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Whole blood RNA-seq analysis reveals the immunomodulatory effects of a supplemental multi-strain direct-fed microbial in the diet of newly weaned beef steers[1]

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We examined the effects of a blend of live *Saccharomyces cerevisiae*, multiple probiotic bacteria, and their fermentation products on the whole blood transcriptome of newly weaned beef steers during a 56-d receiving period. Forty newly weaned Angus crossbred steers (12-h postweaning; 217 ± 4.6 kg of body weight [BW]; 202 ± 4 d of age) from three different sources were stratified by BW and randomly assigned to one of the two treatments: 1) basal diet with no additive (CON; n = 20), and 2) the basal diet supplemented with 9 g/steer/d of a multi-strain microbial additive (PRO; n = 20). The PRO additive was a blend of *S. cerevisiae* and the fermentation products of *Enterococcus faecium*, *Bacillus licheniformis*, *B. subtilis*, *Lactobacillus animalis*, and *Propionibacterium freudenreichii*. On day 56, 10 mL of blood was collected from 10 randomly selected beef steers from each treatment group prior to morning feeding. Total RNA was isolated from the whole blood samples for the determination of gene expression profiles. Differentially expressed genes (DEGs) were identified using a false discovery rate (FDR) ≤ 0.10. A total of 41 DEGs were detected; 21 genes, including *TLR10*, *GPR183*, *LGR4*, and *FCRL1*, were upregulated in steers fed the PRO additive compared to CON, while 20 genes, such as *C3*, *DDIT4*, and *ADCY8* were downregulated. Gene ontology analysis of the DEGs revealed the enrichment (FDR< 0.05) of pathways related to positive regulation of inflammatory response, regulation of cytokine secretion, positive regulation of defense response, and positive regulation of response to external stimuli in beef steers fed PRO additive. No significant differences (P > 0.05) in growth performance (BW, DMI, or ADG) were observed between CON and PRO steers. In conclusion, this study revealed that beef steers fed the PRO additive exhibited differential expression of genes related to immune function and inflammatory

response, suggesting an effect on immunity and stress resilience. These findings highlight the potential of multi-strain direct-fed microbials as a nutritional strategy to support immune health, resilience to stress, and overall welfare in beef cattle during the weaning and receiving period.

KEYWORDS

beef cattle, differentially expressed genes, immune competence, inflammatory response, weaning

Introduction

The transition period following weaning is one of the most critical and stressful phases in the life of beef cattle (Justice et al., 2024). This period is often associated with multiple stressors, including transportation, vaccination, pathogen exposure, dietary changes, and adaptation to a new environment (Lynch et al., 2019; Galyean et al., 2022). If not managed properly, these stressors can reduce dry matter intake, impair growth performance, and negatively impact cattle health. Additionally, the incidence of bovine respiratory disease (BRD) remains high during the feedlot receiving phase (de Souza et al., 2018; Beenken-Bobb et al., 2023), despite efforts to reduce stress and the implementation of immunization strategies against BRD pathogens (Snowder et al., 2006). Therefore, identifying effective strategies to mitigate these stressors is crucial for enhancing the health and productivity of beef cattle.

Nutrition plays a vital role in animal health, and one promising approach to reducing stress in calves involves the use of direct-fed microbials (DFMs). Direct-fed microbials, which include various microorganisms such as lactic acid-producing and spore-forming bacteria, yeast, and fungi have gained attention for their ability to modulate gut microbiota, enhance nutrient absorption, improve immunocompetence, and reduce morbidity in newly weaned beef cattle (Adeyemi et al., 2019; Guimaraes et al., 2024; Treon et al., 2024). Additionally, microbial fermentation by-products such as short-chain fatty acids (SCFAs) have been shown to provide further benefits by modulating the immune system, reducing inflammation, and improving gut barrier function (Prajapati et al., 2023; Rafique et al., 2023). Recent strategies have focused on combining live microorganisms with their fermentation products to enhance efficacy, although the effectiveness of these approaches can vary depending on factors such as microbial strain selection, dosage, and diet composition (AlZahal et al., 2014; Ogunade et al., 2020; Ban and Guan, 2021). Treon et al. (2024) examined the effects of adding a blend of *Saccharomyces cerevisiae*, multiple live probiotic bacteria, and their fermentation products on the performance and health of newly weaned beef steers during a 56-d receiving period. The results from Treon et al. (2024) demonstrated that beef steers supplemented with the additive exhibited improved growth performance and reduced stress response, as indicated by a lower white blood cell concentration during the initial days after weaning compared to non-supplemented steers. However, the exact mode of action of this additive remains unknown.

RNA sequencing provides a comprehensive method for analyzing gene expression, offering critical insights into the molecular pathways influenced by DFMs, particularly those involved in immune response, inflammation, nutrient absorption, and stress regulation (Mejia-Garcia et al., 2024; Shemery et al., 2025). Additionally, RNA-seq facilitates the detection of novel gene expression patterns and the identification of biomarkers associated with improved health and productivity in cattle (Wickramasinghe et al., 2014; Perera et al., 2022). Recent applications of RNA-seq in beef cattle have identified differentially expressed immune-related genes in response to weaning (O'Loughlin et al., 2012) and dietary interventions (Wang et al., 2023), further supporting its value for evaluating nutritional strategies. Whole blood serves as a readily accessible and minimally invasive tissue for transcriptomic analysis, providing a practical approach for identifying biomarkers related to immune function and stress responses (Bai et al., 2016). Compared to tissue biopsies, blood sampling enables real-time, on-farm monitoring of physiological changes, allowing for faster assessments of cattle health and the effectiveness of dietary interventions (Melchizedek and Maria, 2023; Zoratto et al., 2024). We hypothesized that dietary supplementation of a multispecies DFM would modulate the gene expression profile, particularly those involved in immune response, inflammation, nutrient absorption, and stress regulation of newly weaned beef steers. Therefore, the objective of this study was to evaluate the effects of dietary supplementation with a blend of live *Saccharomyces cerevisiae*, multiple probiotic bacteria, and their fermentation products on whole blood transcriptome in newly weaned beef steers during a 56-day receiving period.

