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Highly Targeted Metabolomics Coupled With Gene Expression Analysis by RT–qPCR Improves Beef Separation Based on Grass, Grain, or Grape Supplemented Diet

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ABSTRACT

The objective of this study was to use a multi-omics (i.e., gene expression quantification, metabolomics, and fatty acid [FA] profiling) approach to separate and authenticate beef from three different dietary groups. In this 2-year study, Red Angus steers ($n = 54$) were randomly allocated to one of three treatments: (1) complex biodiverse pasture (GRASS), (2) total mixed ration (TMR) in feedlot (GRAIN), or (3) TMR in feedlot supplemented with 5% (dry matter) grapeseed extract for the last 30 days (GRAPE). FAs were measured by gas chromatography-mass spectrometry (GC–MS), secondary metabolites were identified using ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS), and gene expression analysis was performed using quantitative reverse transcription polymerase chain reaction (RT–qPCR). All target genes were upregulated in beef from GRASS compared to the other two groups. Multivariate analyses showed that long-chain *n*-3 polyunsaturated FAs, the *n*-6:*n*-3 ratio, vitamin E, organic acids, amino acid derivatives, and the nephronectin isoform X1 (*NPNT-1*) gene were the most important compounds for group separation. These compounds, considered to be beneficial for human health, showed higher concentrations in beef from GRASS. The success of beef separation by dietary treatment was highlighted by the 90.4% prediction accuracy of the random forest model, with beef from GRASS being 100% accurately predicted and beef from GRAPE being 94.4% accurately predicted. Beef from GRAIN was 76.5% accurately predicted. In conclusion, coupling gene expression analysis to metabolomics and FA profiling allowed for the separation of beef samples from varying dietary backgrounds with a high degree of confidence.

1 | Introduction

Amid mounting concerns about beef production practices (Godfray et al. 2018; Spratt et al. 2021), consumers are interested in agricultural commodities produced through regenerative farming practices, including grass-finished beef (GFB) (Cheung et al. 2017; Regenified 2024). Such interest is motivated by health claims, environmental metrics, and animal welfare factors (Krusinski,

Sergin et al. 2022; Xue et al. 2010). GFB is generally greater in beneficial omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs), conjugated linoleic acid (CLA), vitamin E, minerals, and antioxidants, and displays a lower omega-6:omega-3 (*n*-6:*n*-3) ratio compared to grain-finished beef (Krusinski et al. 2022b; Krusinski et al. 2023). Grazing cattle on pasture also allows animals to express their natural behavior and engage in dietary selectivity, leading to improved animal welfare (Provenza et al. 2019; Stampa et al. 2020). Managed grazing can also restore soil health and ecosystem function, improve rancher resiliency, and increase biodiversity (Spratt et al. 2021; Teague and Kreuter 2020).

All beef is not equal, and production practices (i.e., grazing pattern, diets, climate) impact meat quality (Evans et al. 2024; Krusinski et al. 2022c). More importantly, not all GFB is nutritionally equal; Bronkema et al. (2019) conducted a national nutritional survey of commercially available GFB and found wide variations in the fatty acid (FA) and micronutrient profiles of meat coming from different producers. In some instances, the *n*-6:*n*-3 ratio was almost three times higher in GFB than grain-finished beef (Bronkema et al. 2019). Currently, GFB only accounts for a small percentage of the US supply, and imports far outweigh the domestic US GFB production (imports represent ~80% of the total US GFB sales) (Cheung et al. 2017; Hayek and Garrett 2018). Such a dynamic may lead US producers to increase productivity while cutting costs, resulting in practices that may or may not be allowed under GFB protocols. Additionally, GFB labeling rules are unclear, with no current legislative definition or guidelines for GFB in the US or the European Union (O'Brien et al. 2023; Stampa et al. 2020).

From 2007 to 2016, the United States Department of Agriculture (USDA) carried voluntary marketing claim standards to help regulate grass-fed products. These standards were discontinued, but producers can still seek approval from the USDA to market GFB (O'Brien et al. 2023). According to the USDA, GFB can only come from meat derived from cattle fed 100% forage, but it also allows for partial claims (e.g., 50% grass-fed). Participating producers can define their own claim and need to comply with written protocols and sign an affidavit, but no audits are conducted (Cheung et al. 2017; Food Safety and Inspection Service 2019). Third-party certifications also apply their labels to meat products, such as the American Grassfed Association (AGA). According to the AGA, grass-finished cattle must only consume fresh grass and forages throughout their lifetime, with the inclusion of conserved forages in case in inclement weather or low forage availability (American Grassfed Association 2022). The AGA conducts on-farm inspections to check for compliance, but no analytical methods are employed to ensure the authenticity of grass-finished products. Moreover, these inspections do not apply to GFB imported to the United States.

With consumers willing to pay premiums for GFB and producers spending time and resources using grass-finished protocols, it becomes crucial to develop reliable beef separation and authentication methods (Stampa et al. 2020). Some producers may rely on agricultural by-products to supplement the diet of their animals, such as grapeseed extract (GSE), which may make authentication more challenging. GSE is rich in polyphenols (anthocyanins, proanthocyanidins, and flavanols), which may transfer to meat when consumed by animals (Krusinski et al. 2023; Muñoz-González et al. 2019). Authentication protocols usually use mass spectrometry (MS) and genomics methods, but discrimination efficiency may be performance-limited when these methods are used separately (Prache et al. 2020). FA, micronutrients, secondary metabolites, and gene expression data can all be used synergistically using a "hurdle approach" to separate beef by diet (Carrillo et al. 2016; Sweeney et al. 2016). Targeted metabolomics and gene expression analysis measure predefined classes of metabolites and sets of genes compared to untargeted methods that aim to analyze all measurable metabolites/genes (Kuemmerle et al. 2024; Roberts et al. 2012). Therefore, the objective of this study was to use a multi-omics approach employing metabolomics, FA profiling, and gene expression quantification to identify key biomarkers and separate beef from three diets: (1) grazing on biodiverse pastures, (2) a total mixed ration (TMR) in feedlot, or (3) a TMR supplemented with GSE in feedlot.

2 | Materials and Methods

2.1 | Sample Collection and Processing

This study lasted 2 years (2019–2020) and was conducted at the Michigan State University Upper Peninsula Research and Extension Center (Chatham, MI, USA). The dietary feeding periods were June to September 2019 and June to October 2020. The experimental design was described in detail previously (Krusinski et al. 2022b, 2023). Briefly, a total of 54 beef samples from Red Angus steers (14–20 months old) assigned to one of three diets were collected (n = 18 per treatment): (1) complex biodiverse pasture (GRASS), (2) TMR in feedlot (GRAIN), or (3) TMR in feedlot supplemented with 5% (dry matter—DM) GSE for the last 30 days (GRAPE). For each year, the goal was to have nine samples per diet (three samples per pen per year). Due to lower male births in 2020, only seven beef samples were available for GRAIN; therefore, two additional beef GRAIN samples from 2019 were randomly selected. All animals for the GRAPE group were housed in a single feedlot pen. The number of samples was therefore nine per group per year (n = 54).

Plant species found in the biodiverse pasture diet (GRASS) were meadow fescue (*Schedonorus pratensis* (Huds.) P. Beauv.), red clover (*Trifolium pretense* L.), timothy grass (*Phleum pratense*), alfalfa (*Medicago sativa*), white clover (*Trifolium repens* L.), birdsfoot trefoil (*Lotus corniculatus*), chicory (*Cichorium intybus*), orchardgrass (*Dactylis glomerata* L.), and dandelion (*Taraxacum officinale* L.). The TMR used for the GRAIN and GRAPE groups was made of orchard grass hay, high-moisture corn, dry corn, and pellets (36% crude protein). For the GRAPE group, 5% of GSE (DM), provided by Pioneer Enterprises (Lewiston, ID, USA), was added to the TMR for the last 30 days of the finishing period. Feed samples were collected every 2 weeks throughout the experimental periods for quality control purposes. The nutritional composition of the diets was previously reported (Krusinski, Maciel et al. 2022; Krusinski et al. 2022b, 2023), and an overview of the nutritional profile is shown in Figure 1.

