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^{1}H-NMR-Based Plasma Metabolomic Profiling of Crossbred Beef Cattle with Divergent RFI Phenotype

Godstime Taiwo [1,*], Modoluwamu Idowu [1], Taylor Sidney [1], Emily Treon [1], Deborah Ologunagba [1], Yarahy Leal [1], Samanthia Johnson [1], Rhoda Olowe Taiwo [2], Anjola Adewoye [3], Ephraim Ezeigbo [3], Francisca Eichie [1] and Ibukun M. Ogunade [1,*]

[1] Division of Animal and Nutritional Science, West Virginia University, Morgantown, WV 26506, USA; tss0020@mix.wvu.edu (T.S.); edt00002@mix.wvu.edu (E.T.); doo00005@mix.wvu.edu (D.O.); ygl0001@mix.wvu.edu (Y.L.); srj00005@mix.wvu.edu (S.J.); foe00001@mix.wvu.edu (F.E.)
[2] The Institute for Drug Research, The School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel; rhoda.olowe@mail.huji.ac.il
[3] Department of Chemistry, West Virginia University, Morgantown, WV 26506, USA; awa00005@mix.wvu.edu (A.A.); ece00007@mix.wvu.edu (E.E.)
* Correspondence: gat00005@mix.wvu.edu (G.T.); ibukun.ogunade@mail.wvu.edu (I.M.O.)

Abstract: This study focused on exploring the metabolomic profiles of crossbred beef cattle with varying levels of residual feed intake (RFI), a measure of feed efficiency in beef cattle. Sixty-seven crossbred growing beef steers (BW = 277 ± 29.7 kg) were subjected to a high-forage total mixed ration for 64 days to determine their RFI phenotypes. At the end of the 64d feeding trial, 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). Blood samples were collected, and plasma samples were analyzed using Nuclear Magnetic Resonance spectroscopy, resulting in the identification of 50 metabolites. The study found a distinct metabolomic signature associated with RFI status. Eight metabolites, including amino acids (tyrosine, glycine, valine, leucine, and methionine) and other compounds (dimethyl sulfone, 3-hydroxy isovaleric acid, citric acid, creatine, and L-carnitine), showed differential abundance between low- and high-RFI groups. Specifically, tyrosine, glycine, and dimethyl sulfone exhibited significant specificity and sensitivity, which produced a discriminatory model with an area under the receiver operating characteristic (ROC) curve of 0.7, making them potential markers for RFI. A logistic regression model incorporating these biomarkers effectively distinguished between high- and low-RFI steers, with a threshold cutoff point of 0.48, highlighting a distinctive metabolite profile associated with efficient nutrient utilization in low-RFI cattle. The logistic regression model, incorporating these biomarkers, holds promise for accurately categorizing RFI values, providing insights into the metabolic basis of feed efficiency in beef cattle.

Keywords: feed efficiency; omics; metabolites; amino acids; logistic regression

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1. Introduction

Enhancing the feed efficiency of beef cattle with the primary objective of improving profitability, productivity, health, and environmental sustainability of livestock production has become a vital subject in the past few decades [1–3]. 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 [4,5]. Low-RFI cattle consume less feed than expected for the same level of production compared to high-RFI cattle [3,6]. Metabolomics has played a significant role in characterizing markers associated with the RFI trait.

Metabolomics provides robust and holistic insights into metabolites and their interactions in a biological system [3,7,8]. Among the analytical tools used for metabolomics investigations, proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) requires minimal sample preparation and provides robust and highly reproducible information [9], which compensates for its lower sensitivity in comparison to other analytical platforms. Utilizing ^{1}H Nuclear Magnetic Resonance (NMR), a metabolomic strategy has demonstrated efficacy in examining diverse physiological events, providing valuable information for blood-based signatures in livestock [10,11]. Metabolites, particularly of amino acids or their derivatives, including creatine, methionine, choro-lysine, and urea, have been reported to be associated with RFI phenotypes in several studies [3,12,13]. These earlier studies were focused on identifying the differentially abundant metabolites in beef cattle with divergent RFI values and consequently identifying potential biomarkers for RFI without exploring further mathematical modeling techniques capable of confirming the predictive capabilities of these blood-based signatures [14–17]. This highlights the importance of appropriate screening by combining metabolomics approaches and other analytical techniques in elucidating and identifying blood markers.

