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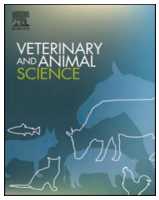

Genes involved in the cholecystokinin receptor signaling map were differentially expressed in the jejunum of steers with variation in residual feed intake

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ABSTRACT

The jejunum is a critical site for nutrient digestion and absorption, and variation in its ability to take up nutrients within the jejunum is likely to affect feed efficiency. The purpose of this study was to determine differences in gene expression in the jejunum of beef steers divergent for residual feed intake (RFI) in one cohort of steers (Year 1), and to validate those genes in animals from a second study (Year 2). Steers from Year 1 ($n = 16$) were selected for high and low RFI. Jejunum mucosal tissue was obtained for RNA-seq. Thirty-two genes were differentially expressed ($P_{FDR} \leq 0.15$), and five were over-represented in pathways including inflammatory mediator, cholecystokinin receptor (CCKR) signaling, and p38 MAPK pathways. Several differentially expressed genes (*ALOX12, ALPI, FABP6, FABP7, FLT1, GSTA2, MEF2B, PDK4, SPP1*, and *TTF2*) have been previously associated with RFI in other studies. Real-time qPCR was used to validate nine differentially expressed genes in the Year 1 steers used for RNA-seq, and in the Year 2 validation cohort. Six genes were validated as differentially expressed ($P < 0.1$) using RT-qPCR in the Year 1 population. In the Year 2 population, five genes displayed the same direction of expression as the Year 1 population and 3 were differentially expressed ($P < 0.1$). The CCKR pathway is involved in digestion, appetite control, and regulation of body weight making it a compelling candidate for feed efficiency in cattle, and the validation of these genes in a second population of cattle is suggestive of a role in feed efficiency.

Introduction

There are three sections of the small intestine that are important for proper digestion and absorption of nutrients: the duodenum, jejunum, and ileum. The duodenum is the section located directly after the abomasum and is the site of addition of bile and pancreatic secretions that increase the pH of the digesta and aid in digestion of nutrients. The jejunum section of the small intestine is an important site of post-ruminal nutrient digestion and absorption. The ileum absorbs vitamins, bile salts and any nutrients passed from the jejunum. Additionally, the small intestine, of which the jejunum accounts for approximately 40 %, uses a large proportion of energy and nutrients due to its high metabolic activity and rapid turnover (Ferrell, 1988; Johnson et al., 1990). There are examples in the literature illustrating a correlation between size and morphology of the small intestine with feed efficiency (Montanholi et al., 2013; Meyer et al., 2014; Zhang et al., 2019a), including the same steers used in the current study (Cunningham-Hollinger, 2022). In addition, there are reports of RNA-sequencing studies of the jejunum and duodenum sections of the small intestine of pig and chickens with variation in residual feed intake (Liu et al., 2019; Reyer et al., 2018; Wang et al., 2019; Yi et al., 2015). However, there are no reports of differences in the jejunum transcriptome in beef steers with

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high and low residual feed intake (RFI) phenotypes.

There is a growing body of evidence to suggest that feed efficiency is linked to inflammatory pathways. In the rumen and liver tissues of cattle with variation in RFI, inflammatory response pathways have been previously identified (Elolimy et al., 2018; Paradis et al., 2015; Weber et al., 2016). Additionally, genes related to inflammation have been identified as differentially expressed in the duodenum, jejunum and ileum of beef steers divergent for gain and intake (Lindholm-Perry et al., 2016). The metabolic and inflammatory responses relationships with feed efficiency have been reported to result from an increase in oxidative stress and mitochondrial dysfunction or inefficiency which can result into systemic inflammation among inefficient animals (Ferronato et al., 2024; Fonseca et al., 2019)

The purpose of this study was to identify differences in the small intestinal gene expression of finishing steers with variation in RFI. We hypothesized that genes involved in the absorption of nutrients and in immune and inflammatory responses would be identified as contributing to feed efficiency.

Methods

Institutional care and animal use

The University of Wyoming Animal Care and Use Committee approved the ethics and the experiment (Protocol #20140603SL00109–01). The procedures for handling cattle complied with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010).