Beef samples were collected in September 2019 and October 2020 in a USDA-regulated slaughter facility. All animals were slaughtered on the same day at 16–18 months old for GRAIN and GRAPE and 24–26 months old for GRASS. Ribeye samples were

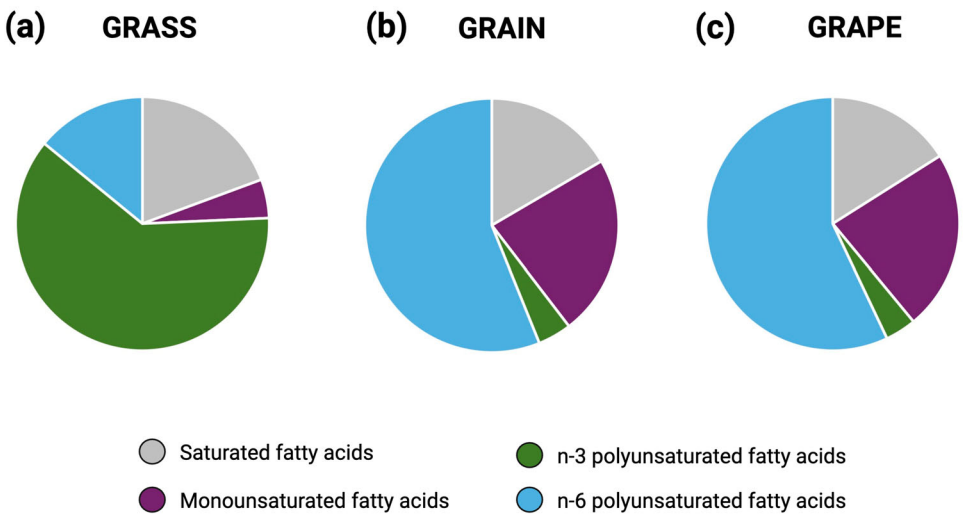

FIGURE 1 | **Main fatty acid profile of the diets adapted from Krusinski et al.** (2023). (a) Fatty acid profile of the biodiverse pasture diet (GRASS); (b) fatty acid profile of the total mixed ration used in feedlot (GRAIN); (c) fatty acid profile of the total mixed ration used in feedlot with the inclusion of 5% (dry matter) grapeseed extract.

collected from the left side of the carcass between the 11th and 13th rib by trained personnel. Steaks were then cut in 1×1 cm^2 cubes before being flash-frozen in liquid nitrogen. Beef samples were stored at −80°C until further analysis.

2.2 | Gene Expression Quantification

Beef samples were ground into a fine powder using a mortar and pestle on dry ice. Then, frozen and pulverized tissue samples (50 mg) underwent RNA extraction in duplicate. Total RNA was extracted using the PureLink RNA Mini Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions for the purification of RNA from animal tissues with the on-column DNase treatment. Tissue lysis was performed using the provided lysis buffer mixed with 2-mercaptoethanol and 2.8 mm sterile ceramic beads in a FastPrep-24 bead beating lysis system (MP Biomedicals, Irvine, CA, USA) at 6 m/sec for 60 s. RNA was then eluted with 30 µL of RNase-free water. After elution, duplicate extracts were combined prior to measuring RNA concentration and purity on a NanoDrop One UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All extracts were then diluted using Tris-EDTA (TE) buffer and standardized to 1.5 ng/µL. Extracts were stored at −20°C until cDNA synthesis. Each extraction batch contained a reagent blank in which the tissue sample was excluded for quality control purposes.

Quantitative reverse transcription polymerase chain reaction (RT–qPCR) was used to quantify the gene expression of four target genes: (1) eukaryotic translation initiation factor 4E-binding protein 1 (*EIF4EBP1*), (2) delta-aminolevulinic acid dehydratase (*ALAD*), (3) nephronectin isoform X1 (*NPNT-1*), and (4) peroxisome proliferator activated receptor gamma (*PPARG*). Additionally, two reference genes (1) tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and (2) ribosomal protein lateral stalk subunit P0 (*RPLP0*) were tested. Standardized RNA extracts (1.5 ng/µL) underwent cDNA synthesis using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA) per the manufacturer's instructions. The reaction mixture contained 4 µL of 5x iScript Reaction Mix, 1 µL of iScript Reverse Transcriptase, and 15 µL of RNA template for a final reaction volume of 20 µL. Samples were amplified using an Eppendorf Mastercycler Nexus Gradient Thermal Cycler (Hamburg, Germany) under the following conditions: 25°C for 5 min, 46°C for 20 min, 95°C for 1 min, and hold at 4°C upon completion. cDNA concentration and purity were measured using a Qubit fluorometer (Invitrogen, Waltham, MA, USA) then stored at −80°C until RT–qPCR. Each cDNA synthesis reaction batch included two negative controls: (1) a reagent blank from RNA extraction and (2) a non-template control using RNase-free water.

qPCR assays targeting *EIF4EBP1*, *ALAD*, *NPNT-1*, and the two reference genes were carried out using the primers described by Sweeney et al. (2016), whereas primer sequences for *PPARG* were from Duckett et al. (2009). Gene names, main functions, and primer sequences are shown in Table 1. A targeted RT–qPCR method was employed to quantify gene expression of a predefined set of genes selected as described above. Each reaction mixture contained 10 µL iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA, USA), 1 µL of each forward and reverse primer (10 µM), and 8 µL cDNA template for a total reaction volume of 20 µL. RT–qPCR was performed on a Rotor-GeneQ (Qiagen, Hilden, Germany) under the following cycling conditions: 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min for *EIF4EBP1*, *ALAD*, *NPNT-1*, and the two reference genes (Sweeney et al. 2016). The cycling conditions for *PPARG* were 95°C for 15 min, 40 cycles for 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C (Duckett et al. 2009). For all genes, amplification concluded with a melt curve analysis step performed at 65°C–95°C with increasing increments of 0.5°C at 2–5 s/step. Target gene amplification was validated using a melt curve threshold unique to each gene. Each qPCR batch contained two negative controls containing (1) a reagent blank carried over from cDNA synthesis and (2) a non-template control containing

TABLE 1 | Target and reference genes used in this study.

Gene symbol	Gene name	Related class(es)	Pathway(s) and functions[a]	Primers (5′–3′)[b]
ALAD	Delta-aminolevulinic acid dehydratase	Vitamins and co-factors	Porphyrin metabolism, metabolic pathways, biosynthesis of co-factors	F:GCCCCGTCGGACATGA R:ATCCATGTGCCATCAGAGCTT
EIF4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1	Animal health	HIF-1 signaling pathway, mTOR signaling pathway, longevity regulating pathway	F:GGCGGCACGCTCTTCA R:AGGAACTTCCGGTCATAGATGATC
NPNT-1	Nephronectin isoform X1	Fatty acids and secondary metabolites	ECM-receptor interaction, PUFA and bioflavonoids may regulate its expression	F:TGATCGACAGGCCCACTTCT R:GGCTCTGGTATTGGCTTTGG
PPARG	Peroxisome proliferator activated receptor gamma	Fatty acids, secondary metabolites, physical activity	Regulation of fat cell differentiation, expression is regulated by exercise and polyphenols	F:AGGATGGGGTCCTCATATCC R:GCGTTGAACTTCACAGCAAA
RPLP0	Ribosomal protein lateral stalk subunit P0	Reference gene	Reference gene	F:CAACCCTGAAGTGCTTGACAT R:AGGCAGATGGATCAGCCA
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	Reference gene	Reference gene	F:GCATCCCACAGACTATTTCC R:GCAAAGACAATGACAGACCA

Abbreviations: HIF-1, hypoxia-inducible factor 1; mTOR, mammalian target of rapamycin; PUFA, polyunsaturated fatty acids.

[a]Pathways are related classes were taken from the Kyoto Encyclopedia of Genes and Genomes (KEGG) *Bos taurus* library (https://www.genome.jp/kegg/).

[b]Primer sequences were taken from Sweeney et al. (2016) and Duckett et al. (2009).

RNase-free water in place of cDNA template. Positive control standards containing cDNA extract from either commercially available Angus grass-finished ribeye (*EIF4EBP1*, *ALAD*, *PPARG*, and the two reference genes) or an Angus grain-finished ribeye sample (*NPNT-1*) were prepared by serial dilution at 1:1, 1:10, and 1:100 ratios. Previous work showed up-regulation of these genes in either grass- (*EIF4EBP1*, *ALAD*, *PPARG*) or grain-finished beef (*NPNT-1*), determining the positive controls used in this study (Buchanan et al. 2013; Sweeney et al. 2016).

