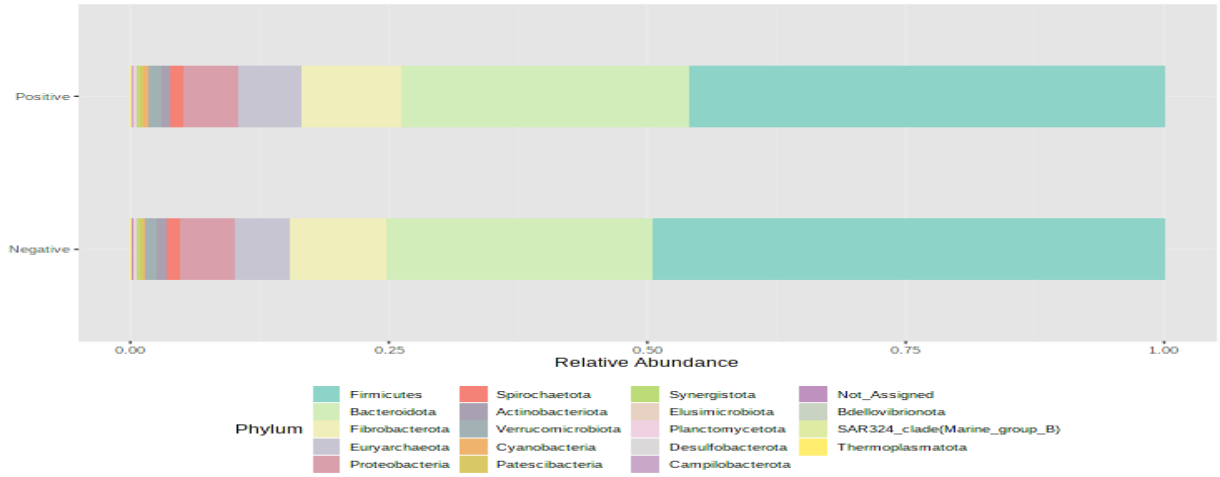

Figure 5. 1 Relative abundance of rumen microbial taxa at the phylum level in beef steers with divergent residual feed intake phenotypes.

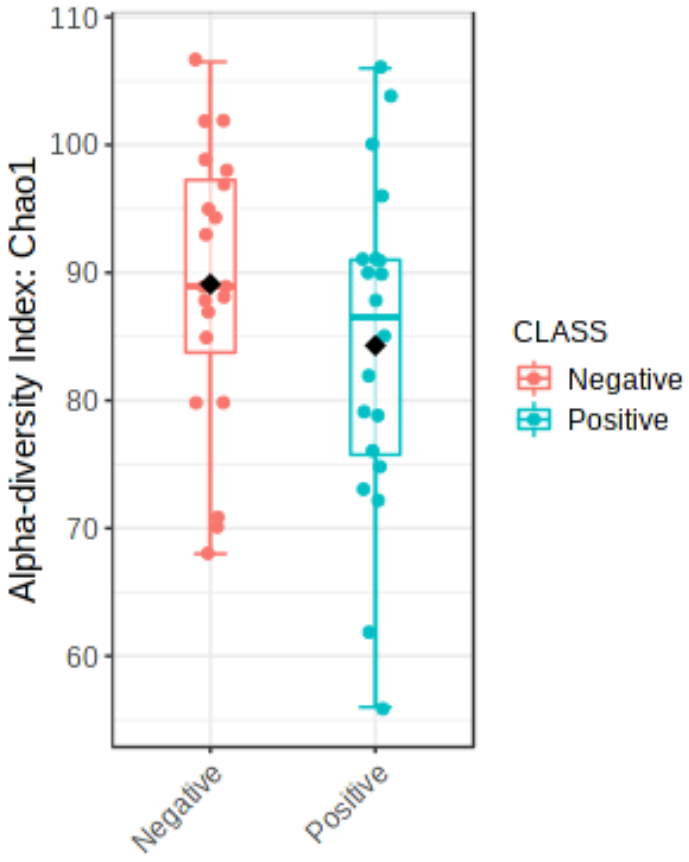

Figure 5. 2 Alpha diversity index (Chao1) of rumen microbial taxa in beef steers with divergent residual feed intake phenotypes (P-value = 0.31).