Utilizing diverse analytical tools in metabolomics and a mathematical model, a study by [18] revealed that serum metabolites have the potential to predict and categorize bovine RFI values in a cost-effective manner. Exploring the combined approaches could better explain downstream biology, capable of providing relevance to feed efficiency phenotype in beef cattle, especially with regression analysis, which is often used to demonstrate associations among variables believed to be biologically related [19]. To our knowledge, a mathematical model for predicting RFI in crossbred beef steers from plasma biomarkers has not been fully explored and thus, concrete models that could further confirm the predictive capability of these metabolic signatures toward accurate phenotypic assessments would be needed. We therefore hypothesize that plasma biomarkers can serve as reliable indicators for predicting residual feed intake in crossbred beef steers. Our objective, therefore, was to investigate the potential of applying NMR analytical approaches and logistics regression models to identify a distinct plasma metabolomic profile of beef cattle for predicting RFI phenotype in crossbred beef cattle.

2. Materials and Methods

2.1. 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 (Angus × Hereford) beef steers with an average BW of 277 ± 29.7 kg were fed a high-forage total mixed ration (TMR; the TMR primarily consisted of corn silage, cracked corn, grass baleage, and a ration balancing; see Supplementary Table S1) 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, AB, Canada) to monitor individual feed intake. Additionally, two In-Pen Weighing Positions (IPW Positions, developed by Vytelle LLC, Lenexa, KS, USA) were installed in each pen to measure the daily BW of the steers [20,21].

At the end of the feeding trial, the RFI values of the beef steers were determined as described previously by [22]. 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 the difference between the predicted value of the regression and the actual measured value using the following equation: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$, where Y is the standardized 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 residuals [23]. 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). 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. These were immediately kept in ice 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 at −80 °C until further analysis.

2.2. NMR-Based Metabolome Analysis of Plasma Samples

Nuclear Magnetic Resonance (NMR) spectroscopy was utilized to conduct metabolome analysis on all plasma samples. The procedures for plasma sample preparation and NMR spectral analysis followed the previously published protocols by [24]. Briefly, a deproteinization process was carried out using ultra-filtration, following the method outlined by [25], 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% D_2O and 46% 250 mM KH_2PO_4 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, Billerica, MA, USA) equipped with a pulsed-field gradient cryoprobe. The obtained 1H-NMR spectra were processed and analyzed using Bayesil (http://www.bayesil.ca), an analysis software for quantitative analysis of NMR spectra, as outlined by [26]. An additional examination and verification process was conducted by an NMR spectroscopist to ensure accuracy in compound identification and quantification. Fifty (50) metabolites, including amino acids, hexoses, organic acids, carnitines, and lipids, were generated (Table S2).

2.3. Data Modelling and Statistical Analysis

Variables such as initial and final BW, ADG, DM intake, and RFI values were analyzed using the GLIMMIX procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA), with RFI status included as a fixed effect. Significant effects were declared at $p \leq 0.05$. Values of initial body weight were included as a covariate for the final body weight. Metabolome data was analyzed using MetaboAnalyst 5.0 software [27]. Before 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 area under the curve (AUC > 0.70). Partial least squares discriminant analysis (PLS-DA) was also performed to visualize the difference between the two groups of 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. A logistic regression model was performed to derive a parsimonious discriminant low- versus high-RFI biomarker model. 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.

3. Results and Discussion

The average RFI values of low- and high-RFI steers were −1.54 and 1.49 kg/d, respectively ($p = 0.01$). Although the initial BW, final BW, and ADG were similar between the two groups ($p > 0.05$), the high-RFI group had greater ($p = 0.01$) DMI than low-RFI beef steers (Table 1).