Animal population

Animal management has previously been described in detail by Cunningham-Hollinger (2022). Hereford × Angus steers ($n = 16$, Year 1; $n = 14$, Year 2) with high and low RFI were selected over two years from larger populations from a single contemporary group from birth to slaughter in each year (Year 1, $n = 59$ and Year 2, $n = 75$). For this study, Year 1 was treated as the discovery population, and Year 2 was treated as the validation population.

At the start of the study, the initial body weights were 461 ± 4.5 kg and 412 ± 3.8 kg for steers from Year 1 and 2, respectively, and initial ages were 379 ± 1.5 days and 370 ± 1.1 days for Year 1 and 2, respectively. Animals were transitioned to a corn-based finishing diet (Table 1). Diets varied by year due to feed availability; however, crude protein and Mcal concentrations were similar. Individual feed intakes were monitored using the GrowSafe system (model 4000E, GrowSafe Systems Ltd. Airdrie, AB, Canada). Feed intake was monitored for 57 days in Year 1 and 80 days in Year 2. For steers with a 12th rib fat thickness (determined by ultrasound) of ≥1.02 within each year, RFI was calculated as expected daily dry matter intake (DMI) subtracted from actual daily DMI. Actual daily DMI was regressed on average daily gain (ADG) and metabolic midweight to determine expected DMI. Average daily gain was calculated from initial and final steer body weights. At the end of each trial period, the 20 % most efficient ($n = 8$, low RFI) and 20 % least efficient ($n = 8$, high RFI) steers were selected for tissue collection at harvest in each year. Fat thickness was used to prevent selection of animals that were earlier in the growth curve as more efficient, given this would likely be caused by composition of gain more than most other physiological differences. Performance data by contemporary group is shown in Table 2.

Table 1
Ration provided to steers for both years of the study.

	Year 1 (%)	Year 2 (%)
Shelled corn	84.7	62.5
Alfalfa hay	5.1	
Hay		5.8
Alfalfa haylage	6.8	23.7
Straw		4.3
Protein & Micronutrient supplement	3.4	3.7
Crude Protein (%)	11.4	13.3
Mcal NEm/kg	2.0	1.91
Mcal NEg/kg	1.34	1.25

RNA isolation and library preparation

All jejunum tissues were collected within 30 min of slaughter. A 15-cm long section of jejunum was sampled as described in Meyer et al. (2014) and Cunningham-Hollinger et al. (2022), rinsed with PBS, and immediately placed in aluminum foil and frozen on dry ice. Samples were stored at −80 °C until ribonucleic acid (RNA) isolation was performed. Briefly, RNA was extracted with the RNeasy Plus Mini kit and Qiashredder columns (Qiagen, Valencia, CA, USA). A total of 800 μL of Qiagen lysis plus buffer with β-mercaptoethanol was added to 50–100 mg of tissue and homogenized for 40 s using an Omni Prep 6-station homogenizer (Omni International, Kennesaw, GA, USA). The homogenate was centrifuged through a QiaShredder column on 16,000 x g for 3 min. Genomic DNA was removed from the total RNA with the Qiagen RNeasy Plus mini-kit, according to the manufacturer's protocol, and the total RNA was eluted in 50 μL of RNase free water. Total RNA was quantified with a NanoDrop One spectrophotometer (Thermo Scientific). An Agilent Bioanalyzer RNA 6000 nano kit (Santa Clara, CA, USA) was used to determine the RNA integrity number (RIN). The average RIN was 6.95 with a range of 6.3–7.6.

Ribonucleic acid samples (250 ng) from the 16 steers from Year 1 were prepared for RNA sequencing with the Illumina TruSeq stranded messenger ribonucleic acid (mRNA) library preparation kit following the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). The libraries were quantified with reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) using the NEBNext Library Quant Kit (New England Biolabs, Inc., Beverly, MA, USA) on a CFX384 thermal cycler (Bio-Rad, Hercules, CA, USA). The size and quality of the library was evaluated with an Agilent Bioanalyzer deoxyribonucleic acid (DNA) 1000 kit (Santa Clara, CA, USA). The libraries were diluted to 4 nM and were paired-end sequenced with 150 cycle high output sequencing kits on an Illumina NextSeq 500 instrument.