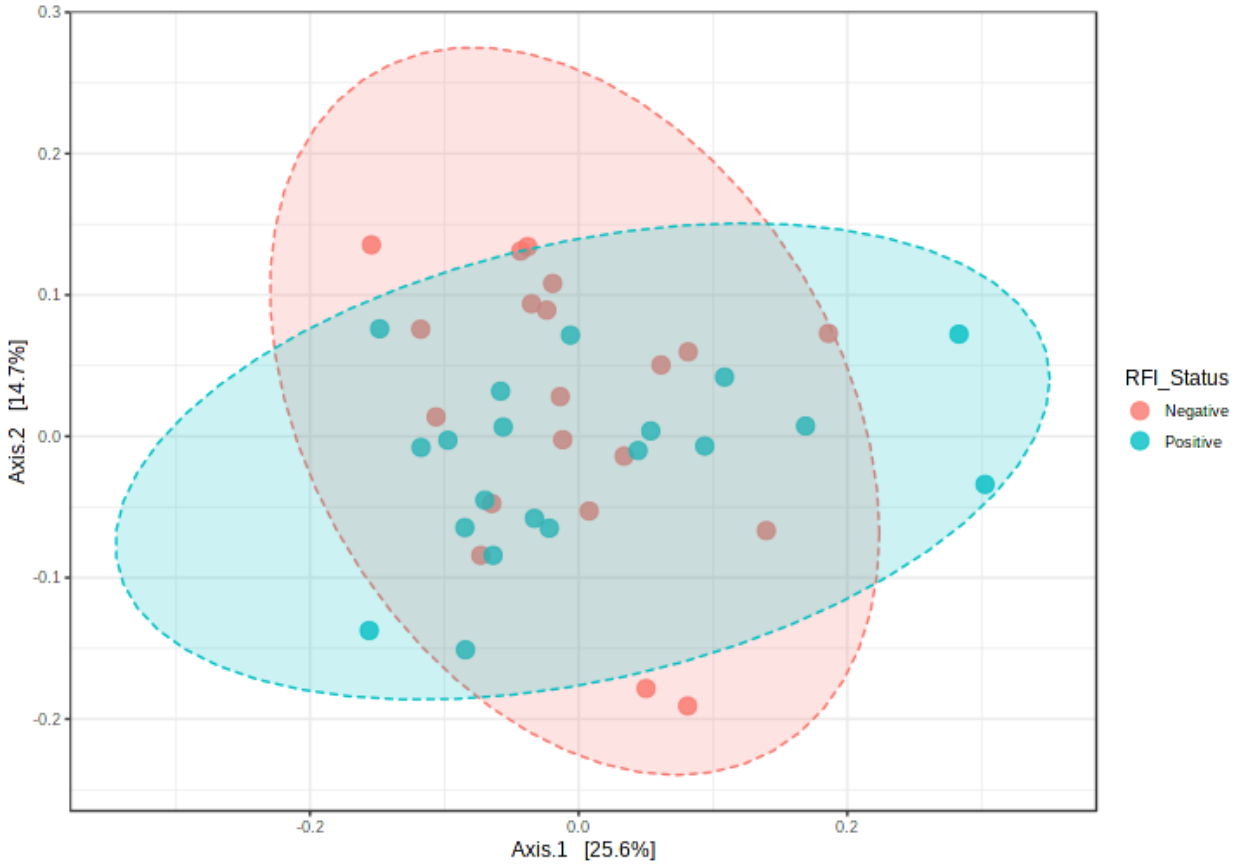

Figure 5. 3 Principal coordinates analysis (PCoA) of ruminal microbiota based on an unweighted unifrac distance (Beta diversity P = 0.53).

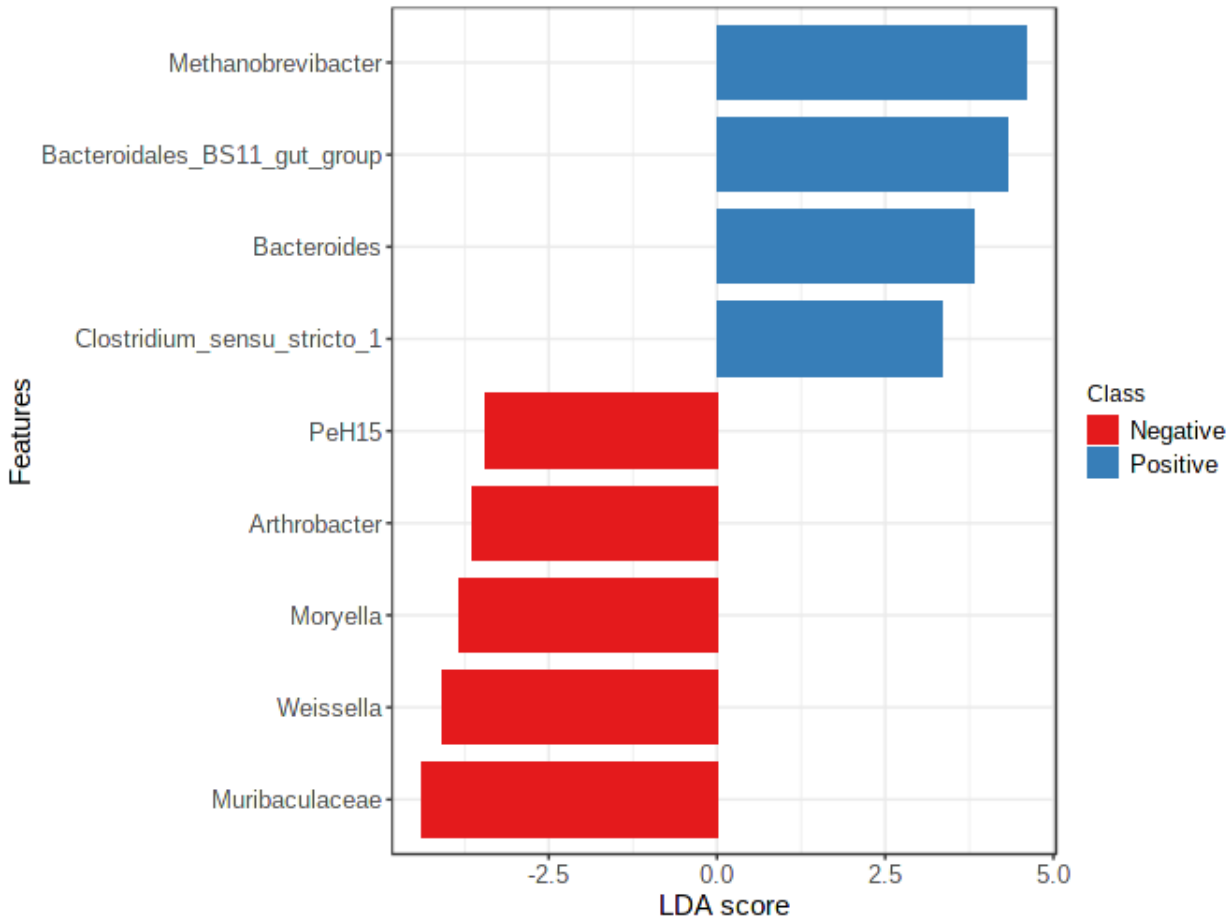

Figure 5. 4 Linear discriminant analysis effect size (LEfSe) of rumen microbiota of beef steer with divergent residual feed intake phenotypes. The linear discriminant analysis plot indicates the most differentially abundant taxa found by ranking according to their effect size (≥ 2.0) at the genus.