Table 1. Growth performance of the beef steers with divergent residual feed intake.

Item	Low-RFI	High-RFI	SEM	*p*-Value
RFI (kg/d)	−1.54	1.49	0.03	0.01
Initial weight (kg)	280	272	8.90	0.63
Final weight (kg)	336	327	5.93	0.41
ADG (kg/d)	1.13	1.11	0.04	0.86
DMI (kg/d)	7.08	8.23	0.38	0.01

SEM, standard error of the mean; ADG, average daily gain; DMI, dry matter intake.

A total number of 50 metabolites were quantified (Supplementary Table S2). 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 1), indicating altered metabolome of the beef steers based on their RFI status. A total of nine differentially abundant ($p \leq 0.05$) metabolites were detected between the low- and high-RFI steers (Table 2; Figure 2). Compared to high-RFI steers, plasma concentrations of six 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 three metabolites (creatine, L-carnitine and glycine) were lower (p-value ≤ 0.05; Table 2). 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 3). Our findings highlight the role of amino acid metabolism in enhancing the feed efficiency of beef cattle. These results align with previous studies that showcased an intricate relationship between amino acid metabolism and the RFI status of beef cattle, as evidenced by [3,14]. For instance, ref. [14] revealed that plasma metabolites related to the urea cycle, such as ornithine, aspartate, lysine, and valine, were associated with RFI in Charolais heifers. To ensure the reliability of the ROC curve model, a permutation test was conducted with 1000 randomized permutations for validation purposes. The logistic regression equation incorporating the three candidate biomarkers yielded an ROC curve with an AUC value of 0.789 (Table 3; 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:

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

where P is the probability of an animal belonging to the low-RFI classification. According to the analysis, the threshold cutoff point for the above equation is 0.48. In other words, any beef steers with values greater than or equal to 0.48 are 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.

Table 2. 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

Table 2. *Cont.*

Metabolites (mM)	FC (Low-RFI/High-RFI)	*p*-Values
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.

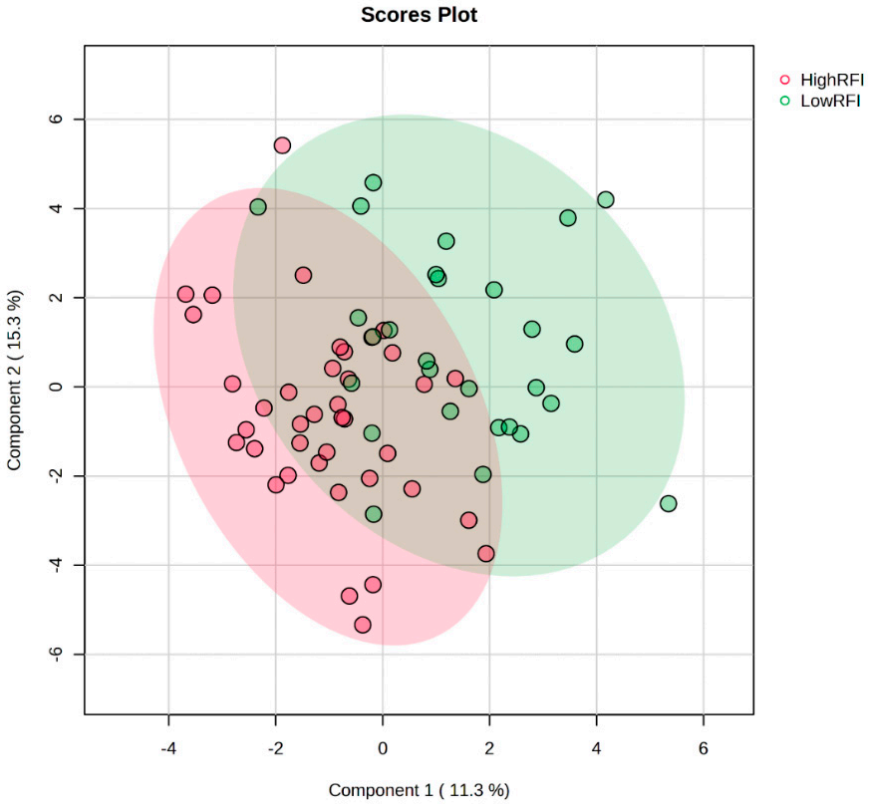

Figure 1. Partial least square discriminant analysis (PLS–DA) scores plot of the plasma metabolome of all the beef steers.