Materials and methods

Animals, housing, and feeding

The use of animals in this experiment was approved by the Institutional Animal Care and Use Committees of West Virginia

University (protocol number #2108046615.1). Forty newly weaned Angus crossbred beef steers (12-h postweaning; 217 ± 4.6 kg of body weight [BW]; 202 ± 4 d of age) from three different sources (West Virginia Department of Agriculture, Ben Wetzel Farm, Isaiah Smith Farm) were used. Steers were vaccinated 5 months prior to the start of the experiment and received the booster shots 2 weeks prior. The beef steers were transported approximately 160 kilometers to the research feedlot barn and immediately weighed, processed, and placed on a corn silage-based diet on the day of arrival (d 0). Processing included ear tag placement for unique radiofrequency identification and administration of appropriate vaccines, as well as an injection of dewormer. The vaccine protocol included Alpha-7/MB-1 Cattle Vaccine (Boehringer Ingelheim Animal Health, Duluth, GA), Pyramid-5 + Preresponse SQ Cattle Vaccine (Boehringer Ingelheim Animal Health), and the dewormer used was Safeguard Dewormer Suspension (Merck Animal Health, Summit, NJ). Based on d 0 BW, the steers were stratified by BW into two weight blocks. For each weight block, the beef steers were randomly assigned into one of two pens (20 steers per pen) such that each pen had a similar average BW at the beginning of the experiment. Each pen (dimensions = 11.89 × 9.75 m^2) was equipped with two GrowSafe intake nodes (GrowSafe Systems Ltd., Airdrie, AB, Canada) to measure individual feed intake. Starting from d 1, the pens were randomly assigned to receive a corn silage-based diet with no additive (CON; 1 pen; n = 20) or a basal diet supplemented with 9 g per head of a PRO additive (PRO; 1 pen; n = 20) for a period of 56 d. The PRO additive (Papillon, Easton, MD) is a blend of live *S. cerevisiae* (1.41 billion CFU/g), multiple live bacteria (*E. faecium*, *B. licheniformis*, *B. subtilis*, *L. animalis*, and *P. freudenreichii*) and their fermentation products (total bacterial count = 120 million CFU/g). The basal diet was fed as a total mixed ration (TMR; Table 1), and the additive was blended into the TMR at a specific percentage, calculated based on the previous day's average feed intake for each pen (day × intake was utilized to determine the inclusion rate for day 'x +1'). This procedure ensured that each beef steer in every pen received the required quantity of the additive, amounting to an average of 9 g (12.7 billion CFU of *S. cerevisiae* and 1.08 billion CFU of total bacteria) of PRO per head per day. To prevent any risk of cross-contamination, pens for the CON and PRO groups were physically separated by barriers and the CON and PRO diets were prepared separately in dedicated feed trucks. The diets were provided *ad libitum* to the steers, and they had unrestricted access to water.

Body weight and intake measurement

Steers were weighed prior to morning feeding on days 0, 1, and 56. Individual feed intake was recorded using Grow-Safe intake nodes (GrowSafe Systems Ltd., Airdrie, Alberta, Canada). Average daily gain (ADG) was calculated by subtracting the initial body weight (average of days 0 and 1) from the final body weight on day 56 and dividing by the total duration of the experiment (56 days). Total mixed ration samples were collected daily, weighed, and oven-dried at 55°C for 72 hours to determine dry matter (DM) content. Dried TMR subsamples were composited within treatment, ground using a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) to pass through a 2-mm sieve, and analyzed for nutritional composition at a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY).

Blood sample collection and RNA extraction

On day 56, 10 mL of blood were collected from 10 randomly selected beef steers per treatment before morning feeding (after overnight feed withdrawal). The blood samples were taken from the jugular vein into tubes containing sodium heparin. Subsequently, subsamples of 500 µL each were immediately transferred into RNA-protect tubes (Cat. No. 76,554; Qiagen) per sample. RNA-protect tubes contained a reagent capable of lysing blood cells and stabilizing intracellular RNA. The samples were stored at -80 °C for later analyses. The use of 10 animals per treatment group for RNA-seq is consistent with established practices in livestock transcriptomics, where 6 to 10 biological replicates per group are commonly used to achieve acceptable statistical power for detecting

TABLE 1 Ingredient and chemical composition of the basal diet.

Ingredient (% DM)	% of dietary DM
Corn silage	94.3
Concentrate supplement[1]	5.20
Vitamin and mineral premix[2]	0.50
Nutrient analysis[3]	
DM, %	42.8
CP, %	13.3
aNDF, %	27.8
ADF, %	16.5
Starch, %	37.5
Ca, %	1.08
P, %	0.46
TDN, %	72.5
NEm, Mcal/kg	1.60
NEg, Mcal/kg	1.04

[1]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.

[2]Guaranteed analysis: 15% Ca; 7.5% P; 20% salt; 1% Mg; 1% K; 3,600 mg/kg Mn; 12 mg/kg Co; 1,200 mg/kg Cu; 3,600 mg/kg Zn; 27 mg/kg Se; 60 mg/kg I; 660,000 IU/kg vitamin A; 660 IU/kg vitamin E; and 66,000 IU/kg vitamin D.