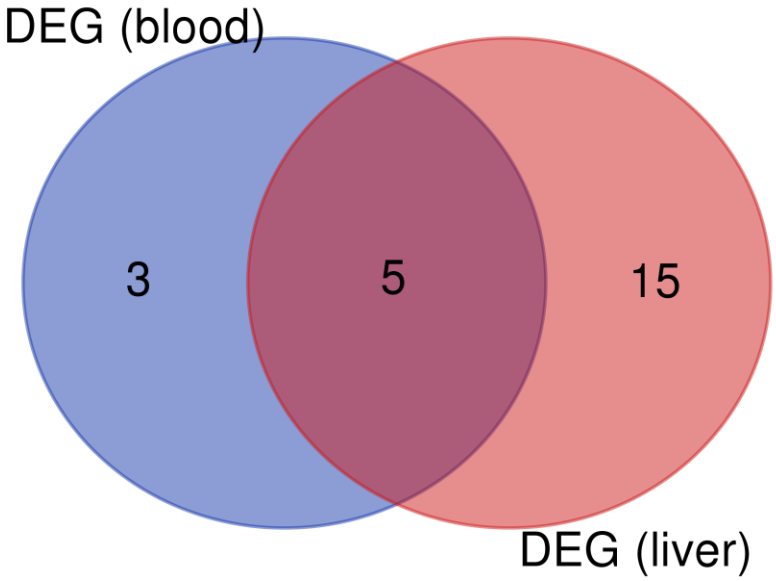

Figure 5. 5 Differentially expressed whole blood and liver innate and adaptive immune genes in low- compared with high-RFI steers. The overlapping region of the diagram represents the differentially expressed genes (IL17A, CXCL10, MPO, IL2, and LY96) detected in both the whole blood and liver of low-RFI steers.

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Chapter 6

Chapter 6. Plasma metabolites as predictive biomarkers for residual feed intake phenotype in crossbred beef cattle

Abstract

Objective: To investigate the potential of plasma metabolites as predictive biomarkers for Residual Feed Intake (RFI) in beef cattle.

Methods: A total of 67 crossbred growing beef steers (BW = 277 ± 29.7 kg) were fed high-forage total mixed ration for 64 days intake system to determine their RFI phenotype. On d 0 and 1, blood samples (5 mL) were collected from all the beef steers in the morning before feeding from the jugular vein into blood tubes containing sodium heparin for the preparation of plasma. At the end of the 64-d feeding trial, all the beef steers were divided into two groups based on their RFI values: low (or negative)-RFI beef steers (n = 28; RFI = -1.08 ± 0.88 kg/d) and high (or positive)-RFI beef steers (n = 39; RFI = 1.21 ± 0.92 kg/d). Plasma samples from all the beef steers were analyzed using Nuclear Magnetic Resonance spectroscopy to quantify 46 metabolites. Data analysis involved univariate and multivariate statistical methods, including volcano plot analysis, area under the receiver operating characteristic (ROC) curve analysis, and partial least squares discriminant analysis (PLS-DA).

Results: The results revealed a distinct metabolomics profile associated with RFI status. Eight differentially abundant metabolites were identified between low- and high-RFI groups, including amino acids (tyrosine, glycine, valine, leucine, and methionine) and other compounds (dimethyl sulfone, 3-hydroxy isovaleric acid, citric acid, creatine, and L-carnitine). Plasma concentrations of tyrosine, glycine, and dimethyl sulfone exhibited sufficient specificity and sensitivity, making

them eligible as predictive markers for RFI in this study. Logistic regression analysis using these biomarkers generated a model that effectively distinguished high- and low-RFI steers, with a threshold cutoff point of 0.48.

Conclusions: The use of plasma metabolites, particularly amino acids such as tyrosine, glycine, and dimethyl sulfone hold economic significance as cost-effective tools for predicting RFI in beef cattle. The logistic regression model incorporating tyrosine, glycine, and dimethyl sulfone shows promise for categorizing RFI values.

Introduction

Significant research efforts have been dedicated to enhancing the feed efficiency of beef cattle with the primary objective of improving profitability, productivity, health, and environmental sustainability of livestock production (Arthur et al., 2001; Maia de Souza et al., 2017; see chapter 2). Among the various aspects of feed efficiency, residual feed intake (RFI) has gained considerable attention for several years. Residual feed intake (RFI) as a measure of feed efficiency accounts for the difference between an animal's actual intake and its expected intake requirements for growth and maintenance (Koch et al., 1963; Archer et al., 1999). Low-RFI cattle consume less feed than expected for the same level of production compared to high-RFI cattle (Richardson et al., 2001). Despite the positive attributes associated with RFI as a feed efficiency measure, obtaining RFI data is laborious and expensive, and this has limited its spread as a feed efficiency measurement (Basarab et al., 2003; Wang et al., 2006; Foroutan et al., 2020).

Exploring a simple and low-cost measure that could comparatively predict the efficiency of animals using an analytical technique in animals offers a cost-effective alternative to manual RFI measurement. Metabolomics and its associated statistical analyses provide robust and holistic

insights into metabolites and their interactions in a biological system (Wang et al., 2010; See chapter 2; Qiu et al., 2023). Several studies have applied metabolomics to identify potential serum or plasma biomarkers that are associated with RFI in beef cattle (Kelly et al., 2010; Fitzsimons et al., 2013). In fact, metabolites associated with amino acid metabolism such as creatine, methionine, choro-lysine, and urea are reported to be associated with RFI in several studies (Karisa et al., 2014; Jorge-Smeding et al., 2019; See chapter 2). Earlier studies were focused on identifying the differentially abundant metabolites in beef cattle with divergent RFI values. However, a mathematical model for predicting RFI in beef steers from plasma biomarkers has not been fully described. Therefore, the objective of this study was to evaluate plasma metabolites as potential biomarkers for predicting RFI in crossbred beef cattle.