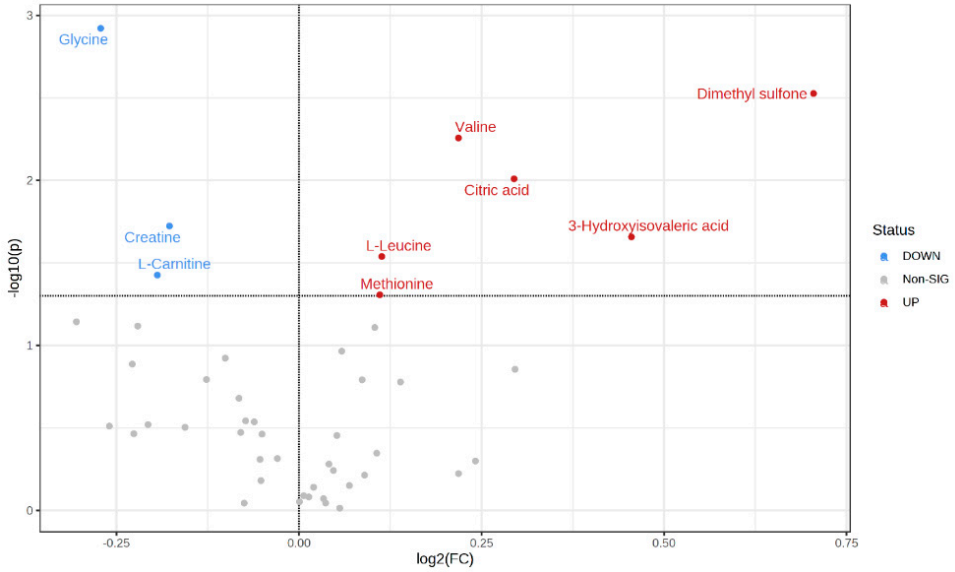

Figure 2. Volcano plot showing the differential plasma metabolites in beef steers with low- or high-residual feed intake.