[3]Values other than DM are expressed as a percentage of dietary DM; DM, dry matter; CP, crude protein; aNDF, neutral detergent fiber (amylase-treated); ADF, acid detergent fiber; NEm, net energy of maintenance; NEg, net energy of gain.

differential gene expression, while balancing practical and economic limitations (Schurch et al., 2016; Conesa et al., 2016).

Total RNA was isolated from the whole blood samples using RNeasy Micro Kit (Cat No: 130 74,004; Qiagen, Germantown, MD), following the manufacturer's instructions. RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, 132 MA). RNA samples were screened for quality using the Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA), ensuring all samples exhibited satisfactory RNA integrity (> 8), and were used to construct sequencing libraries (n = 10 beef steers/treatment).To enhance transcriptome coverage and reduce interference from highly abundant globin transcripts, globin mRNA was depleted before library preparation using a commercial globin reduction protocol (Choi et al., 2014). Library preparation was performed by Novogene (Sacramento, CA, USA), and 150-bp pair-ended sequencing was conducted on an Illumina 1.9 HiSeq, resulting in ~19–22 million clean reads per sample. The raw data were filtered out for low-quality reads and adapters. All samples had an effective rate greater than 93.5% (Clean reads/Raw reads × 100). Samples read mapped to their input read produced an average of 97%.

RNA-seq data analysis and quality control

All the growth performance data, including initial and final BW, DMI, and ADG were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). Each animal was considered the experimental unit. The statistical model included the treatment as a fixed effect (CON vs PRO) and the random effect of animals. The initial BW was used as a covariate for the final BW. Comparisons of least square means were performed using Tukey test with significance set at $P \leq 0.05$. All paired-reads FASTQ files were quality-checked using Fastqc (version 0.12.1). This step produced sequencing files with an average per base Phred score > 35, which indicates a high-quality base call. Cleaned reads were aligned to the *Bos taurus* genome (GCF_002263795.3_ARS-UCD2.0_genomic.fna) using STAR, Version 2.7 and the resulting sam files were converted to bam format using samtools v1.22 Reads mapping to the genes (GCF_002263795.3_ARS-UCD2.0_genomic.fna) were counted using featureCounts v1.4.6-p1 (Liao et al., 2014). Differential expression analysis was performed using PyDESeq2 in Python. Gene expression changes were visualized by Volcano plots using SRplot. Phenotypes of the differentially expressed genes identified were obtained from the Ensembl BioMart tool and filtered based on gene ID, gene name, and phenotype description. The expression with adjusted *P*-value ≤ 0.10 were considered to be differentially expressed. This FDR threshold was selected *post hoc* to balance the discovery of meaningful biological effects with statistical rigor. No additional log2 fold-change cutoffs were applied. Batch effect correction was not performed as sample processing was consistent and sequencing was conducted in a single batch. Gene ontology (GO) terms and pathways analyses of DEGs were performed using a web-based gene-ontology software (http://www.geneontology.org) as described by Ashburner et al., 2000. Significantly enriched pathways were catalogued using false discovery rate-adjusted *P*-values (FDR ≤ 0.05) (Benjamini and Hochberg, 1995).

Results

Growth performance

The effects of PRO supplementation on the growth performance of the beef steers are presented in Table 2. There were no differences ($P > 0.05$) between the two treatments for initial BW, final BW, ADG, and DMI.

Sequencing coverage and read counts

After quality control and data filtering, 79,336,266 ± 5,789,255 clean reads per sample were obtained. For each sample, 95.55% to 97.83% of the reads were uniquely mapped to the *Bos taurus* reference genome, indicating sufficient coverage (Supplementary Table 1).

Differentially expressed genes and gene ontology analysis

A total of 41 DEGs were detected (FDR ≤ 0.10) between the two groups (Table 3), with 20 downregulated and 21 upregulated in beef steers fed the PRO additive (Figure 1). Several downregulated genes, including *C3* (FDR = 0.01; FC = -1.70), *DDIT4* (FDR = 0.04; FC = -1.45), and *STAB1* (FDR = 0.003; FC = -1.69), are associated with immune and inflammatory responses. In contrast, upregulated genes such as *TLR10* (FDR = 0.0008; FC = 1.57), *MYTIL* (FDR = 0.08; FC = 4.18), *GPR183* (FDR = 0.03; FC = 1.34), and *LGR4* (FDR = 0.09; FC = 1.43) are involved in cytokine regulation and toll-like receptor signaling. Functional analysis and GO enrichment of these DEGs revealed significant overrepresentation of biological processes such as positive regulation of inflammatory response, cytokine secretion, response to external biotic stimuli, and lipid localization (Figure 2).

TABLE 2 Growth performance and dry matter intake of beef steers fed diet supplemented with a blend of *Saccharomyces cerevisiae* and multiple live probiotic bacteria on day 56 of the receiving period.

Parameters	PRO	CON	SEM	*P*-value
Initial Body Weight (kg)	203	205	9.07	0.84
Final Body Weight (kg)	269	278	5.52	0.15
ADG (kg/d)	1.17	1.32	0.10	0.14
DMI (kg/d)	6.73	6.66	0.45	0.88

PRO, a blend of Saccharomyces cerevisiae, Enterococcus faecium, Bacillus licheniformis, Bacillus subtilis, Lactobacillus animalis, Propionibacterium freudenreichii, and their fermentation products fed at 9 g/steer/d (Papillon, Easton, MD); CON, control; SEM, standard error of mean; ADG, average daily gain; DMI, dry matter intake.