Materials and Methods

Animals, feeding, RFI determination

The research procedures employed in this study were approved by the Institutional Animal Care and Use Committees of West Virginia University (protocol number 22-103). A total of 67 growing crossbred beef steers with an average BW of 277 ± 29.7 kg were fed a high-forage total mixed ration (TMR; primarily consisting of corn silage; cracked corn; grass baleage and a ration balancing; Data not shown) for 64 d (including a 15-d adjustment period) to determine their RFI phenotype. The beef steers were housed in four confinement dry lot pens measuring 15 by 47 m^2 each. Each pen was equipped with two GrowSafe 8000 feeding nodes (manufactured by GrowSafe Systems Ltd., located in Airdrie, Alberta, Canada) to monitor individual feed intake. Additionally, two In-Pen Weighing Positions (IPW Positions, developed by Vytelle LLC) were installed in each pen to measure the daily BW of the steers (Wells et al., 2021; MacNeil et al., 2021), respectively.

On d 0 and 1 of the adjustment period, blood samples (5 mL) were collected from all the beef steers in the morning before feeding from the jugular vein into blood tubes containing sodium heparin. Blood samples were immediately place in ice onsite, transported to the lab and centrifuged at 2500× g at 4 ◦C for 15 min for plasma preparation. Plasma samples from each beef steer were composited and subsequently stored -80°C until further analysis.

At the end of the feeding trial, the RFI values of the beef steers were determined as described previously (See chapter 2). Briefly, daily BW was regressed on time to calculate the beginning BW, mid-test BW, and average daily gain (ADG) of each animal. Thereafter, ADG and metabolic mid-test BW (mid-test $BW^{0.75}$) were regressed against individual daily DMI, and RFI was calculated as described by Durunna et al. (2011). At the end of the experiment, 28 beef steers were identified as low (negative) RFI (-1.08 ± 0.88 kg/d) and 39 beef steers were identified as high (positive) RFI (1.21 ± 0.92 kg/d)

NMR-based metabolome analysis of plasma samples

Nuclear Magnetic Resonance (NMR) spectroscopy was utilized to conduct metabolome analysis on all plasma samples. This technique enabled the measurement of 47 metabolites, including amino acids, hexoses, organic acids, carnitines, and lipids. The procedures for plasma sample preparation and NMR spectral analysis followed the previously published protocols by Ogunade et al. (2018). Briefly, a deproteinization process was carried out using ultra-filtration, following the method outlined by Psychogios et al. (2011), to eliminate larger molecules such as proteins and lipoproteins. Then, 160 µL of the sample was mixed with 40 µL of a standard buffer solution composed of 54% D2O and 46% 250 mM KH2PO4 at pH 7.0. The resulting plasma sample (200 µL) was transferred to an NMR tube for spectral analysis. All 1H-NMR spectra were acquired using a 700 MHz Avance III spectrometer (Bruker) equipped with a pulsed-field gradient

cryoprobe. The obtained 1H-NMR spectra were processed and analyzed using Bayesil, an analysis software for quantitative analysis of NMR spectra, as outlined by Ravanbakhsh et al. (2015). An additional examination and verification process was conducted by an NMR spectroscopist to ensure accuracy in compound identification and quantification.

Data and statistical analysis

Metabolome data was analyzed using MetaboAnalyst 5.0 software (Chong et al., 2019). Prior to the statistical analysis, the data were log-transformed and autoscaled. A volcano plot analysis (univariate analysis: t-test and fold-change values) was combined with the area under the receiver operating characteristic (ROC) curve analysis (multivariate statistics) to identify the differentially abundant biomarkers that distinguish the beef steers with low RFI from those with high RFI. Differentially abundant predictive biomarkers were identified at $P \leq 0.05$ and AUC > 0.70. Partial least squares discriminant analysis (PLS-DA) was also performed to visualize the difference between the two groups of beef steers. A logistic regression model was employed to investigate the predictive ability of the biomarkers. The performance of the biomarker regression models was assessed by interpreting the area under the ROC curve, aiming to identify the optimal cut-off point that maximizes both sensitivity and specificity. To ensure the reliability of the ROC curve model, a permutation test was conducted with 1000 randomized permutations for validation purposes.

Results

A total number of 46 metabolites were quantified (Data not shown). The PLS-DA plot showed a slight separation between the two groups of beef cattle using the first two principal components with 15.3% and 11.3% of explained variance (Figure 6.1), indicating altered

metabolome of the beef steers based on their RFI status. A total of 8 differentially abundant ($P \leq 0.05$) metabolites were detected between the low- and high-RFI steers (Figure 6.2). Compared to high-RFI steers, plasma concentrations of 6 metabolites (dimethyl sulfone, 3-hydroxy isovaleric acid, citric acid, valine, leucine, and methionine) were greater ($P \leq 0.05$) in low-RFI beef steers whereas 3 metabolites (creatine, L-carnitine and glycine) were lower (P-value ≤ 0.05; Table 6.1), compared to high-RFI beef steers. To identify the differentially abundant metabolites with the greatest contribution to the separation between the two groups of beef steers, we applied a biomarker analysis using the ROC curve as calculated by the ROCCET web server. The results of the ROC analysis revealed that three metabolites (tyrosine, glycine, and dimethyl sulfone) with respective AUC values of 0.747, 0.728, and 0.720 had sufficient specificity and sensitivity to qualify as the biomarkers for predicting RFI in this study (Figure 6.3).