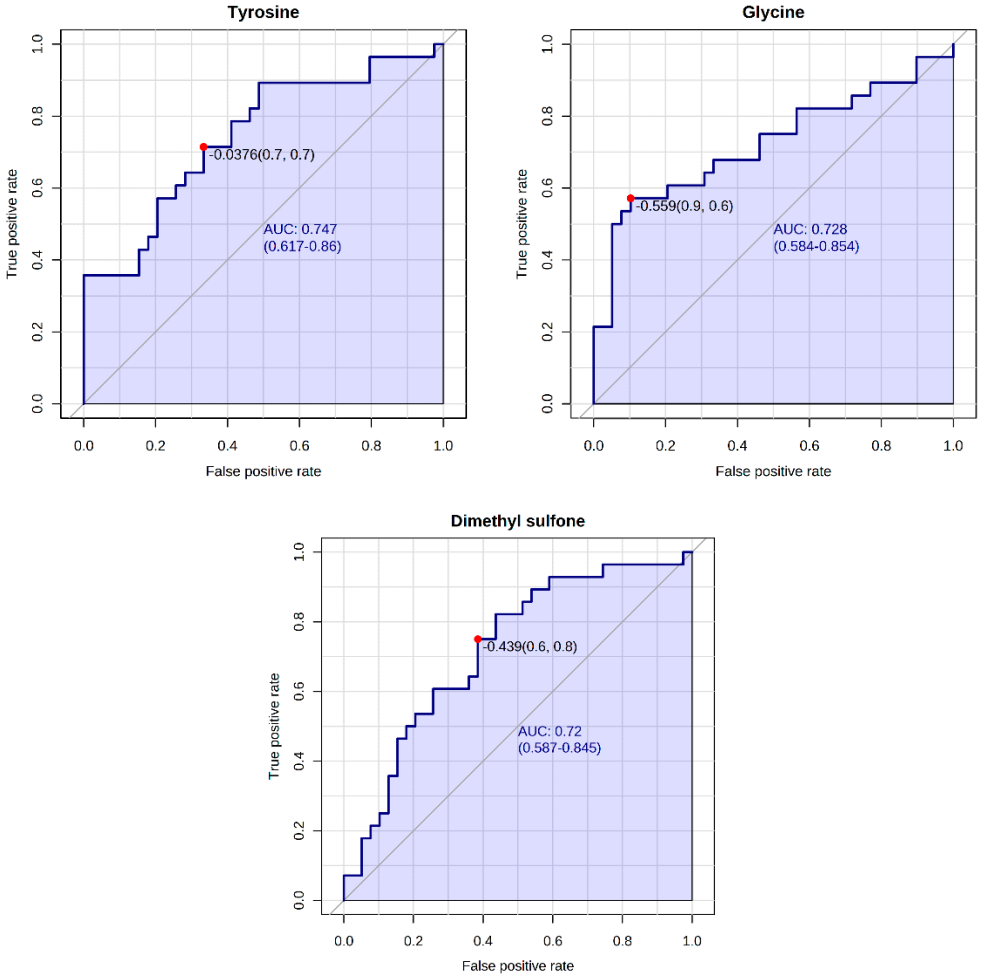

Figure 3. Biomarker analysis of plasma metabolome. ROC curve analysis of candidate plasma bio-markers (glycine, tyrosine, and dimethyl sulfone) of beef steers with low- or high- RFI.

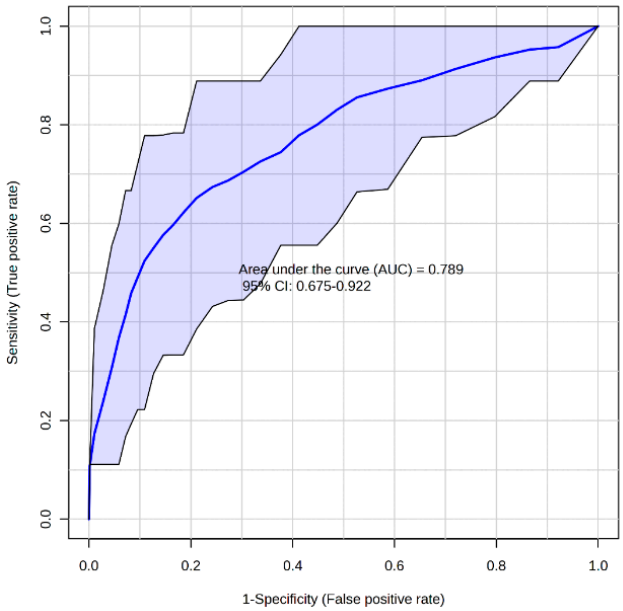

Figure 4. A smooth ROC curve (100 cross-validations) showing the performance of the logistic regression model having accurate sensitivity and specificity for dimethyl sulfone, tyrosine, and glycine.

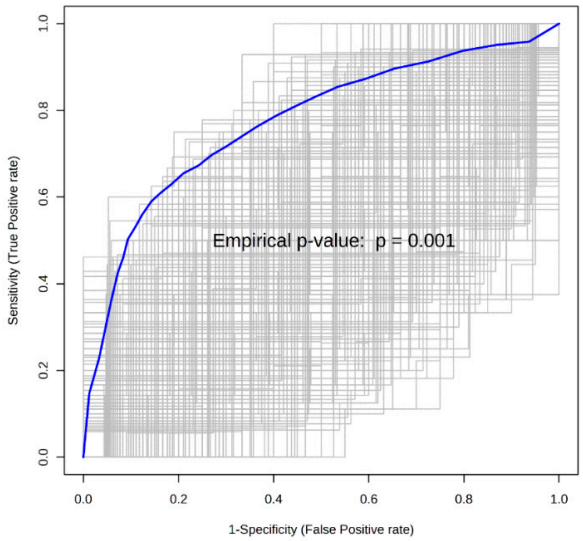

Figure 5. Logistic regression receiver operating characteristic (ROC) curve analysis of the candidate biomarkers (dimethyl sulfone, tyrosine, and glycine).