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TABLE 3 The list of differentially expressed genes in PRO compared to CON[1].

Gene Symbol	Gene name	Fold change	FDR P-value[2]
HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	7.26	0.08
MYT1L	Myelin transcription factor 1 like	4.18	0.08
THOC7	THO complex 7	1.90	0.03
GRB7	Growth factor receptor bound protein 7	1.82	0.05
VPREB3	V-set pre-B cell surrogate light chain 3	1.82	0.03
EPHX4	Epoxide hydrolase 4	1.62	0.07
TNFRSF13C	TNF receptor superfamily member 13C	1.58	0.05
TLR10	Toll like receptor 10	1.57	0.0008
PTCH1	Patched 1	1.51	0.05
HHEX	Hematopoietically expressed homeobox	1.50	0.03
MAP4K3	Mitogen-activated protein kinase 3	1.47	0.05
RUNX1T1	RUNX1 partner transcriptional co-repressor 1	1.47	0.1
LGR4	Leucine-rich repeat-containing G protein-coupled receptor 4	1.43	0.09
CXCR5	C-X-C motif chemokine receptor 5	1.43	0.05
FCRL1	Fc receptor like 1	1.41	0.02
STX12	Syntaxin 12	1.39	0.05
HLTF	Helicase like transcription factor	1.36	0.01
SESN1	Sestrin 1	1.36	0.09
HABP4	Hyaluronan binding protein 4	1.36	0.04
SINHCAF	SINHCAF – SIN3-HDAC complex associated factor	1.34	0.1
GPR183	G protein-coupled receptor 183	1.34	0.03
LSS	Lanosterol synthase	-1.38	0.02
DDIT4	DNA damage inducible transcript 4	-1.45	0.04
HK3	Hexokinase 3	-1.48	0.03
GIMAP5	GTPase, IMAP family member 5-like	-1.50	0.09
OSBPL5	Oxysterol binding protein like 5	-1.51	0.05
XPNPEP2	X-prolyl aminopeptidase 2	-1.55	0.07
NLRP1	NLR family pyrin domain containing 1	-1.57	0.05
LPL	Lipoprotein lipase	-1.59	0.09
NIPAL4	NIPA like domain containing 4	-1.67	0.02
KCNT1	Potassium sodium-activated channel subfamily T member 1	-1.67	0.07
GZMB_1	Granzyme B.	-1.68	0.05
STAB1	Stabilin 1	-1.69	0.003
C3	Complement C3	-1.70	0.01
SULF2	Sulfatase 2	-1.80	0.03
ADCY8	Adenylate cyclase 8	-3.64	0.003
IGHV4-59	Immunoglobulin heavy variable 4-59	-4.46	0.07
ADYC8	Adenylate cyclase 8	-3.64	0.004
LOX	Lysyl oxidase	-10.61	0.08
MEGF11	Multiple EGF like domains 11	-51.87	0.0007
FN1	Fibronectin 1	-2.25	0.08

[1]PRO, a blend of Saccharomyces cerevisiae, Enterococcus faecium, Bacillus licheniformis, Bacillus subtilis, Lactobacillus animalis, Propionibacterium freudenreichii, and their fermentation products fed at 9 g/steer/d (Papillon, Easton, MD); CON, control.
False discovery rate-adjusted *P*-value.
Positive fold change indicates that the expression of the gene is upregulated in beef steers fed the PRO additive.
Negative fold change indicates that the expression of the gene is upregulated in beef steers fed the control diet.

Discussion

Growth performance metrics such as BW, DMI, and ADG are critical indicators of how dietary interventions affect the health and productivity of beef steers. In the present study, supplementation with the PRO additive did not result in statistically significant differences in final BW, DMI, or ADG between treatment groups. These findings are consistent with several previous studies where supplementation with DFMs or yeast-based products showed limited or no effects on performance during the receiving period, particularly in lower-stress environments (Deters et al., 2018; Hall et al., 2018).

The immune system plays a critical role in maintaining health and resilience in newly weaned beef steers, especially during stressful periods like weaning, often associated with increased susceptibility to infections and immune dysregulation (Munteanu and Schwartz, 2022). Dietary interventions, such as supplementation with DFMs have shown the potential to modulate immune responses and enhance animal health by influencing key immune pathways (Ban and Guan, 2021). The microbial additive used in this study contains several microbes, including *S. cerevisiae*, lactic acid bacteria, and their fermentation products, known to directly or indirectly interact with components of the immune system.

Yeast (*Saccharomyces cerevisiae*) and its derivatives are widely recognized for their ability to modulate the immune system in farm animals (Maturana et al., 2023). *Saccharomyces cerevisiae* and its cell wall components, such as β-glucans and mannans, interact with immune cells via pattern recognition receptors

TABLE 4 Enriched biological processes (FDR ≤ 0.05) highlighted in the over-representation analysis of the differentially expressed genes in PRO compared to CON[1].