Discussion

These results align with previous studies that have shown a correlation between amino acid metabolism and the RFI status of beef cattle. Jorge-Smeding et al. (2017) revealed that plasma metabolites related to the urea cycle, such as ornithine, aspartate, lysine, and valine, were associated with RFI in Charolais heifers. Furthermore, our previous study in chapter 2 demonstrated that the amino acid metabolic pathway was the most significant pathway linked to divergent RFI phenotypes in beef cattle fed a high-forage diet.

In a separate study conducted in our lab as shown in chapter 4, whole blood transcriptome analysis and gene set enrichment analysis were utilized to identify pathways linked to divergent selection for low or high RFI in beef cattle. The results demonstrated that amino acid metabolism is the most significantly affected metabolic pathway, as indicated by the number of leading-edge genes associated with this pathway. Compared to high-RFI beef steers, plasma concentrations of

tyrosine and dimethyl sulfone were greater in low-RFI beef steers and were identified as predictive biomarkers of RFI in this study. Tyrosine, plays a vital role in protein synthesis and serves as a precursor for the synthesis of neurotransmitters (Kim et al., 2006), consequently initiating important roles in appetite regulation, energy metabolism, and stress response (Church et al., 2020; Yoo et al., 2021). Tyrosine is also involved in the production of antibodies, cytokines, and other immune factors which can help protect animals from infections and diseases (Mohagheghpour et al., 2000; Kin and Sander, 2006) and ultimately has a role in improving overall health, performance, and efficiency (Konashi et al., 2000; Li et al., 2007). Dimethyl sulfone, a sulfur-containing compound, was identified as a predictive biomarker of RFI in this study as well. Sulfur is an essential nutrient for ruminants, playing a role in various metabolic functions. Sulfur is involved in the synthesis of sulfur-containing amino acids, such as methionine and cysteine (Wu et al., 2006; Kim et al., 2006), which supports the increased plasma concentration of methionine in low-RFI observed in this study. Previous studies conducted on growing beef cattle fed high-forage diets have identified methionine as the primary limiting amino acid, highlighting its critical role in influencing the feed efficiency of beef cattle consuming high-forage diets (Kerley, 2016; Cantalapiedra-Hijar et al., 2020). Dimethyl sulfone has been investigated for its antioxidant potential and has shown promise in reducing inflammation (Sanmartín-Suárez et al., 2011; Butawan et al., 2017). Antioxidants are crucial to animal health by neutralizing harmful free radicals and reducing oxidative damage in cells and tissues (Ponnampalam et al., 2022). Oxidative stress has been associated with a range of pathophysiological conditions that are significant for growth, reproduction, and overall health in ruminants. (Lykkesfeldt and Svendsen, 2007; Celi, 2011; Sies and Jones, 2020). It has been demonstrated through multiple studies that oxidative damage to cell organelles and biomolecules serves as an energy drain and detrimentally impacts

several cellular processes (Cao and Kaufman, 2014; Coleman et al., 2020). Based on these findings, it is plausible to speculate that elevated plasma levels of dimethyl sulfone in low-RFI beef steers could signify an enhanced adaptive mechanism for mitigating oxidative stress.

We employed logistic regression to develop a model that distinguishes high- from low-RFI steers. The logistic regression equation incorporating the three candidate biomarkers yielded a ROC curve with an AUC value of 0.789 (Figure 4). Through permutation testing (n = 1000), the significance of this model was confirmed (P = 0.001; Figure 5). The logistic regression model is presented below:

$$\text{logit}(P) = \log\left(P / (1 - P)\right) = -0.437 + 1.035 \text{ dimethyl sulfone} + 0.248 \text{ tyrosine} - 1.152 \text{ glycine}$$

Where P is the probability of an animal belonging to low-RFI classification. According to the analysis, the threshold cutoff point for the above equation is 0.48. In other words, any beef steer with values greater than or equal to 0.48 is potentially classified as a low-RFI group, while beef steers with values < 0.48 belong to the high-RFI group. Owing to the normalization measures adopted in this study, metabolites were log-transformed and thereafter auto-scaled. Therefore, the values for dimethyl sulfone, tyrosine and glycine in the above equation correspond to their log-transformed values. Our study highlights the potential of plasma metabolites as predictive markers for RFI in beef cattle. Although the association observed between the metabolite biomarkers and RFI in this study provides valuable insights into the metabolic pathways underlying feed efficiency, it is important to note the inherent biological variability within beef cattle populations poses a challenge for predicting RFI. Variations in diet, genetics, gut microbiota, and other environmental factors can significantly influence the metabolome of animals (Fujisaka et al., 2018; Clemmons et al., 2018) and RFI. Moreover, the limited availability of standardized protocols and varying sensitivity and resolution of the analytical methods hinder the comparability and

reproducibility of results across studies. This lack of standardization impedes the establishment of a consistent and reliable set of metabolomic markers for predicting RFI. Nonetheless, our results provide preliminary evidence for the cost-effective prediction and categorization of RFI values using plasma metabolites. Further validation using larger and more diverse cattle populations is necessary to confirm these findings.

Conclusion

Our study demonstrates that plasma metabolites, specifically amino acids such as tyrosine, glycine, and dimethyl sulfone, hold potential as predictive biomarkers for RFI in lean growth phase beef cattle. The logistic regression model incorporating these biomarkers shows promise in distinguishing high- from low-RFI steers, with a threshold cutoff point of 0.48. However, the inherent biological variability within beef cattle populations and the lack of standardized protocols pose challenges in predicting RFI consistently across studies. To establish a dependable set of metabolomic markers for predicting RFI, further validation using larger and more diverse cohorts is essential. Nonetheless, our findings provide early evidence for the use of plasma metabolites as cost-effective tools for predicting RFI.