Cycle-threshold (Ct) values for the two reference genes were used to analyze their suitability for target gene expression normalization using RefFinder (https://www.ciidirsinaloa.com.mx/RefFinder-master/) (Xie et al. 2012). This software integrates geNorm, Normfinder, BestKeeper, and the comparative ΔCt method to compare and rank the tested candidate reference genes. Target gene expression quantification was then calculated according to Sweeney et al. (2016) as follows: Ct-values of the target genes and most stable housekeeping gene (*YWHAZ*) were converted to relative quantities (Q) using the equation $Q = E\Delta Ct$, where ΔCt is the Ct-value for a given sample subtracted by the minimum Ct-value of all samples run for a single target gene, and (E) is the PCR efficiency of the assay. The reference gene normalization factor was calculated by taking the geometric mean of the Q values for the most suitable housekeeping gene. The Q values of the target genes were then divided by the reference gene normalization factor to determine the final normalized expression for each sample.

2.3 | FA Profiling

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Microwave-assisted extraction was performed on beef tissue samples using a CEM Mars 6 Microwave (CEM Corp., Matthews, NC, USA) as described previously (Bronkema et al. 2019; Krusinski et al. 2022c). To extract FAs, 400 mg of minced beef was added to 8 mL of 4:1 (v/v) ethyl acetate:methanol with 0.1% butylated hydroxytoluene (BHT) in a microwave vessel. The microwave was set to 55°C for 15 min with initial ramp of 2 min at 400 W. Following microwave extraction, samples were filtered into another set of tubes containing 3.5 mL of HPLC water before being centrifuged at 2500 rpm (840 × g) for 6 min. The upper layer was transferred to another set of tubes and dried under a stream of nitrogen gas. The oil was then resuspended in 4:1 (v/v) dichloromethane:methanol with 0.1% BHT to bring the concentration to 20 mg of oil/mL.

For the creation of fatty acid methyl esters (FAMEs), 2 mg of oil was resuspended in toluene and 20 μg of internal standard (methyl 12-tridecenoate, U-35 M, Nu-Chek Prep, Elysian, MN, USA). After resuspension, 2 mL of 0.5 N anhydrous potassium methoxide was added, and samples were heated to 50°C for 10 min. After cooling to room temperature, 3 mL of methanolic HCl (5%) was added before heating the samples to 80°C for 10 min. Tubes were allowed to cool down, and 2 mL of HPLC water and 2 mL of hexane were added to the samples before being centrifuged at 2500 rpm (840 × g) for 5 min. The upper layer was transferred to another set of tubes and dried under a stream of nitrogen gas. FAMEs were then resuspended in 1 mL of isooctane to reach a concentration of 2 mg/mL. Samples were then transferred to gas chromatography–mass spectrometry (GC–MS) vials with inserts. Modified methods from Jenkins (2010) were used for the creation of FAMEs.

The PerkinElmer (Waltham, MA, USA) 680/600S GC–MS set to electron impact (EI) mode (70 eV) equipped with an Agilent Technologies (Santa Clara, CA, USA) HP-88 column (100 m, 0.25 mm ID, 0.2 μM film thickness) was used for the quantification of FAMEs according to Kramer et al. (2008). Beef samples were analyzed by injecting 1 μL twice (20:1 split) at 175°C and 150°C with the following temperature settings: (1) initial temperature at 80°C for 4 min; ramp 13°C/min to 175 °C; hold 27 min; ramp 4°C/min to 215°C; hold 35 min, and (2) initial temperature at 80°C for 4 min; ramp 13°C/min to 150°C; hold 47 min; ramp 4°C/min to 215°C; hold 35 min. Beef samples were injected a third time in splitless mode (0.75 min splitless hold time, 40 mL/min flow). Helium was used as the carrier gas with a flow rate of 1 mL/min. Data were acquired in full MS scan mode (m/z 70–400 amu), and the MS transfer line and ion source were kept at 180°C.

To identify FAs on the basis of retention time, MassLynx V4.1 SCN 714 (Water Corp., Milford, MA, USA) was used. EI mass fragmentation was done with comparison to the reference standard (Supelco 37 Component FAME Mix with mead acid, docosatetraenoic acid, *n*-3 docosapentaenoic acid (DPA), *n*-6 DPA, and palmitelaidic acid) (Cayman Chemical, Ann Arbor, MI, USA). The CLA reference standard UC–59 M (Nu-Chek Prep, Elysian, MN, USA) was used for identification of CLA isomers. To identify FAs not included in the reference standard, EI mass fragmentation and elution order were used (Kramer et al. 2008). The concentration of each FA was determined by comparing the peak area of the internal standard and analyte with the standard curve. FAs were reported in percent of total.

2.4 | Metabolomics

All chemicals were LC−MS grade (LiChrosolv) and purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Metabolomics methods were previously described (Krusinski et al. 2024; van Vliet et al. 2023). Beef samples were first ground into a fine powder using a mortar and pestle on dry ice. Then, 200 mg of pulverized beef was mixed with 990 μL of methanol and 10 μL of QReSS internal standards (mixture of isotopically labeled metabolites, Cambridge Isotope Laboratories, Tewksbury, MA, USA). Samples were homogenized for 10 min at 20 Hz using a Qiagen TissueLyser II (Qiagen Sciences, Germantown, MD, USA) with two 5 mm glass beads and then kept at −20 °C for 1 h for protein freeze-out. The supernatant was harvested in fresh tubes after samples were centrifuged at 15,000 rpm (23,000 × g) for 10 min. The supernatant was then diluted with 2 mL of 1% formic acid in water (v/v) and loaded on preactivated Strata C18-E cartridges (Phenomenex, Torrance, CA, USA) for solid-phase extraction (SPE). The cartridges were activated with 1 mL of 1% formic acid in methanol (v/v) and rinsed with 1 mL of 1% formic acid in water (v/v). The diluted supernatant was eluted under 5 psi for 10 min, followed by rinsing with 2 mL of 1% formic acid in water (v/v). Samples were harvested in 1.2 mL of 0.1% formic acid in methanol (v/v) under 5 psi for 5 min. Finally, eluents were dried down under a stream of nitrogen gas and transferred into high-

performance liquid chromatography (HPLC) amber vials with 0.25 mL glass inserts.

Identification of metabolites was done by monitoring precursor ion/product ion pair using multiple reaction monitoring (MRM) via ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). A front-end Shimadzu Nexera LC–40 Series (Kyoto, Japan) liquid chromatography system was used. Samples (5 µL injections) were separated over a Kinetex F5 100 Å column (2.1 mm × 150 mm, 1.7 µM) (Phenomenex, Torrance, CA, USA) at 30°C. The mobile phases were programmed with binary pumps (SIL-40D X3, Shimadzu, Japan) at 0.2 mL/min. The gradient started at 5% B, increased to 95% B over 14 min, and returned to 5% B at 16 min. Mobile phase A was 0.1% formic acid in water (v/v), and mobile phase B was 0.1% formic acid in acetonitrile (v/v), all LC−MS grade. The system was equilibrated for 4 min before the next injection. Samples were kept at 10°C in a SIL–40C autosampler (Shimadzu, Japan). A secluded multiple reaction monitoring (sMRM) method was run on a SCIEX Hybrid Triple Quad 7500 (Framingham, MA, USA) in both positive and negative ionization modes. The OptiFlow electrospray ionization ion source was operated at 400°C. Detailed equipment settings were described previously (Krusinski et al. 2023). In both modes, the maximum cycling time was 1000 ms, with dwell times ranging from 3 to 250 ms depending on the number of MRM triggered at specific retention times. We ran two MRM for each analyte, one quantifier and one qualifier, with a few exceptions. All MRM are shown in Table S1. A 16-point standard curve was run for quantification, and purified standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA).

For quality control, a pooled sample, a double blank (100% methanol), and an internal standard blank sample were injected after every 15 sample injections. The internal standard blanks were used to subtract background from samples, whereas double blanks were used to assess the processing quality of samples. All samples were injected and analyzed in random order using the Analyst 3.1 software (AB SCIEX, Framingham, MA, USA). The integration of peaks was done using the MQ4 method, and all chromatograms were checked manually and corrected if needed. Data normalization was done using QReSS internal standards on the basis of compound class and retention groups, and block corrected for by setting the medians to one (1.00) and normalizing each data point proportionally. Metabolomics data were expressed as arbitrary units (AU).

2.5 | Micronutrient Profiling

Iron, zinc, copper, and selenium were measured using an Agilent 7900 inductivity coupled plasma–mass spectrometer (ICP–MS) (Agilent Technologies Inc., Santa Clara, CA, USA). Controls included a six-point calibration curve and known standards of bovine liver and mussels (National Institute of Standards and Technology, Gaithersburg, MD, USA). Detailed methods were previously described (Krusinski et al. 2024; Wahlen et al. 2005).