Table 3. 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

Furthermore, our previous study [3] 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 by [22], 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, although a non-essential amino acid whose derivatives (peptide tyrosine-tyrosine (PYY)) have been implicated in playing a vital role in protein synthesis, is secreted from intestinal enteroendocrine cells in response to a meal [28,29], and is capable of initiating important roles in appetite regulation, energy metabolism, and stress response [30,31]. Tyrosine is also involved in the production of antibodies, cytokines, and other immune factors which can help protect animals from infections and diseases [32,33], thereby improving their overall health, performance, and feed efficiency [34,35]. Dimethyl sulfone—a sulfur-containing compound—was identified as a predictive biomarker of RFI in this study. Sulfur is involved in the synthesis of sulfur-containing amino acids, such as methionine and cysteine [28,36], 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 [37,38]. Dimethyl sulfone has been investigated for its antioxidant potential and has shown promise in reducing inflammation [39,40]. Antioxidants are crucial to animal health because they neutralize harmful free radicals and reduce oxidative damage in cells and tissues [41]. Oxidative stress has been associated with a range of pathophysiological conditions that are significant for

growth, reproduction, and overall health in ruminants [42–44]. 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 [45,46]. Interestingly, dimethyl sulfone has been implicated as a main metabolite substrate activating catalytic antioxidation of many key endogenous enzymes (methionine sulfide reductase A) with consequent cytoprotective functions and extension of organism lifespan [47]. Based on these findings, it is possible to speculate that elevated plasma levels of dimethyl sulfone in low-RFI beef steers potentially enhanced adaptive mechanisms for mitigating oxidative stress. This adaptation might result in reduced energy expenditure and enhanced energy availability, potentially contributing to enhanced feed efficiency.

This observational study has limitations. We utilized a pragmatic sample size of roughly 30 beef cattle in each group. A small sample size limits the power to detect a difference, and conversely, differences detected may be spurious. We therefore explored the use of logistic regression to develop a model that distinguishes high- from low-RFI steers.

Our study highlights the potential of plasma metabolites as predictive markers for RFI in beef cattle. However, it is important to note that variations in diet, genetics, gut microbiota, and other environmental factors can significantly influence the metabolome of beef cattle populations [48,49] and thus pose a challenge for predicting RFI. The association observed between the metabolite biomarkers and RFI in this study provides valuable insights into the metabolic pathways underlying feed efficiency. 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. Similarly, this lack of standardization impedes the establishment of a consistent and reliable set of metabolomic markers for predicting RFI. Nonetheless, our results provide further evidence of vital biomarkers associated with RFI using plasma metabolites. Should the three-metabolites model (logistic regression model) be successfully validated across studies using a larger beef cattle cohort, then point-of-care diagnostics capable of identifying feed-efficient cattle from the population could be developed, such as a simple dipstick or laboratory assay.

4. Conclusions

Our study demonstrates that plasma metabolites, specifically amino acids such as tyrosine, glycine, and dimethyl sulfone, hold potential as predictive biomarkers for RFI in 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. Yet due to the inherent biological differences within beef cattle groups and the absence of uniform procedures, anticipating RFI consistently across various studies presents a challenge. To develop a reliable collection of metabolomic markers for RFI prediction, it is crucial to conduct further validation using broader and more varied cohorts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ruminants4020012/s1, Table S1: GrowSafe ration; Table S2: Relative intensity values of 47 plasma metabolites of the 67 steers.

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