Description	Associated genes	FDR P-value[2]
Interspecies interaction between organisms	TLR10/C3/THOC7/ DDIT4/LPL/LGR4	0.01
Positive regulation of inflammatory response	TLR10/C3/LPL	0.01
Regulation of cytokine secretion	TLR10/LGR4	0.02
Positive regulation of defense response	TLR10/C3/LPL	0.02
Response to other organism	TLR10/C3/DDIT4/ LPL/LGR4	0.02
Response to external biotic stimulus	TLR10/C3/DDIT4/ LPL/LGR4	0.02
Response to biotic stimulus	TLR10/C3/DDIT4/ LPL/LGR4	0.02
Regulation of lipid storage	C3/LPL	0.03
Cytokine secretion	TLR10/LGR4	0.03
Regulation of inflammatory response	TLR10/C3/LPL	0.04
Regulation of response to external stimulus	TLR10/C3/GPR183/LPL	0.04
Positive regulation of lipid localization	C3/LPL	0.04
Positive regulation of response to external Stimulus	TLR10/C3/LPL	0.04
Defense response to other organism	TLR10/C3/DDIT4/LGR4	0.04
Positive regulation of response to stimulus	TLR10/C3/GPR183/ LPL/LGR4	0.05
Acute inflammatory response	C3/FN1	0.05
Toll-like receptor signaling pathway	TLR10/LGR4	0.05

[1]PRO, a blend of Saccharomyces cerevisiae, Enterococcus faecium, Bacillus licheniformis, Bacillus subtilis, Lactobacillus animalis, Propionibacterium freudenreichii, and their fermentation products fed at 9 g/steer/d (Papillon, Easton, MD); CON, control.
[2]False discovery rate *P*-value.

(PRRs) like Dectin-1 and TLR2, thereby activating innate immune responses (Lesage and Bussey, 2006; Ogunade et al., 2021). These interactions stimulate the production of pro-inflammatory cytokines (e.g., IL-6, TNF-α) and enhance the phagocytic activity of macrophages and neutrophils (Levin, 2005). Additionally, yeast cell wall polysaccharides improve gut barrier integrity by promoting the production of tight junction proteins, reducing intestinal permeability, and supporting gut-associated lymphoid tissue (GALT), a central component of the immune system (Zhou et al., 2023). Studies have shown that supplementing animals with *S. cerevisiae* enhances immune cell proliferation and function, particularly increasing the activity of macrophages, dendritic cells, and T lymphocytes (Chou et al., 2017; Mahmoud et al., 2020). For example, dietary supplementation with a hydrolyzed mannan- and glucan-rich yeast fraction in newly received feedlot cattle improved the immune response of beef steers (Pukrop et al., 2018). Furthermore, yeast supplementation has been associated with improved gut microbiota composition, fostering a beneficial microbial environment that supports both local and systemic immune responses (Pascual et al., 2020).

Lactic acid-producing bacteria (LAB), including *Lactobacillus* and *Bifidobacterium* species, along with their fermentation products, also play a crucial role in immune modulation and inflammation control (Fijan, 2014). Lactic acid-producing bacteria produce organic acids, such as lactic acid and acetic acid, which lower the intestinal pH, creating unfavorable conditions for pathogenic bacteria while promoting the growth of beneficial microbes (de Vrese and Schrezenmeir, 2008; Fidan et al., 2022). This shift in gut microbiota composition strengthens the intestinal barrier and enhances mucosal immunity. Moreover, LAB stimulate the production of antimicrobial peptides, cytokines, and immunoglobulins (IgA) in the gut, contributing to infection defense (Hernández-González et al., 2021; Anjana and Tiwari, 2022). Fermentation products from LAB, such as short-chain fatty acids (SCFAs), further promote anti-inflammatory responses by regulating immune cell functions (Fusco et al., 2023). In particular, butyrate has been shown to reduce the production of pro-inflammatory cytokines and promote a regulatory T-cell phenotype, supporting immune homeostasis (Shin et al., 2023). Studies indicate that supplementation with LAB or their fermentation products in livestock improves immune function, reduces inflammation, and enhances resilience to stress-induced immunosuppression (García-Burgos et al., 2020; Lin et al., 2022).

The results of our study revealed the differential expression of five genes (*TLR10*, *LGR4*, *GPR183*, *C3*, and *DDIT4*) from critical immune pathways, including the positive regulation of inflammatory response, regulation of cytokine secretion, positive regulation of defense response, and response to external biotic stimuli in beef steers fed PRO feed additive compared with CON (Table 4). The expressions of *TLR10*, *GPR183*, and *LGR4* were upregulated, while the expressions of *C3* and *DDIT4* were downregulated in beef steers fed the PRO feed additive compared with CON. To initiate an effective host defense against microbial pathogens, immune cells must first detect specific molecular patterns from invading microbial pathogens (Li and Wu, 2021). Toll-like receptors (TLRs) are a class of pattern-recognition receptors that play a critical role in recognizing microbial pathogens by detecting pathogen-associated molecular patterns (Janssens and Beyaert, 2003; Duan et al., 2022). Among them, TLR10 is expressed in immune cells such as neutrophils, macrophages, and dendritic cells and has been implicated in modulating innate immune responses through intracellular signaling pathways involved in immune cell recruitment (Balachandran et al., 2015). Additionally, studies have also reported that TLR10 exhibits anti-inflammatory properties (Fore et al., 2020; Mourits et al., 2020; Knez et al., 2023). For instance, Hess et al. (2017) showed that *TLR10* acts as an inhibitor of MyD88-dependent and independent pathways. In their experiment, they used monoclonal Abs (activator of *TLR10*) and observed a