For vitamin E analysis, 1 g of minced beef was mixed and homogenized in 5 mL of water before freezing as described in Bronkema et al. (2019). Homogenized samples were frozen at −20°C for 1 h to lyse cells and start precipitating proteins—an essential step to facilitate chromatographic separation (Salina and Regazzoni 2024). Beef samples were then thawed, and a measured aliquot was taken out. Samples underwent only one freeze-thaw cycle to prevent tocopherol degradation. Ethanol was added to the aliquot to fully precipitate proteins, whereas hexane was added to extract fat-soluble vitamins. Samples were centrifuged, the hexane layer was removed and evaporated, and the residue was resuspended in the chromatographic mobile phase in vials. For chromatographic analysis, the Waters Acquity system and Water Empower Pro Chromatography Manager software (Water Corp., Milford, MA, USA) were used. A mobile phase made of acetonitrile:methylene chloride:methanol (70:20:10, v/vv) and a Symmetry C18, 1.7 µm, 2.1 × 50 mm^2 analytical column (Waters Corp., Milford, MA, USA) were used for isocratic elution. The flow rate was 0.5 mL/min, and vitamin E was quantified at UV absorption 295 nm.

2.6 | Data Analysis

A linear regression model to test the effect of diet on gene expression, metabolites, and FAs in beef was performed using RStudio (R version 4.4.1) (R Core Team, Vienna, Austria). Diet, year, and pen were fixed effects, and the experimental unit was each animal. Tukey's adjustment was performed for post-hoc comparisons, whereas p values for multiple comparisons were corrected using the Dunn–Šidák correction. All tests were two-tailed, and the data followed the normality and equal variance assumptions of the model (checked with *qqplot* in RStudio). For all analyses, values below the limit of detection (LOD) were treated as zeroes. All results were considered significant at $p < 0.05$ and reported as mean ± standard error from the mean (SEM).

MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/) was used for unsupervised data visualization. Data were first normalized to mass and then log-transformed. To visualize relationships between each expressed gene and measured compounds, correlation analyses (using each gene as feature of interest) were performed using the Pattern Hunter feature, reporting Pearson r values and the top 25 correlated compounds. Sparse partial least squares discriminant analysis (sPLS-DA) reduces the number of variables to produce robust and easy-to-interpret models. In this analysis, the number of components was 5, with 25 variables per component. The scores plot used component 1 for the x-axis and component 2 for the y-axis, displaying 95% confidence intervals. A loading plot with variables selected by sPLS-DA for a given component was made. Variables were ranked by absolute values of their loadings. To measure the performance of the sPLS-DA model, cross validations with increasing numbers of components were created using the specified number of variables. A hierarchical, ranked clustering heatmap was produced to provide an intuitive visualization of the top 50 compounds between groups. For the heatmap, Pearson distance measure and Ward clustering were performed, and data were reported using the PLS-DA variable importance in projection (VIP) score. Random forest analysis was performed to test the ability of the measured variables to separate beef by dietary group. The number of trees used was 500, the number of predictors (estimators) was 7, and randomness was turned on. Out-of-bag (OOB) data were used

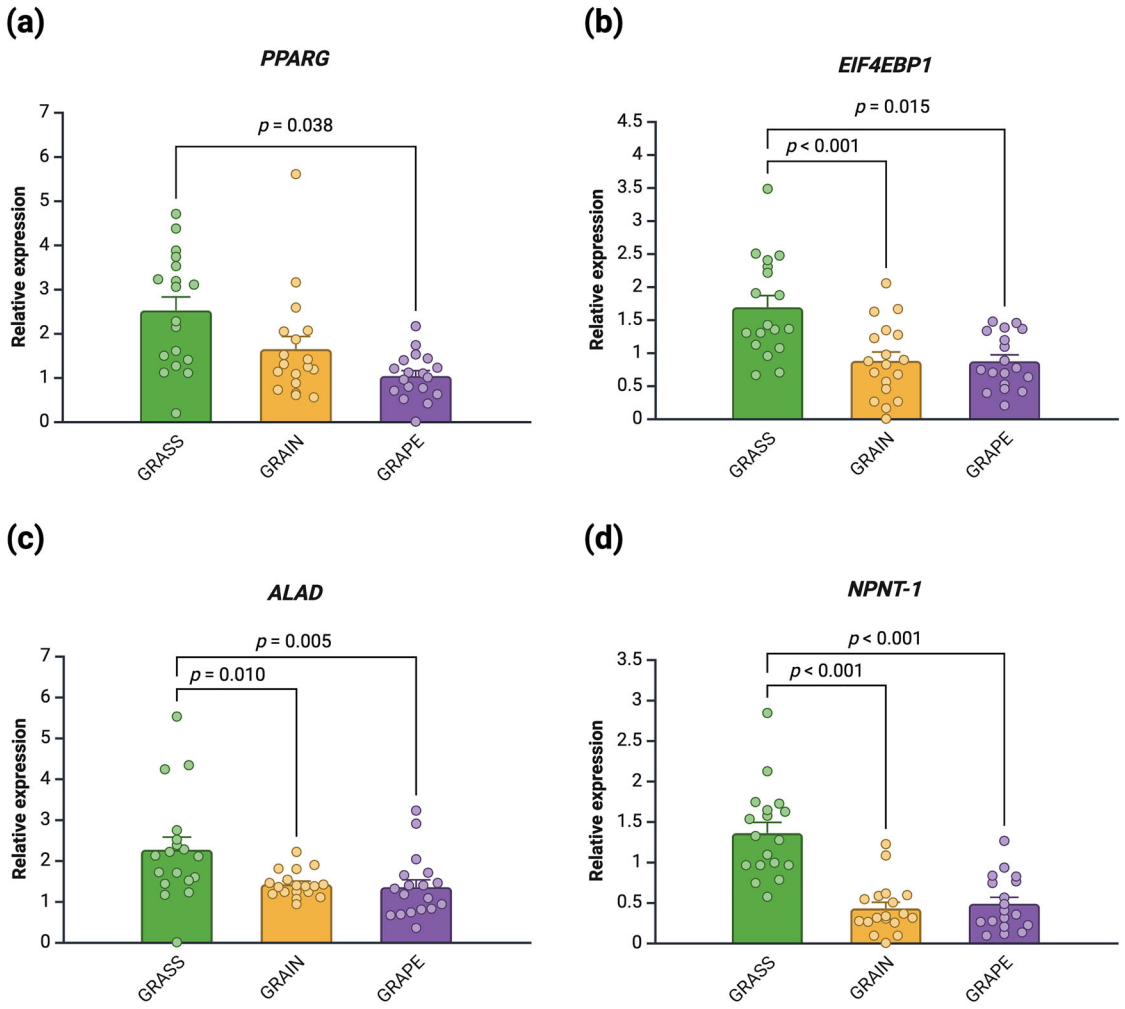

FIGURE 2 | Relative gene expression of the target genes in beef by diet. (a) Peroxisome proliferator activated receptor gamma (*PPARG*) relative expression in beef by diet; (b) Eukaryotic translation initiation factor 4E-binding protein 1 (*EIF4EBP1*) relative expression in beef by diet; (c) Delta-aminolevulinic acid dehydratase (*ALAD*) relative expression in beef by diet; (d) Nephronectin isoform X1 (*NPNT-1*) relative expression in beef by diet. The data shown represent the results from the linear regression model and Tukey's post-hoc comparisons with Dunn–Šidák correction as means (± standard error from the mean) and individual data points. Results were considered significant at $p < 0.05$. Brackets show significant differences in gene expression between dietary groups.

to evaluate the quality of the random forest model to prevent overfitting. All data from targeted metabolomics, gene expression quantification, and FA profiling were included in these analyses.

3 | Results

3.1 | Gene Expression in Beef by Diet

The beef gene expression data for the four measured target genes are shown in Figure 2. For *PPARG*, beef from the GRASS group demonstrated significantly greater relative expression compared to beef from the GRAPE group ($p = 0.038$). Regarding *EIF4EBP1*, *ALAD*, and *NPNT-1*, beef from GRASS consistently showed greater relative expression compared to the other two groups. No significant differences were observed in relative expression for four target genes between beef from GRAIN and GRAPE ($p > 0.05$).