Sequence analysis

Paired end read Illumina sequence data was imported into the CLC Genomics workbench 19 (Qiagen). Reads were trimmed for adapter sequences and quality. Trimmed reads were mapped to the ARS-UCD1.2

Table 2
Feed efficiency phenotypes for the high and low residual feed intake groups for each population of cattle.

	Discovery Population (Year 1)[1]		Validation Population (Year 2)[1]	
	High-RFI	Low-RFI	High-RFI	Low-RFI
Average RFI*	1.27	−1.42	1.31	−1.17
Min	0.89	−1.98	0.85	−2.11
Max	1.98	−0.93	2	−0.58
ADG (kg/d)[2]	1.51	1.56	1.46	1.51
Min	1.12	1.11	1.20	1.07
Max	2.09	1.83	1.69	1.88
DMI (kg/d)*[3]	12.16	9.62	12.18	9.78
Min	10.14	8.38	11.42	8.92
Max	14.5	10.6	13.6	10.6

[1] $n = 16$ animals in Year 1 (8 low RFI, 8 high RFI) and $n = 14$ animals in Year 2 (7 low RFI, 7 high RFI).
[2] ADG = average daily gain.
[3] DMI = dry matter intake.
*= $P < 0.05$ within years.

bovine genome assembly using the RNA-Seq analysis tool using the default parameters allowing for a mismatch cost of 2, and insertion and deletion cost of 3. Genes differentially expressed among animals with high and low RFI class were identified using the Differential Expression for RNA-Seq tool for whole transcriptome RNA-Seq. The CLC Genomics Workbench was used to identify differentially expressed genes. The exact test analysis of the edgeR Bioconductor package with default values are used by CLC, except the estimateCommonDisp parameter used was 1e-14, which is more stringent than the edgeR parameter. Data are reported for genes with False discovery rate adjusted P-value (P_{FDR}) ≤ 0.15 as a cutoff. This level implies an 85 % probability that significant signals are real across the transcripts. Raw sequence data can be accessed at the NCBI Sequence Read Archive (SRA) database with accession number PRJNA912183.

Gene ontology and pathway analyses

To annotate and group the biological functions of the differentially expressed genes, we used the database for annotation, visualization, and integrated discovery (DAVID) v6.8 to perform gene ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Differentially expressed genes (DEG) were uploaded into DAVID (https://david.ncifcrf.gov/tools.jsp). Annotation clusters and KEGG pathway analyses were assessed using the official gene symbols of the differentially expressed genes and *Bos taurus* as the species. The *p-values* associated with each annotation term inside each clusters are Fisher Exact/EASE Scores.

Functions of DEG were determined using the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (Version 14.0). Enrichment analysis of gene function was performed using PANTHER's implementation of the binomial test of over-representation. Significance of GO terms were assessed using the default Ensembl *Bos taurus* GO annotation as background for the enrichment analysis. Data from PANTHER was considered statistically significant at Bonferroni corrected $P \leq 0.05$.

Real-time quantitative polymerase chain reaction

Reverse transcriptase quantitative polymerase chain reaction were performed on both the Year 1 samples (discovery; $n = 16$) and the Year 2 samples (validation; $n = 14$, missing 1 sample per RFI class due to a labeling error) with high and low RFI phenotypes. A total of nine of the genes identified as differentially expressed using RNA-seq were assayed by RT-qPCR. PrimePCR (Bio-Rad) arrays were designed for the target genes *acyl-coA thioesterase 2* (*ACOT2*), *arachidonate 12-lipoxygenase, 12S type* (*ALOX12*), *arachidonate lipoxygenase, epidermal* (*ALOX12E), fatty acid binding protein 6* (*FABP6*), *glutathione S-transferase A2* (*GSTA2*), *myocyte enhancer factor 2B* (*MEF2B*), *fms-related tyrosine kinase 1* (*FLT1), pyruvate dehydrogenase kinase 4* (*PDK4*), and *trefoil factor 2* (*TFF2),* and the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). These genes were selected because some had been identified in other studies, some were involved in key pathways identified in this study, and they included up- and down-regulated genes. The iScript complimentary deoxyribonucleic acid (cDNA) synthesis kit (Bio-Rad, Hercules, CA, USA) was used to generate cDNA from 1ug of RNA according to the manufacturer's protocol. The resulting cDNA was diluted 1:10 for real-time PCR which was performed in triplicate for all samples and genes with SsoAdvance SYBR Green master mix (Bio-Rad) on a Bio-Rad CFX384 (BioRad) instrument. The real-time PCR reaction was performed at 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and a final melting curve from 65 to 95 °C. Relative transcript abundance of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) with the reference gene and a pooled sample. Values were log transformed and the relative abundance expression values for the target genes were tested for association with RFI class using Analysis of Variance (ANOVA). Log transformation more closely resembled normality of the data for ANOVA analysis. The model predicted relative abundance as the dependent variable relative to RFI class as the independent variable along with residual error.