Tables and Figures

Table 6. 1 The differentially abundant plasma metabolites in beef steers with low or high residual feed intake.

Metabolites (mM)	FC (Low RFI/High-RFI)	*P*-values
Dimethyl sulfone	1.63	0.01
3-Hydroxyisovaleric acid	1.37	0.02
Citric acid	1.23	0.01
Valine	1.16	0.01
L-Leucine	1.08	0.03
Methionine	1.08	0.05
Creatine	0.88	0.02
L-Carnitine	0.87	0.04
Glycine	0.83	0.01

FC: fold change (Low-RFI/High-RFI). Low-RFI - beef steers with negative residual feed intake; High-RFI - beef steers with positive residual feed intake. Only metabolites with levels of significance with p-value ≤ 0.05 are shown.

Table 6. 2 Biomarker analysis of beef cattle RFI showing the summary of features of the logistic regression model

Intercept	Estimate	Std. Error	z value	Pr(>|z|)	Odds
(Intercept)	-0.437	0.306	-1.426	0.154	-
Dimethyl sulfone	1.035	0.346	2.996	0.003	2.82
Tyrosine	0.248	0.317	0.782	0.434	1.28
Glycine	-1.152	0.354	-3.256	0.001	0.32

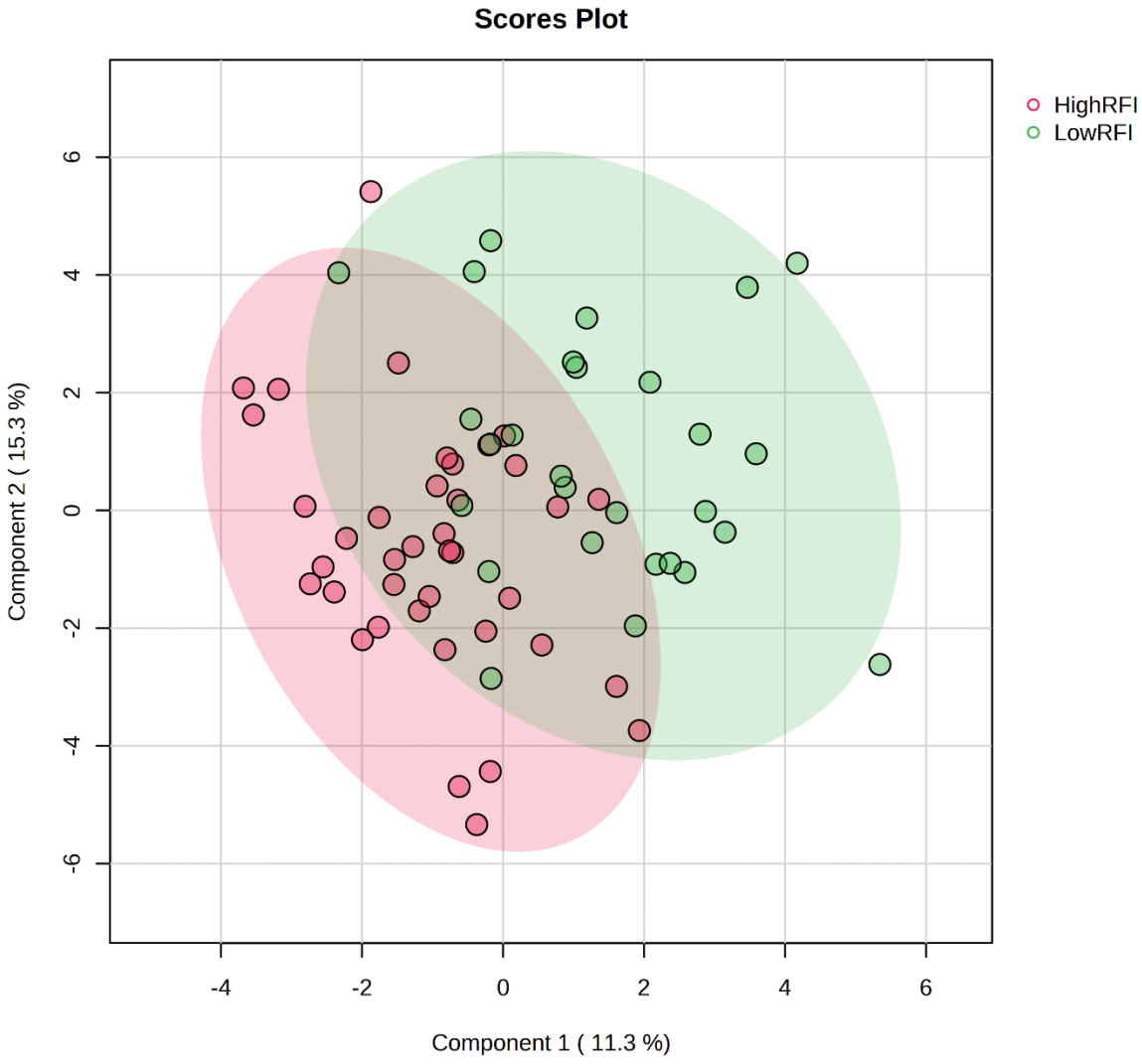

Figure 6. 1 Partial least square discriminant analysis (PLS-DA) scores plot of the plasma metabolome of all the beef steers