3.2 | Correlations Between Target Genes and Measured Compounds

Correlations between each of the four target genes and the top 25 measured compounds (including FAs, secondary metabolites, and micronutrients) are displayed in Figure 3. *PPARG* was positively correlated with long-chain *n*-3 PUFAs, such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), DPA, and docosahexaenoic acid (DHA), as well as organic acids, including citraconic and fumaric acid. On the other hand, *PPARG* was negatively correlated with palmitoleic acid (C16:1 9c), the *n*-6:*n*-3 ratio, spermine, indole, and homocitrulline. *EIF4EBP1* was positively correlated with *n*-3 PUFAs, vitamin E, and cholic acid but was negatively correlated with multiple *cis*-MUFAs, ethanolamine, trimethylamine, and citrulline. *ALAD* was also positively correlated with numerous long-chain *n*-3 PUFAs, total conjugated linolenic acid (CLnA), fumaric acid, and hippuric acid, whereas negative correlations were sparse and included cyclo-C17:0 and *cis*-MUFAs. Finally, *NPNT-1* followed similar trends by showing

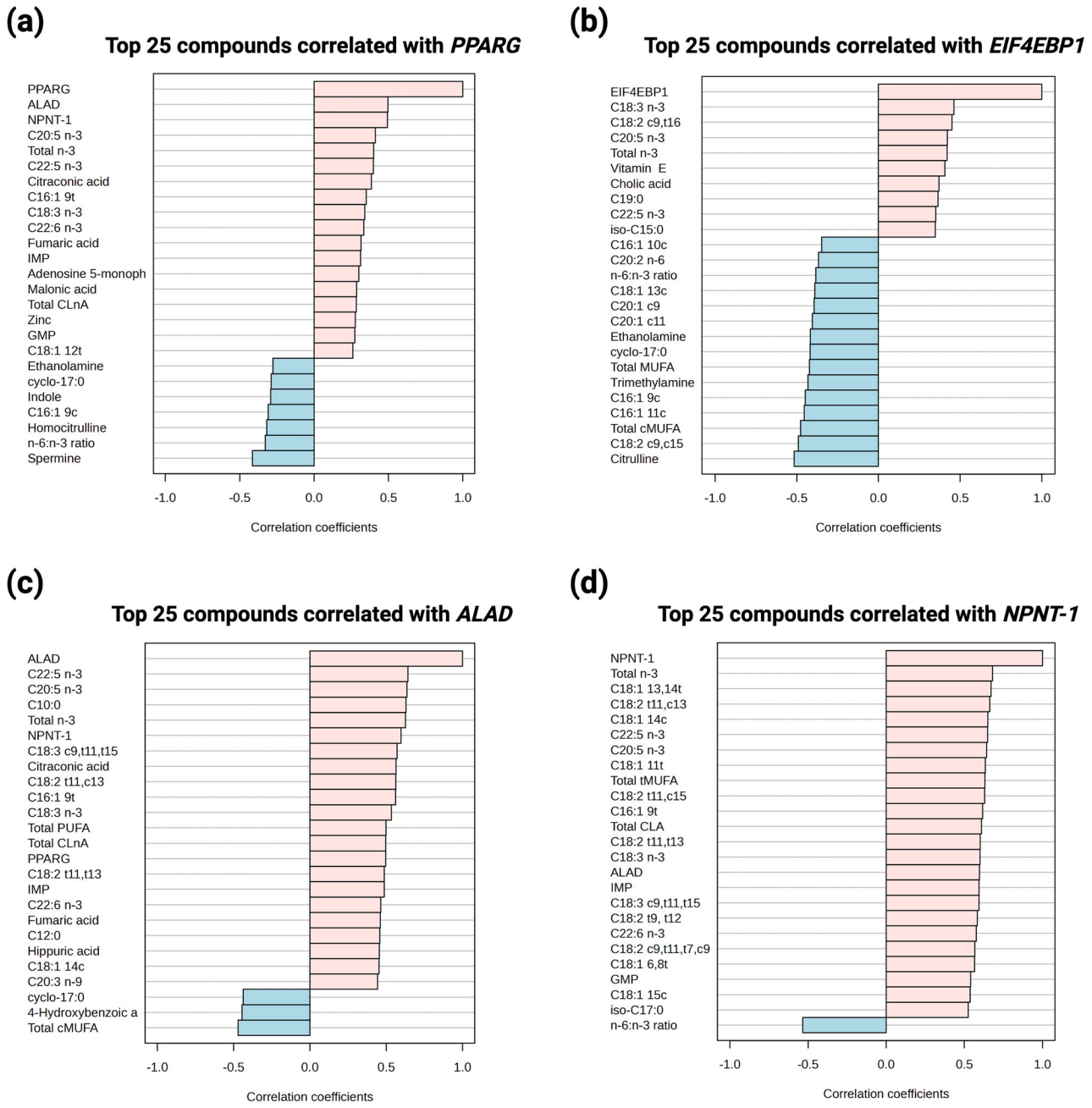

FIGURE 3 | **Correlations between target genes and measured compounds in beef**. Correlation analyses (using each gene as feature of interest) performed using the Pattern Hunter feature reporting Pearson *r* values and the top 25 correlated compounds in MetaboAnalyst (https://www.metaboanalyst.ca/) for (a) Peroxisome proliferator activated receptor gamma (*PPARG*); (b) Eukaryotic translation initiation factor 4E-binding protein 1 (*EIF4EBP1*); (c) Delta-aminolevulinic acid dehydratase (*ALAD*); and (d) Nephronectin isoform X1 (*NPNT-1*).

positive correlations with *n*-3 PUFAs, *trans*-MUFAs, and total CLA, among others. The only negative correlation reported for *NPNT-1* was with the *n*-6:*n*-3 ratio.

3.3 | Multi-Omics Data Visualization for Group Separation

The sPLS-DA scores plot and the loading plot for group separation are shown in Figure 4. The scores plot shows clear separation for beef samples from the GRASS group, whereas beef from GRAIN and GRAPE showed slight overlap with 18.5% variation along component 1. The sPLS-DA loading plot displayed the top 25 compounds capable of group discrimination, with the *n*-6:*n*-3 ratio, ALA, total *n*-3 PUFA, and EPA being the most important. Secondary metabolites such as vitamin E, stachydrine, erythronic acid, succinic acid, and citric acid were also included in the top 25. Regarding target genes, only *NPNT-1* was included. Beef from the GRASS group contained greater levels of the majority (96%) of variables included in the loading plot when compared to the GRAIN and GRAPE groups, with the exception of the *n*-6:*n*-3 ratio. The ranked heatmap showing the top 50

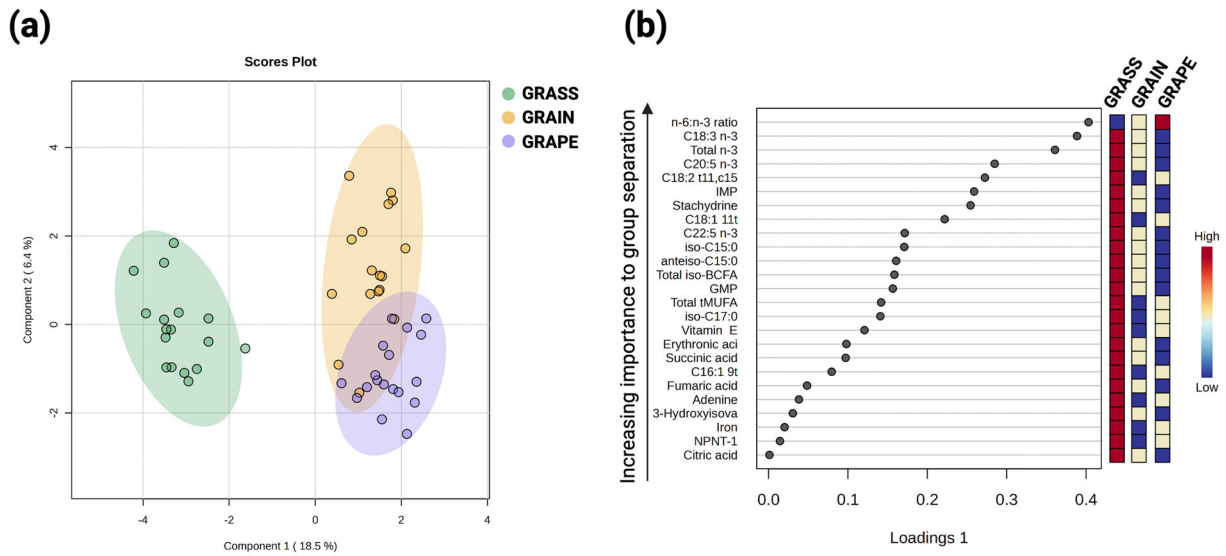

FIGURE 4 | **Beef separation plot and variables of importance for group separation in beef by diet**. (a) Sparse partial least squares discriminant analysis (sPLS-DA) scores plot showing separation of beef by diet. In this analysis, the number of components was 5, with 25 variables per component. The scores plot used component 1 for the *x*-axis and component 2 for the *y*-axis, displaying 95% confidence intervals; (b) Loading plot showing variables selected by sPLS-DA for a given component. Variables were ranked by absolute values of their loadings, with increasing importance to group separation. Analyses were performed in MetaboAnalyst (https://www.metaboanalyst.ca/). GRASS, beef fed a biodiverse pasture diet; GRAIN, beef fed a total mixed ration in feedlot; GRAPE, beef fed a total mixed ration supplemented with 5% (dry matter) grapeseed extract for the last 30 days in feedlot.

compounds varying between dietary groups according to PLS-DA VIP scores is displayed in Figure 5. Similar trends were observed with *n*-3 PUFAs, the *n*-6:*n*-3 ratio, MUFAs, CLA, organic acids, phytochemicals, amino acid derivatives, and *NPNT-1*, showing variations between beef from different dietary groups. The full lists of FAs (Table S2), secondary metabolites (Table S3), and micronutrients (Table S4) measured in beef by diet are included in the Supporting Information section.