Cytokine real-time polymerase chain reaction assay

Real-time PCR using the Bovine Inflammatory Cytokine and Receptor RT^2 PCR Array kit (Catalog #PABT-011Z; Qiagen, Germantown, MD) was used to test the expression of 84 different cytokine and cytokine receptor genes on jejunum RNA from the validation population (Year 2). Briefly, genomic DNA was eliminated, and reverse transcription was performed with 1μg of RNA using the RT^2 first strand cDNA synthesis kit (Qiagen). The cDNA was diluted (1:10) with water and added to RT^2 SYBR Green Mastermix. A total of 25 μL of the master mix with cDNA was placed into each well of the RT^2 Profiler PCR Array plate to assess the transcript abundance of 84 different genes. Thermal cycling was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The thermal cycling conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The Qiagen RT^2 profiler software was used for data analysis (http://pcrdataanalysis.sabiosciences.coms/pcr/arrayanalysis.php). The gene *beta-actin* (*ACTB*) was chosen as the housekeeping gene for normalization because it was the most stable of the five housekeeping genes among the 16 animals. The $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was used to calculate the relative expression values for all 84 genes. Values were log transformed and the relative abundance expression values for the target genes were tested for association using ANOVA with RFI class.

Results

Within each year, steers were not different in ADG ($P > 0.67$, Table 2) but did differ in RFI and DMI phenotypes ($P < 0.002$). Sequenced libraries generated an average of 36.83 million reads and 91.6 % of the reads mapped to the ARS-UCD1.2 genome assembly.

A total of 32 genes were identified as differentially expressed ($P_{FDR} \leq 0.15$) by RNA-seq in the 16 Year 1 animals. Of these, 20 genes were down-regulated in the low RFI (efficient) animals, and 12 were up-regulated (Table 3). These genes were evaluated by DAVID for over-representation in biological pathways and the inflammatory mediator regulation of Transient Receptor Potential (TRP) channels was identified due to the differential expression of genes *ASIC3, ALOX12*, and *ALOX12E* ($P = 0.013$). The PANTHER database revealed three additional pathways using the list of DEG: p38 mitogen-activated protein kinase (MAPK), cholecystokinin receptor (CCKR) signaling map, and inflammation mediated by chemokine and cytokine signaling pathways (Table 4).

Nine genes were evaluated by RT-qPCR in both Year 1 and Year 2 animals with high and low RFI phenotypes (Fig. 1). In the Year 1 discovery population, all genes assayed displayed the same direction of expression detected by RNA-seq. The RT-qPCR expression of two genes (*ALOX12* and *PDK4*) were associated ($P \leq 0.05$) with high RFI and three (*FLT1, MEF2B,* and *GSTA2*) showed a tendency ($P \leq 0.1$) towards association with low RFI. In the Year 2 validation population of animals with high and low RFI, five genes (*ALOX12e, FLT1, TFF2, PDK4, MEF2B*) displayed the same direction of expression compared to the RNA-seq data on the discovery population. In addition, the RT-qPCR expression of *TFF2* was associated ($P \leq 0.05$) with low RFI and *ALOX12* and *ALOX12E* showed a tendency ($P \leq 0.1$) towards an association with high RFI.