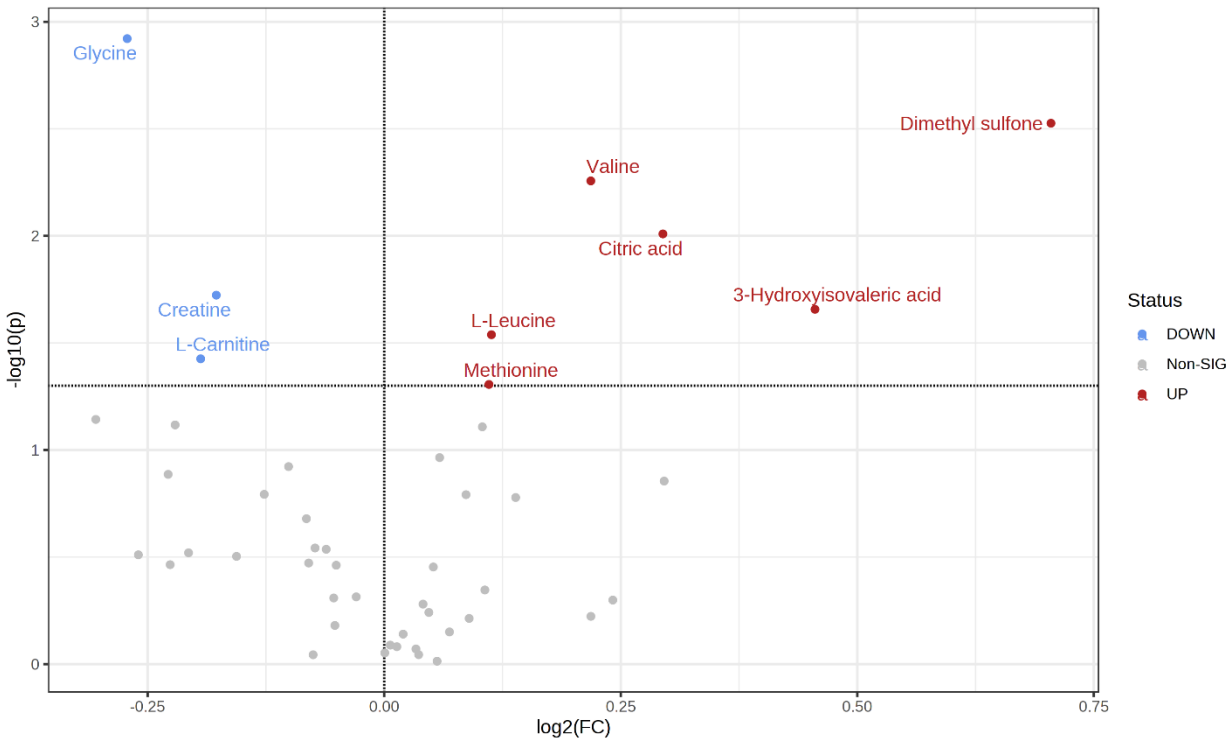

Figure 6. 2 Volcano plot showing the differential plasma metabolites in beef steers with low or high residual feed intake.