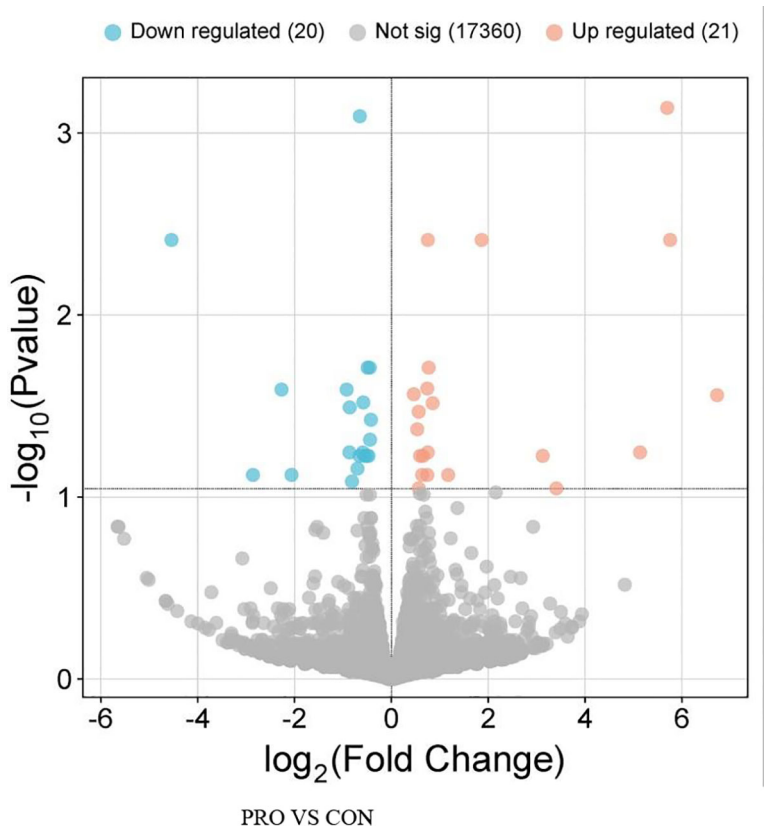

FIGURE 1
Volcano plot showing the number of differentially expressed genes in beef steers fed PRO, a blend of Saccharomyces cerevisiae, Enterococcus faecium, Bacillus licheniformis, Bacillus subtilis, Lactobacillus animalis, Propionibacterium freudenreichii, and their fermentation products fed at 9 g/steer/d (Papillon, Easton, MD) compared with CON, control.

reduction in the production of pro-inflammatory cytokines, *IL-6*, and *TNF-α*. Thus, the upregulation of this gene in beef steer-fed PRO additive may suggest that the additive may prepare the innate immune system for a more effective response to pathogens and promote a more regulated inflammatory environment.

The ability of immune cells to move to the sites of infection or inflammation is essential for effective defense and clearance of invading pathogens (Alberts et al., 2002). G Protein-Coupled Receptor 183 (*GPR183*), also known as *EBI2*, is a G-protein-coupled receptor that plays a key role in directing immune cell migration, particularly for B cells, T cells, and dendritic cells, to the sites of infection or inflammatory response (Barington et al., 2018; Emgård et al., 2018). In mice, it has been found that deficiencies of *GPR183* resulted in impaired positioning of T and B cells within secondary lymphoid organs, leading to defects in T cell-dependent immune response (Hannedouche et al., 2011; Bartlett et al., 2020). Similarly, Chu et al. (2018) demonstrated that *GPR183* knockout mice show increased susceptibility to *Citrobacter rodentium* infection, highlighting the receptor's role in intestinal immune defense. In addition to its role in immune cell migration, *GPR183* has pro-inflammatory activities, particularly in the intestine (Misselwitz et al., 2021). Leucine-rich repeat-containing G-Protein-Coupled Receptor 4 (*LGR4*) is known to be enriched in various organs, including the liver, kidney, intestine, bone, reproductive system (ovary, testis, and mammary gland), intestinal tract, pancreas to nervous system cells (Ordaz-Ramos et al., 2021; Zhang et al., 2023). The *LGR4* gene has been demonstrated to be one of the few GPCRs upregulated during macrophage M2-type polarization, indicating a potential function in modulating macrophage-mediated immunological responses (Wang et al., 2019). Taken together, the increased expression of *TLR10*, *GPR183*, and *LGR4* in beef steers fed the supplemental PRO additive suggests that these animals had a better ability than CON for immune monitoring, pathogen detection, and controlled inflammatory responses, which are crucial for maintaining immune homeostasis and resilience to infections in newly weaned beef steers.

The observed downregulation of *C3* and *DDIT4* in the beef steers fed supplemental PRO additive compared with the CON provides further insight into the immunomodulatory effects of the supplemental diets. Complement Component 3 is a central component of the complement system, which plays a key role in innate immunity activation and inflammation (Peng et al., 2016; Bai et al., 2022). It is an integral part of the complement system and a key molecule of the three complement reaction pathways: the classical pathway (triggered by antibodies), the lectin pathway