In order to further evaluate the role of inflammatory cytokines identified as a pathway involved in RFI from the RNA-seq data, we tested the gene expression of 84 cytokine and cytokine receptor genes with the validation group of animals (Year 2). Three of the 84 genes were differentially expressed between the high and low RFI classes of animals ($P \leq 0.05$; Table 5). These genes were *C5, CCL26* and *CSF3*. Another five

Table 3
List of differentially expressed genes identified in the discovery population (Year 1) of animals with low versus high residual feed intake.

Gene Symbol[1]	Log_2 fold change[2]	*P*-value	FDR *P*-value[3]
FABP6	3.92	3.82E-09	1.31E-04
ACOT2_2	−3.12	1.068E-07	1.83E-03
LOC515601	−9.09	6.99E-07	6.92E-03
BOLA_4	−4.34	8.03E-07	6.92E-03
GSTA2	−2.55	2.82E-06	1.95E-02
APOLD1	−1.44	3.58E-06	2.05E-02
PDK4	−1.34	6.20E-06	3.05E-02
LOC784522	−3.29	8.32E-06	3.18E-02
LOC112445165	−2.67	7.60E-06	3.18E-02
ALPI	4.65	1.27E-05	4.39E-02
ASIC3	2.90	3.05E-05	8.13E-02
MEF2B	2.35	3.31E-05	8.13E-02
ALOX12E	2.82	2.92E-05	8.13E-02
LOC112441469	3.86	3.14E-05	8.13E-02
LOC516742	−3.15	4.46E-05	9.04E-02
MS4A1	2.23	4.39E-05	9.04E-02
CD79B	2.31	4.70E-05	9.04E-02
GP2	2.72	4.73E-05	9.04E-02
BTNL9	−1.36	6.86E-05	1.12E-01
FLT1	−0.84	6.77E-05	1.12E-01
HIST1H1E_2	−2.87	6.21E-05	1.12E-01
FCRLA	2.70	8.83E-05	1.29E-01
SPAAR	−1.80	8.83E-05	1.29E-01
LYVE1	−1.27	9.74E-05	1.29E-01
ALOX12	−0.92	9.42E-05	1.29E-01
GPRC5B	−1.00	9.10E-05	1.29E-01
FABP7	−2.72	1.08E-04	1.32E-01
CCL14	−1.01	1.07E-04	1.32E-01
LOC616942	−0.97	1.16E-04	1.36E-01
TFF2	2.99	1.35E-04	1.5E-01
COL5A1	−0.90	1.32E-04	1.5E-01
AP3B2	2.74	1.43E-04	1.5E-01

[1] Genes are listed in gene symbol nomenclature. Genes in bold were selected for RT-qPCR validation.
[2] Comparison used was low RFI compared to high RFI. Negative values are genes that were down-regulated among the low RFI animals.
[3] False discovery rate–corrected P-value at a 5 % rate.

Table 4
Pathways identified by PANTHER classification system database using the list of differentially expressed genes from the discovery population with variation in residual feed intake.

Pathway	Genes	Expected	Fold Enrichment	*P*-value
p38 MAPK pathway	*MEF2B*	0.05	21.62	4.63E-02
CCKR signaling map	*TTF, MEF2B*	0.20	10.09	1.70E-02
Inflammation mediated by chemokine and cytokine signaling pathway	*ALOX12, ALOX12E*	0.29	6.99	3.34E-02

genes (*CCL20, IL21, OSM, SPP1*, and *TNFSF14*) tended to be differentially expressed ($P \leq 0.1$). Of these genes, five (*C5, CCL26, IL-21, OSM, TNFSF14*) were expressed in higher transcript abundance among the efficient (low RFI) animals.