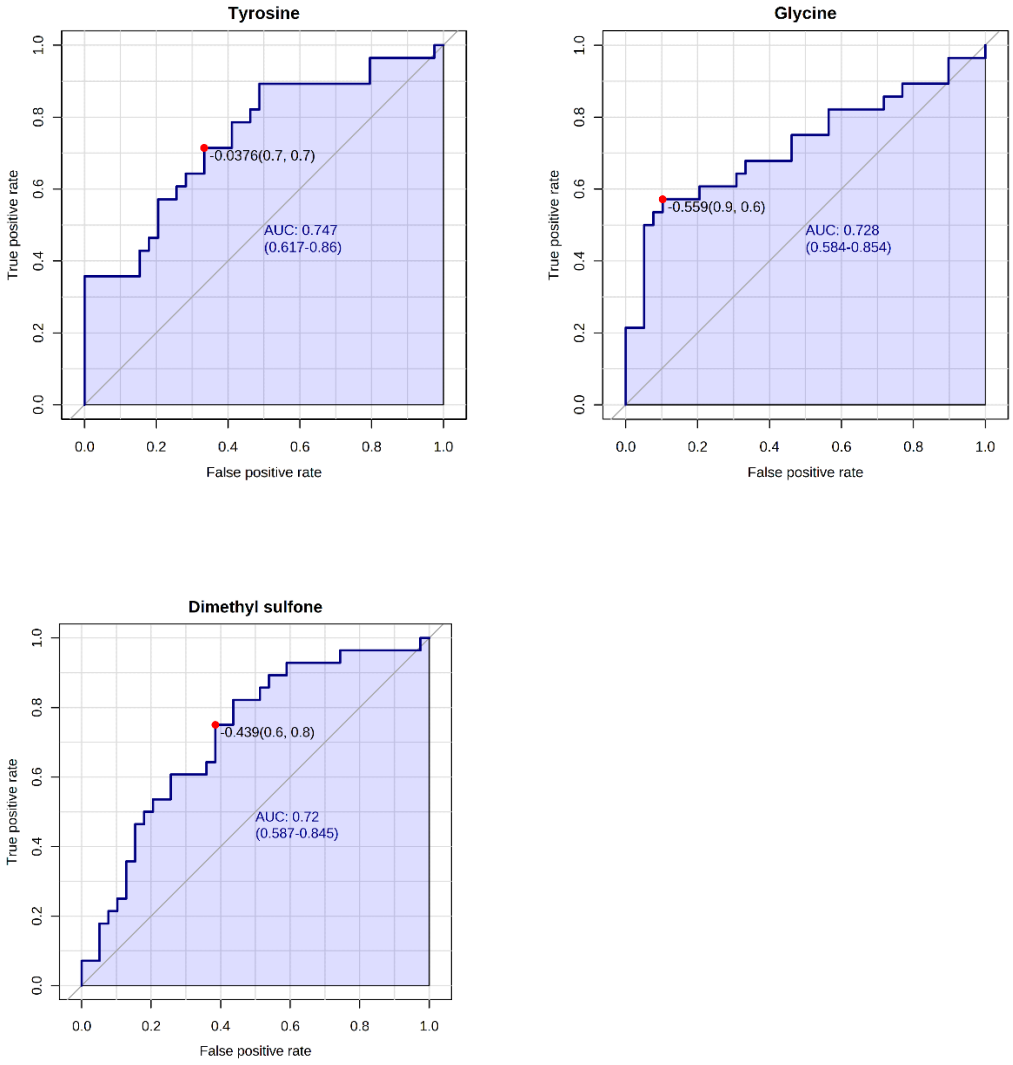

Figure 6. 3 Biomarker analysis of plasma metabolome. ROC curve analysis of candidate plasma biomarkers (glycine, tyrosine, and dimethyl sulfone) of beef steers with low or high RFI.

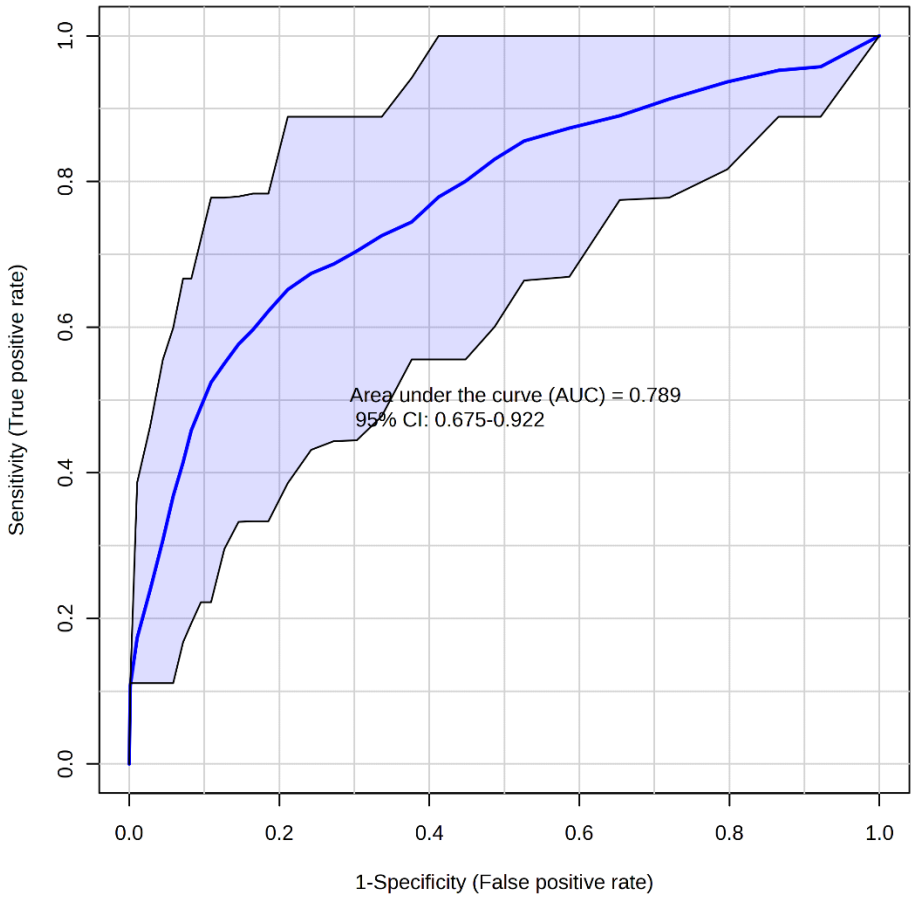

Figure 6. 4 A smooth ROC curve (100 cross-validations) showing the performance of the logistic regression model having accurate sensitivity and specificity for dimethyl sulfone, tyrosine, and glycine.

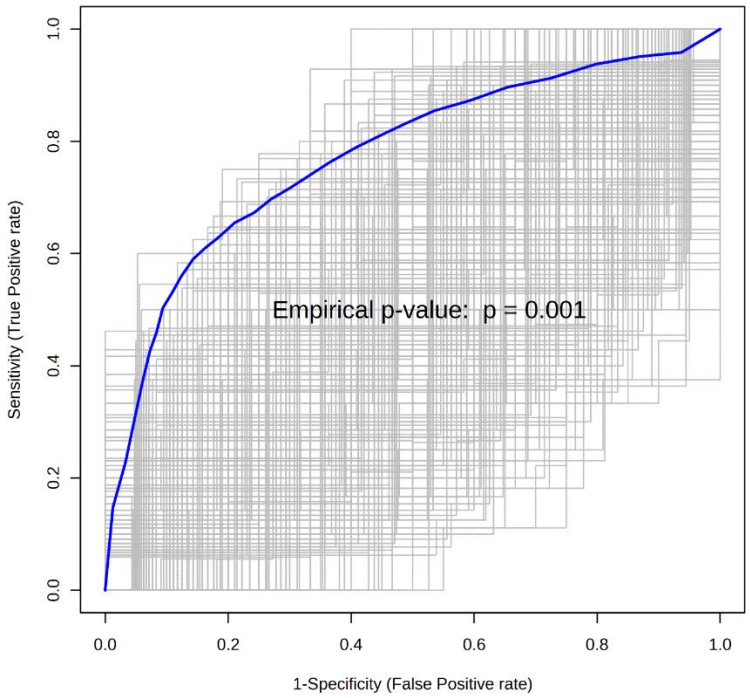

Figure 6. 5 Logistic regression receiver operating characteristic (ROC) curve analysis of the candidate biomarkers (dimethyl sulfone, tyrosine, and glycine).

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