3.4 | Classification Matrix for Beef Dietary Group Prediction Accuracy

The random forest classification and prediction matrix is displayed in Figure 6. The overall accuracy of the random forest algorithm on the basis of OOB error was 90.4%. Beef samples from the GRASS group were all accurately classified with 100% group predication accuracy. Beef samples from GRAPE were separated with 94.4% accuracy, with only one sample being incorrectly predicted as belonging to the GRASS group. Finally, beef samples from GRAIN were separated with 76.5% accuracy, with four samples being incorrectly classified as belonging to the GRAPE group. This analysis used all measured metabolites, gene expression data, and FAs for group prediction.

4 | Discussion

The main objective of this study was to use a multi-omics (i.e., gene expression quantification, metabolomics, and FA profiling) hurdle approach to separate and authenticate beef from different dietary groups. By using this approach, we found that all target genes tested in this study were up-regulated in GFB (GRASS) compared to the other two groups mainly fed a TMR finishing diet. We also uncovered correlations between target genes, FAs, and secondary metabolites. The key variables that allowed dietary group separation of beef were mostly related to *n*-3 PUFAs, the *n*-6:*n*-3 ratio, vitamin E, organic acids, amino acid derivatives, and the *NPNT-1* gene. The listed dietary compounds, considered to be beneficial for human health, were concentrated in beef from GRASS. The success of beef separation by dietary treatment was highlighted by the 90.4% prediction accuracy of the random forest model, with beef from GRASS being 100% accurately predicted and beef from GRAPE being 94.4% accurately predicted.

Sweeney et al. (2016) identified *ALAD*, *EIF4EBP1*, and *NPNT-1* as the top three genes with potential use for authentication and separation of beef production systems. Although not one of the top target genes identified by Sweeney et al. (2016), *PPARG* has significant roles in regulating fat cell differentiation and FA biosynthesis. On the basis of this rationale, we decided to include *ALAD*, *EIF4EBP1*, *NPNT-1*, and *PPARG* in our approach. All four tested genes showed greater normalized relative expression values in GFB compared to the other groups. Sweeney et al. (2016) reported similar findings, except for *NPNT-1*, which was up-regulated in beef from concentrate feeding systems. The difference in *NPNT-1* expression between our study and the study by Sweeney et al. (2016) may originate from the different concentrate diets used, the different breed of cattle, and the longer trial period. The greater expression of *NPNT-1* in beef from GRASS in the current study could be explained by the induction of angiogenesis (i.e., process of new blood vessels formation from existing blood vessels) through exercise. In particular, Kuek et al. (2016) found that NPNT mediated angiogenesis in mice, whereas Y. Zhang et al. (2022) showed the beneficial effects of NPNT on angiogenesis and cardiac repair in mice. Grass-finished animals

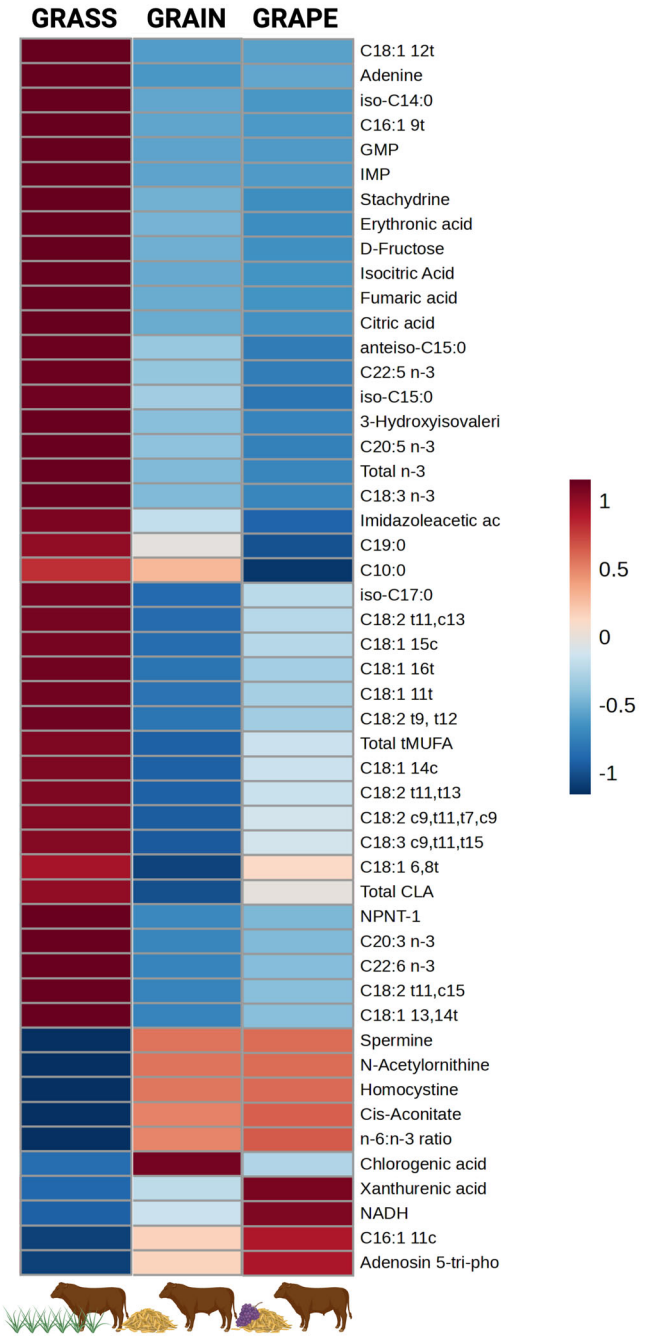

FIGURE 5 | **Ranked heatmap visualizing the top 50 compounds in beef between dietary groups**. Pearson distance measure and Ward clustering were used, and data is reported using the PLS-DA variable importance in projection (VIP) score. The hierarchical heatmap was made in MetaboAnalyst (https://www.metaboanalyst.ca/). GRASS, beef fed a biodiverse pasture diet; GRAIN, beef fed a total mixed ration in feedlot; GRAPE, beef fed a total mixed ration supplemented with 5% (dry matter) grapeseed extract for the last 30 days in feedlot.

spend their whole life on pasture, grazing and moving around, which could potentially explain why *NPNT-1* was upregulated. From the results of the different analyses conducted here, *NPNT-1* appears to be the most influential gene for dietary group separation of the four genes tested.

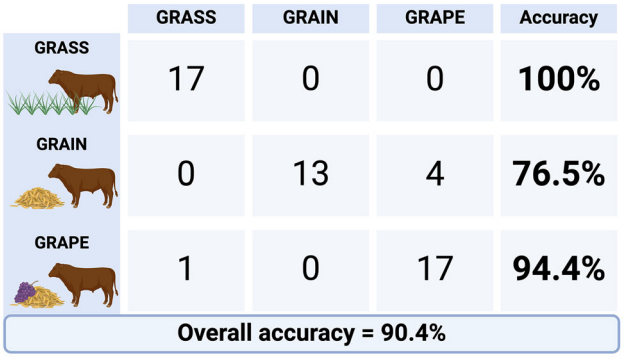

FIGURE 6 | **Random forest classification matrix**. This analysis was used to test the ability of the measured variables to separate beef by dietary group. The number of trees used was 500, the number of predictors (estimators) was seven, and randomness was turned on. Out-of-bag (OOB) data were used to evaluate the quality of the random forest model to prevent overfitting. The analysis was performed in MetaboAnalyst (https://www.metaboanalyst.ca/). GRASS, beef fed a biodiverse pasture diet; GRAIN, beef fed a total mixed ration in feedlot; GRAPE, beef fed a total mixed ration supplemented with 5% (dry matter) grapeseed extract for the last 30 days in feedlot.