Discussion

The jejunum section of the small intestine in cattle is an important site for nutrient digestion and absorption (Liu et al., 2022; Myer et al., 2016), and variation in the ability or efficiency of the tissue to uptake nutrients likely plays a role in feed efficiency. Current literature indicates that variation in small intestinal size, differences in morphology, and gene expression all contribute to feed efficiency in cattle (Cunningham-Hollinger et al., 2022; Foote et al., 2017; Lindholm-Perry et al., 2016; Meyer et al., 2014). A previous study by Cunningham-Hollinger et al. (2022), using the same animals as this study, reported that low RFI steers tended to have less small intestinal mass in Year 1, and had less small intestinal mass relative to body weight and hot carcass weight. Conversely, small intestinal mass did not differ between low and high RFI steers in Year 2. The purpose of this study was to identify differentially expressed genes in the jejunum of beef cattle with high and low RFI, and then verify/validate those gene expression differences in a second population of animals. Variation in small intestinal size differences between efficiency classes in the years should be considered when interpreting gene expression differences or similarities between years. We hypothesize that genes with similar expression profiles (e.g. upregulated in low RFI) between years are likely to be independent of small intestinal size differences, but tat genes with different expression patterns in each year of this study may be affected more by small intestinal size, leading to variation between years.

While this was a preliminary study, the goal was to identify molecular signatures in a tissue that likely contribute to feed efficiency. An understanding of the genes and pathways contributing to feed efficiency may provide us with mechanisms that can be altered to improve feed efficiency. In addition, there may be DNA variants in regulatory regions of these differentially expressed genes that contribute to levels of expression that are associated with optimal feed efficiency phenotypes. These variants could potentially be used as markers for selection.

The CCKR signaling map pathway was one of three pathways identified as associated with RFI in this study. Cholecystokinin (CCK) via its receptors is involved in digestion, appetite control and body weight regulation (Little et al., 2005). The CCKR signaling pathway is regulated by the peptide hormones gastrin and CCK that are released in the gastrointestinal tract and bind to CCK receptors. Gastrin controls the release of gastric acid and plays a role in gastric and colonic mucosal growth and differentiation (Tripathi et al., 2015; Watson et al., 2006). Both gastrin and the cholecystokinin B receptor (CCKBR) have been shown to up-regulate the expression of *MEF2B* and *TFF2* (Guo et al., 2002; Tu et al., 2007). The genes *TFF2* and *MEF2B* were both identified as differentially expressed in the CCKR pathway in this study. They were highly expressed among the more efficient (low RFI) animals in both cohorts evaluated, suggesting that involvement of these genes is independent of small intestinal size. Prior to this study, the *TFF2* gene had been identified as differentially expressed in the duodenum and jejunum in two studies in crossbred beef steers with variation in average daily gain or feed intake (Foote et al., 2017; Lindholm-Perry et al., 2016). The CCR signaling pathway has also been implicated in RFI in species other than beef cattle. In chickens with low RFI, the cholecystokinin gene was identified as down-regulated in the duodenum (Liu et al., 2019), and cholecystokinin octapeptide levels tended to be lower with low RFI in dairy cattle (Xi et al., 2016). The *TFF2* gene is translated into a protein secreted by the gastrointestinal mucosa (Järvå et al., 2020). The trefoil genes are regulated by cytokine expression and are induced after gastric epithelial damage (Taupin et al., 2000; Dossinger et al., 2002; Baus--Loncar et al., 2007; Sands & Podolksky, 1996; Quante et al., 2010). The TFF2 protein may function to stabilize the mucosa and protect it from damage, which in turn could improve the ability of the jejunum to digest and absorb nutrients.

Several of the genes identified as differentially expressed have functions in inflammation or immune responses (i.e., *ALOX12, ALOX12E, ASIC3, CD79B, FLT1, MEF2B*, and *MS4A1;* Chen et al., 2017; Cindrova-Davies et al., 2011*;* Huse et al., 2022; Kulkarni et al., 2021; Mattiola et al., 2021; Morgan et al., 2020). Most were up-regulated in the more efficient (low RFI) animals; only the genes *arachidonate 12-lipoxygenase* (*ALOX12)* and *Fms related receptor tyrosine kinase 1* (*FLT1)* were expressed in lower abundance. Four of these genes were validated in the Year 2 population of animals and three displayed the same direction of expression as Year 1. Inflammation pathways have been previously identified in the small intestine of beef steers divergent for gain and intake (Lindholm-Perry et al., 2016). Small intestinal FLT1