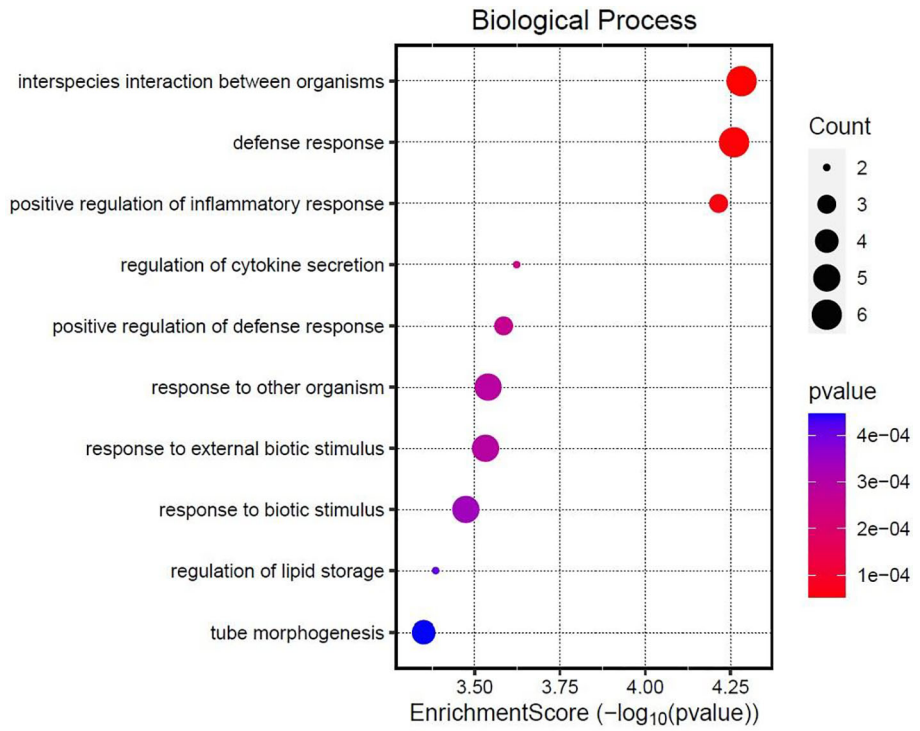

FIGURE 2
Enriched biological processes highlighted in the over-representation analysis of differentially expressed genes identified in PRO, a blend of *Saccharomyces cerevisiae*, *Enterococcus faecium*, *Bacillus licheniformis*, *Bacillus subtilis*, *Lactobacillus animalis*, *Propionibacterium freudenreichii*, and their fermentation products fed at 9 g/steer/d (Papillon, Easton, MD) compared with CON, control.

(triggered by lectin), and the alternative pathway (triggered directly on pathogen surfaces) (Charles et al., 2021). The decreased expression of *C3* in beef steers fed supplemental PRO additive could indicate a more controlled inflammatory state, preventing potential tissue damage caused by overactive immune signaling. Additionally, this modulation might indicate the immunomodulatory properties of the supplemental diet, which could help the animals maintain immune homeostasis under stressful conditions. DNA Damage-Inducible Transcript 4 (*DDIT4*), also known as Regulated in Development and DNA Damage Response 1 (*REDD1*), is involved in cellular stress responses and acts as a negative regulator of the mTOR pathway, which is important for cell growth and metabolism (Tirado-Hurtado et al., 2018). The downregulation of *DDIT4* in beef steers fed the PRO may suggest mitigated stressors or altered stress response pathways, reducing the need for *DDIT4's* regulatory functions and potentially leading to better overall immune function. By modulating the expression of *DDIT4*, the additive may influence pathways associated with immune cell resilience, potentially supporting their function under stress conditions commonly experienced by newly weaned calves. However, these gene expression changes are suggestive and do not confirm functional outcomes.

Beyond the genes identified through GO analysis, several additional genes, including *MEGF11*, *STAB1*, *FCRL1*, *ADCY8*, *HHEX*, *TRAF5*, and *HLTF*, exhibited significant expression changes in beef steers fed the PRO additive compared to CON. Fc Receptor-Like 1, *HHEX*, *TRAF5*, and *HLTF* were up-regulated, while *MEGF11*, *STAB1*, and *ADCY8* were downregulated in beef steers fed the PRO additive compared to CON. Fc Receptor-like 1 (*FCRL1*) is located on chromosome 3 of cattle and plays a role in the immune system by encoding proteins that function as receptors on the surface of immune cells (Daëron, 2016; Zhao et al., 2019);. These receptors are known to interact with antibodies and other molecules involved in regulating immune activation and immune function of B cells (Yousefi et al., 2023). An *in vitro* study revealed that the synaptic accumulation of B cell receptors (BCRs) was significantly impaired in *FCRL1*-knockout primary B cells. Using total internal reflection fluorescence microscopy, the study demonstrated that *FCRL1* plays a critical role in promoting the efficient clustering of BCRs at the immunological synapse during B cell activation, suggesting that *FCRL1* acts as a positive regulator in this process (Zhao et al., 2019). Additionally, in mice, BCR ligation in Fcrl1-deficient primary splenic B cells demonstrated impaired proliferation, as evidenced by lower Carboxyfluoresce in succinimidyl ester dilution compared to wild-type (WT) control B cells (DeLuca et al., 2021). Therefore, the upregulation of *FCRL1* in beef steers fed the PRO additive suggests that the additive may enhance immune function by promoting more efficient B cell receptor (BCR) clustering and activation. Hematopoietically expressed homeobox (*Hhex*) is abundantly expressed in natural killer (NK) cells, and its deletion results in significant impairment in lymphoid development, impacting NK cell maturation and functionality (Goh et al., 2020). Natural killer cells are innate

lymphocytes best known for their functional efficacy against transformed and virus-infected cells (Mujal et al., 2021). Jackson et al. (2017) showed that *Hhex*-deficient mice lack mature B cells because of impaired *IL-7* signaling, dysregulated expression of cell cycle genes, increased apoptosis, and developmental arrests of early progenitors. Consequently, the upregulation of *Hhex* in beef steers fed the PRO additive suggests a potential enhancement of NK cell development and functionality, which is crucial for effective innate immune responses.