The expression of *ALAD* also varied on the basis of dietary treatment. A previous study by Afonso et al. (2020) identified regulators of mineral composition in beef and highlighted that *ALAD* encodes for a metal ion binding protein linked in zinc. The authors also indicated that *ALAD* is a candidate gene linked to beef minerals in general (Afonso et al. 2020). Interestingly, the correlation analysis between *ALAD* and the top 25 measured variables in the present study did not show positive correlation between the target gene and minerals. However, GFB is consistently greater in minerals including iron, zinc, copper, manganese, and molybdenum compared to grain-finished beef (Krusinski et al. 2022b), which could explain the up-regulation of *ALAD* in animals finished on pasture. Additionally, the sPLS-DA loading plot ranked iron as one of the top compounds with important group separation potential, with greater concentrations in beef from GRASS compared to the other groups. Leal-Gutierrez et al. (2020) identified the *ALAD* gene as both expression and splicing master regulator in beef, meaning that this target gene has transcriptional control over a suite of genes in the same metabolic pathway. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), *ALAD* is involved in porphyrin metabolism and the biosynthesis of vitamins and co-factors. The porphyrin ring is the framework for the heme molecule, the pigment in hemoglobin, and is heavily involved with iron (Di Pierro and Granata 2020).

The third target gene identified by Sweeney et al. (2016) for the authentication of GFB against grain-finished beef is *EIF4EBP1*. This gene is involved with the mechanistic target of rapamycin (mTOR) pathway, which plays major roles in protein synthesis, muscle growth, and longevity (Crombie et al. 2023). The mTOR signaling pathway is impaired in skeletal muscles of aging individuals, and the expression of *EIF4EBP1* could mitigate sarcopenia. Crombie et al. (2023) showed that activation of 4EBP1 (a protein encoded by the *EIF4EBP1* gene) relieved oxidative stress in muscle and restored mitochondrial homeostasis and

function in mice. Protein synthesis in skeletal muscle through the mTOR pathway is stimulated by various nutrients, including amino acids, FAs, vitamins, and glucose (Xu and Velleman 2023). Bile acids can also activate mTOR signaling (Chao et al. 2019). Interestingly, our results showed that cholic acid (a bile acid) was positively correlated with the expression of *EIF4EBP1*. This target gene showed greater expression values in beef from GRASS compared to the other two groups, indicating that pasture-based feeding management (along with specific nutrients found in forages) could potentially play a role in protein synthesis and longevity in cattle. It was previously noted that the mTOR pathway can govern adipogenesis of muscle satellite cells by the expression of the *PPARG* gene in mice (Xu and Velleman 2023). Xu et al. (2022) reported a relationship between the mTOR pathway and intracellular lipid accumulation in turkey meat. Zhang et al. (2015) also found that mTOR is essential for satellite cell function and skeletal muscle regeneration in mice. The authors highlighted the potential implications of these findings for promoting meat growth in livestock by targeting the mTOR pathway.

PPARG was positively correlated with long-chain, unsaturated FAs in the present study. It was previously reported that increased FA chain length and unsaturation can regulate *PPARG*, and that this target gene allows for the identification of genetic markers that contribute to differences in FA profiles (Bionaz et al. 2013; Buchanan et al. 2013). *PPARG* usually shows higher expression values in adipose tissue, with expression of this gene being two-thirds that in skeletal muscles (Gu et al. 2021). Upregulation of *PPARG* in pig skeletal muscles was associated with the formation of oxidative muscle fibers associated with endurance exercise (Gu et al. 2021). Greater expression values for *PPARG* were observed for beef from GRASS in the present study, whereas no differences were seen with the inclusion of GSE. Similar results were reported by Milojevic et al. (2020), finding no significant effect with the inclusion of GSE on the expression of genes related to lipid metabolism. Polyphenols prevent inflammation in tissue, with *PPARG* playing a significant role in this mechanism (Gessner et al. 2017). We hypothesized that including GSE in the diet of feedlot cattle would increase the meat polyphenol content and therefore up-regulate the expression of *PPARG* in beef from the GRAPE group. Our results do not show significant differences between the GRAIN and GRAPE groups for this target gene, whereas beef from GRASS showed greater expression values. One explanation could be the low dose of GSE and length of supplementation used in this study (5% DM for the last 30 days of the finishing period). It was previously reported that the effects of GSE on meat are dose-dependent (Krusinski et al. 2023; Manso et al. 2016). The abundance of phenolic compounds in the biodiverse pasture fed to the GRASS group could explain the observed trends for *PPARG*. *PPARG* can also be upregulated in response to exercise in livestock (M. Zhang et al. 2022). Animals in the GRASS group were continuously grazing and, therefore, exercising more than animals in the other two feedlot groups, which could also explain the greater expression of this gene in beef from the GRASS group. These results are consistent with previous studies which also reported upregulation of the *PPARG* gene in grazing animals (Buchanan et al. 2013; Dervishi et al. 2012). FAs (especially PUFAs) are natural ligands of *PPARG* that control the expression of genes related to lipid metabolism (Grygiel-Gorniak 2014). Greater concentrations of PUFAs (especially EPA and DHA) in tissue lead to the activation of *PPARG*. Flavonoids can also act as natural ligands (Malibary 2023). Additionally, heterodimers are formed between *PPARG* and the retinoid x receptor (RXR) (Kilu et al. 2021). Retinoic acid, a vitamin A metabolite, plays a major role in the activation of the *PPARG*/RXR heterodimer, which means that greater concentrations of vitamin A and its metabolites may downregulate the expression of *PPARG* (Malibary 2023). However, in the present study, levels of retinol (a form of vitamin A) were not significantly different across all three treatments. It appears that PUFA levels, flavonoids concentrations, and exercise played a major role in the upregulation of *PPARG* in beef from GRASS compared to the other two groups. All target genes tested as part of this study are related to long-chain *n*-3 PUFAs, vitamins, and secondary compounds metabolism and biosynthesis, compounds that may play beneficial roles in human nutrition.

One of the main motivations for consumers to purchase GFB is its potential health benefits (Xue et al. 2010). From the results shown in the sPLS-DA loading plot, the PLS-DA VIP score ranked heatmap, and the tables in Supporting Information section, compounds with potential health benefits were found in greater concentrations in beef from GRASS compared to the other two groups. For example, *n*-3 PUFAs (including ALA, EPA, and DHA), MUFAs, CLA isomers, phytochemicals, organic acids, vitamin E, and iron all presented greater levels in beef from GRASS. These compounds have numerous human health benefits, including cardiovascular and cognitive advantages (Mendivil 2021; Parolini 2019; van Vliet et al. 2021). Consuming foods containing high concentrations of these metabolites (e.g., *n*-3 PUFAs, polyphenols, vitamins, and micronutrients) can help prevent malnutrition and address nutritional deficiencies in populations (Ahmed et al. 2022). Others also reported greater concentrations of such compounds in GFB compared to grain-finished beef (Evans et al. 2024; van Vliet et al. 2023). Spears et al. (2024) recently reported differences in postprandial response in humans after consumption of grass- or grain-finished beef. The authors reported differences in plasma metabolites between the two groups at different time points, with participants consuming GFB exhibiting greater levels of calamendiol, an anti-inflammatory compound, in their blood (Spears et al. 2024).

Although differences in nutritional composition between grass- and grain-finished animals are usually clear, less is known about agricultural by-product supplementation in cattle diets. In the present study, we included 5% (DM basis) of GSE to a TMR for the last 30 days of the finishing period (GRAPE group), hypothesizing that GSE would increase phytochemical and polyphenolic concentrations in beef. However, only minor differences in nutrient density between the GRAIN and GRAPE groups were observed (lower abundance of some SFAs, slightly greater abundance of some CLA isomers, vitamin E, and iron in GRAPE). One explanation could be the low dose of GSE used in this study (5% DM basis). Previous studies that found a dose-dependent effect only started observing differences in the nutritional composition of animal products after adding at least 10% (DM basis) of grape by-products to the diet (Manso et al. 2016; Serra et al. 2013). Additionally, the modest supplementation period of 30 days could have further limited efficacy. However, agricultural by-products, such as GSE and grape pomace, merit investigation as they are cheap, abundant, and do not compete

with human consumption (Blasi et al. 2024; Brenes et al. 2008; Muñoz-González et al. 2019).

Although no major differences in gene expression, FA, and metabolomics profiles were observed between beef from GRAIN and GRAPE, the prediction models used in the current study were still able to separate beef from the different dietary treatments. The sPLS-DA scores plot showed some overlap between beef from GRAIN and beef from GRAPE, which was expected as the base diet was similar for both groups (TMR). Interestingly, results from the random forest classification matrix showed that the only error with the GRAPE group separation was that one beef sample from the GRAPE group was incorrectly classified as GRASS. This was unexpected, as both the GRAIN and GRAPE groups were fed mainly a TMR. Polyphenols present in pastures and GSE could explain this misclassification. Overall, we identified grass- vs. grain-finished beef with a high degree of accuracy, even when the TMR was supplemented with GSE.

One of the main goals of this study was to identify key biomarkers that would allow for group separation and beef authentication. Food authentication usually involves multiple approaches, including PCR-based methods, MS approaches, mineral traceability, stable isotope traceability technology, and near-infrared spectroscopy (Wang et al. 2022). Prache et al. (2020) noted that FAs, volatile and phenolic compounds, vitamin E, and gene expression can all be used for the authentication of GFB products. Additionally, the authors highlighted the importance of using multiple methods synergistically to obtain better results, especially for less-contrasted feeding regimes (i.e., for comparing more groups than grass-fed vs. grain-fed only) (Prache et al. 2020). It is for this reason that we used a multi-omics hurdle approach to separate beef from three different feeding regimes. By running targeted metabolomics and FA profiling, we uncovered around 175 compounds in beef, and by using multivariate analysis and prediction models, we identified key metabolites that contribute most to dietary background differences in meat; however, these MS-based methods are time consuming, expensive, and require specialized knowledge (Ahuja et al. 2023). Highly targeted methods such as RT–qPCR for gene expression used in this study, are faster, more affordable, and require less technical knowledge. There is a critical need for rapid, reliable food testing methods that are more affordable and require less equipment and preparation.

Given that the main discriminating biomarkers for dietary treatments in beef have been identified, it is worth exploring "bigger picture" methods focusing on these biomarkers (i.e., the *n*-6:*n*-3 ratio, *n*-3 PUFAs, vitamin E, iron, stachydrine, and *NPNT-1*). Instead of focusing on individual compounds, future authentication studies should examine using shorter methods for *n*-6:*n*-3 ratio determination, lipid oxidation analysis, or highly targeted PCR-based methods to separate and authenticate beef products. Although multivariate analyses allowed us to separate beef samples by dietary groups in this study, such statistical analyses usually require large datasets to perform well and are not always generalizable (Hazra and Gogtay 2017). Current methods of testing work well in controlled, laboratory settings, but implementing them in industry settings is challenging. In the US, the AGA provides third-party certifications for grass-fed products. To ensure compliance with set standards and traceability, they perform on-farm visits and audits (American Grassfed Association 2022). However, no analytical testing of products to ensure compliance with dietary and production practices is performed. The Bleu-Blanc-Coeur (2020) initiative in France conducts on-farm audits and analytical testing of animal foods to guarantee animal welfare, soil and environmental health, and nutrient density. Their analyses focus on FAs, vitamins, minerals, and secondary metabolites. For example, to receive the Bleu-Blanc-Coeur label, beef products need to display a *n*-6:*n*-3 ratio of less than 4:1. Our work shows that such analytical methods to ensure labels of quality and authenticity can be implemented in North America. Our recommendation for third-party certifications for grass-fed and finished beef in the US is to include analytical analyses of products coupled with on-farm audits to ensure compliance with set standards and protocols. This would also allow for the standardization of labels and would benefit both producers and consumers.

This study used an integrated approach to differentiate and separate beef from varying dietary backgrounds. Although most studies focus on a limited number of key nutrients, the current study reported more than 175 compounds, including FAs (with multiple isomers), vitamins, minerals, secondary metabolites, and four target genes. Additionally, most studies focus solely on comparing grass- and grain-finished beef. This study included a third finishing diet by including GSE to a TMR, unveiling more subtle differences in beef based on diet. Finally, metabolomics coupled with FA profiling and gene expression quantification uncovered interesting underlying pathways in beef cattle. Our study has some limitations. The dose of GSE used was most likely too low to show major differences. We also only focused on four target genes chosen from previous studies. It is possible that other genes may show greater discrimination power. Finally, the methods used here require expensive equipment, are time-consuming, and require specialized knowledge, making their applicability to the industry challenging. Therefore, faster, reliable, and more affordable authentication methods should be developed and investigated. The production of a beef database gathering all measured variables and production systems would facilitate the implementation of fast, high-throughput segregation methods. Frigerio et al. (2024) noted that chemical analyses require complex sample preparation and expensive equipment. Using our already-gathered data, developing simplified sensor-based tools would accelerate beef authentication. Colorimetric indicator could be developed, but methodological and technological innovations are needed (Frigerio et al. 2024). Finally, deep learning-based and artificial intelligence (AI) tools could lead to new authentication methods when coupled with machine vision systems as portable devices. These advances would make beef segregation methods on the basis of production systems more applicable to the industry.

5 | Conclusion

In this study, we used a multi-omics (i.e., gene expression quantification, metabolomics, and FA profiling) hurdle approach to separate and authenticate beef from three different dietary groups. Results showed that all target genes tested were upregulated in GFB compared to the other two groups mainly fed a TMR. These genes play major roles in animal health, longevity, and metabolism of FAs, minerals, vitamin co-factors, and phy-

tochemicals. The key biomarkers that allowed dietary group separation of beef were mostly related to *n*-3 PUFAs, the *n*-6:*n*-3 ratio, vitamin E, organic acids, amino acid derivatives, purines, nucleotides, plant-derived secondary metabolites, and the *NPNT-1* gene. These compounds, considered to be beneficial for human health, were concentrated in beef from GRASS. The success of beef separation by dietary treatment was highlighted by the 90.4% prediction accuracy of the random forest model, with beef from GRASS being 100% accurately predicted, beef from GRAIN being 76.5% accurately predicted, and beef from GRAPE being 94.4% accurately predicted. These results showed the strong authentication potential of omics-based methods and may be used to develop a robust database that can contribute to future studies. This study reported more than 175 compounds and investigated commonly used finishing diets (grass- and TMR-based) as well as an underutilized agricultural by-product (GSE). It also highlighted the need for stronger and more robust standardized grass-fed and finished certification standards. Analytical methods coupled with multivariate data analyses should be used by certification organizations, and future work should focus on developing faster, more affordable analytical methods using only a few key biomarkers or biomarker classes.

Author Contributions

Lucas Krusinski: conceptualization, methodology, formal analysis, data curation, statistical analysis, writing – original draft preparation, writing – review and editing. **Chloe Castanon**: methodology, formal analysis, data curation, writing – original draft preparation, writing – review and editing. **Rosalee S. Hellberg**: methodology, formal analysis, data curation, writing – review and editing, supervision. **Isabella C. F. Maciel**: writing – review and editing. **Muhammad Ahsin**: methodology, formal analysis, data curation, writing – review and editing. **Stephan van Vliet**: methodology, formal analysis, data curation, writing – review and editing. **Jason E. Rowntree**: conceptualization, methodology, data curation, writing – review and editing, supervision, funding acquisition. **Jenifer I. Fenton**: conceptualization, methodology, formal analysis, data curation, writing – original draft preparation, writing – review and editing, supervision, funding acquisition. All authors read and approved the final manuscript.

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Ethics Statement

The animal use and procedures received approval from the Institutional Animal Care and Use Committee at Michigan State University (IACUC #201800155) from December 18, 2018 to December 19, 2021.

Conflicts of Interest

L.K., J.E.R., and J.I.F., report grants from USDA SARE and the Greenacres Foundation for the enhancement of the healthfulness and authenticity of GFB. They have not accepted personal honoraria from any organization to prevent undue influence in the eye of the public. S.v.V. reports grants from USDA, the Turner Institute of Ecoagriculture, Perdue Foods LLC, Applegate LLC, the Bionutrient Institute, and the Greenacres Foundation. S.v.V. reports having received speaking fees and travel reimbursements for talks related to the topic. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered potential conflicts of interest. The Greenacres Foundation had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data Availability Statement

The datasets supporting the conclusions of this article are included within the manuscript. Metabolomics and fatty acid data used for statistical analysis are included in the Supporting Information. Additional information can be provided by the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.