TNF receptor-associated factor 5 (*TRAF5*) belongs to the *TRAF* family, which plays an important role in transducing intracellular signals via TNF receptor superfamily molecules, particularly in regulating immune and inflammatory responses (Nakano et al., 1997). The *TRAF5* gene functions as an adaptor protein, facilitating the transmission of signals from *TNF* receptors and other immune receptors, activating key pathways such as *NF-κB* and AP-1, both crucial for driving inflammation, immune activation, and cytokine production (Au and Yeh, 2013; Arkee and Bishop, 2020). The upregulation of *TRAF5* in beef steers fed the additive suggests an improved ability to mount stronger immune responses, indicating that the additive has a role in promoting a more efficient immune defense mechanism in these animals. Helicase-like Transcription Factor (*HLTF*) is a DNA helicase that plays a key role in maintaining genomic stability by participating in the repair of damaged DNA, particularly during replication stress (Chavez et al., 2018; Bai et al., 2024). Its upregulation in beef steers fed additives could indicate an enhanced ability of the cells to manage DNA damage and maintain genomic integrity, particularly under stress conditions.

The expressions of *MEGF11*, *STAB1*, and *ADCY8* were downregulated in response to the dietary supplementation PRO additive as compared with the CON. Multiple EGF-Like Domains Protein 11 (*MEGF11*) is involved in cell adhesion and neural development, particularly influencing synaptic connectivity (Kay et al., 2012; Ray et al., 2020). Recent studies indicate that knockdown of *MEGF11* significantly affects Protein kinase B, mTOR, and NF-κB signaling, as well as the decreased expression of transcription factors like *NF-κB p65*, *CREB*, and *AP-1* (Chiu et al., 2020). The downregulation of *MEGF11* may suggest reduced inflammation, modulated immune responses, and possibly improved overall health and resilience in beef steers fed the PRO additive. Stabilin-1 (*STAB1*) encodes a transmembrane receptor primarily involved in clearing extracellular molecules such as modified lipids, dead cells, and pathogens (Arias-Alpizar et al., 2021). In fact, *STAB1*'s presence can promote an anti-inflammatory M2 macrophage phenotype, which is associated with tissue repair and resolution of inflammation (Soler Palacios et al., 2020). In the current study, the downregulation of *STAB1* may serve as a mechanism to return to homeostatic *STAB1* mRNA levels following a transient upregulation. Adenylate cyclase 8 (*ADCY8)* is a member of the adenylyl cyclase (*AC*) family that encodes the enzyme adenylyl cyclase 8 (*AC8*), which plays a critical role in converting *ATP* to cyclic AMP (Guo et al., 2022; Devasani and Yao, 2022). Cyclic AMP is a secondary messenger that modulates various biological processes, including cellular responses to hormones and stress signals (Yan et al., 2016). In mice, it has been found that over-expression of *AC8* in cardiomyocytes increased cellular stress that cardiomyocytes experience during normal physiological aging (Kumar et al., 2024). Thus, the decreased expression of *ADCY8* in beef steers fed the supplemental PRO additive, compared to the CON group, may suggest that feeding the supplemental PRO additive alleviated the stress that the CON group continued to experience.

Although PRO additive supplementation did not affect BW, DMI, and ADG in our study, the RNAseq analysis revealed that beef steers fed PRO exhibited increased expression of immune-related genes, suggesting that DFM may enhance immune competence and resilience. While no measurable improvements in performance were observed, these findings support the role of DFM in priming the immune system, potentially better preparing animals for future stressors, such as pathogen exposure and environmental challenges. However, sampling only at day 56 may have missed the acute post-weaning window, when DFMs are typically most active. Future studies should incorporate earlier and multiple sampling points to better capture the temporal dynamics of DFM-induced effects. Additionally, while independent qPCR validation of key differentially expressed genes was not conducted, particularly given that the FDR threshold was relaxed to 0.10, the transcriptome data provide a comprehensive overview of gene expression changes in response to the PRO additive. These findings offer valuable insights into the role of multi-strain DFM supplements in immune modulation and stress resilience in newly weaned beef steers, which can inform future studies aimed at further validating these gene expression patterns.

Conclusion

Our findings demonstrate that dietary supplementation with the PRO additive was associated with modulation of immune-related gene expression in newly weaned beef cattle. The upregulation of critical genes associated with immune recognition, regulation of defense response, and inflammatory regulation indicates an improved capacity for pathogen defense and a more balanced immune response in beef steers receiving the PRO additive. Additionally, the downregulation of certain genes, such as *C3*, *DDIT4*, *MEGF11*, *STAB1*, and *ADCY8*, suggests a strategic modulation of inflammatory responses and stress-related pathways, potentially promoting better overall health and recovery during the challenging weaning period. This study adds to the growing body of evidence supporting the use of multi-strain DFM supplements to promote immune health and stress resilience in livestock management. Future studies involving larger cohorts of steers are warranted to confirm these effects and assess the translational potential of PRO additive supplementation in commercial beef production settings.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SAMN48310144.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of West Virginia University (IACUC Protocol Number: 2108046615.1). The study was conducted in accordance with the local legislation and institutional requirements. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AA: Data curation, Conceptualization, Writing – review & editing, Methodology, Investigation, Writing – original draft, Formal Analysis, Visualization. SJ: Investigation, Methodology, Writing – review & editing, Data curation. IM: Data curation, Writing – review & editing. YL: Data curation, Writing – review & editing. TS: Data curation, Writing – review & editing. GT: Data curation, Writing – review & editing. IO: Writing – review & editing, Funding acquisition, Visualization, Resources, Project administration, Validation, Conceptualization, Methodology, Supervision, Data curation. CA: Data curation, Validation, Conceptualization, Supervision, Project administration, Methodology, Writing – review & editing, Resources, Investigation, Formal Analysis, Visualization, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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