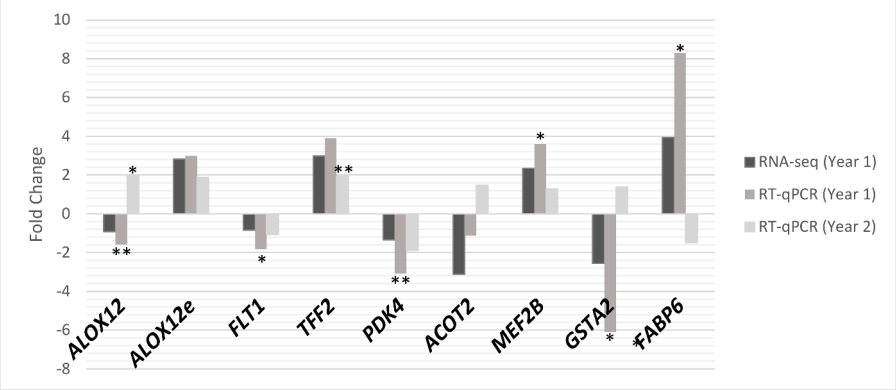

Fig. 1. Comparison of RNA-seq in the Year 1 discovery population of steers with RT-qPCR data the same animals from the Year 1 group, and also with the Year 2 validation populations of steers with low versus high residual feed intake. Each dataset was individually analyzed (*$P \leq 0.1$; **$P \leq 0.05$). All genes were significantly associated with RFI in the RNA-seq dataset.

Table 5
Bovine inflammatory cytokine and receptor gene expression associated with RFI.

Gene Symbol	Gene Name	High RFI LSMEAN (SEM)	Low RFI LSMEAN (SEM)	*P*-value
C5	*Compliment C5*	−0.322 (0.144)	0.127 (0.134)	0.04
CCL26	*C-C Motif Chemokine Ligand 26*	−0.770 (0.254)	0.00226 (0.254)	0.05
CSF3	*Colony Stimulating Factor 3*	0.375 (0.191)	−0.547 (0.191)	0.05
CCL20	*C-C Motif Chemokine Ligand 20*	−0.150 (0.150)	−0.574 (0.150)	0.07
IL21	*Interleukin 21*	−0.428 (0.166)	0.0160 (0.166)	0.08
OSM	*Oncostatin M*	−0.266 (0.143)	0.130 (0.143)	0.07
SPP1	*Secreted Phosphoprotein 1*	−0.414 (0.248)	−1.127 (0.248)	0.06
TNFSF14	*TNF Superfamily Member 14*	−0.127 (0.0665)	0.0510 (0.665)	0.08

mRNA expression has previously been affected by feed intake in ruminants (Meyer et al., 2012), although it was previously not correlated with RFI or gain:feed in steers (Meyer et al., 2014). In other species, such as poultry and swine, inflammatory response pathways have been associated with feed efficiency in duodenal tissue (Liu et al., 2019; Ramayo-Caldas et al., 2018). Similar to our findings, Alexandre et al. (2015) identified both lipid metabolism and inflammatory response genes increased in the liver of inefficient feedlot steers. They concluded that in feedlot steers, genetics by environment interaction with the diet was likely the cause for their observations, and that a change in diet and acidosis were contributing factors (Alexandre et al., 2015). Further, Gozho et al. (2006) found an increase in the inflammatory response of the rumen during the adaptation period when stepping up to a concentrate-based diet. High feed intake leads to an increase in lipid metabolism which results in an increase in inflammation and may thereby reduce feed efficiency (Alexandre et al., 2015), and efficient animals are likely to spend less energy on systemic inflammation. In this study, most of the immune/inflammatory response genes were up-regulated among the efficient animals, which could suggest that these animals are still responding to inflammation, but may have a more optimal and less energetically expensive response compared to inefficient animals.

To examine inflammatory genes further, the jejunum from the validation population of animals in this study was also evaluated for differences in the expression of 84 cytokines and receptors by RT-qPCR. Eight of these genes were either associated or displayed a trend towards association with RFI. Five of these were highly expressed in the low RFI animals (*C5, CCL26, IL21, OSM*, and *TNFSF14*). Three of these genes, *C—C motif chemokine ligand 20* (*CCL20), interleukin 21 (IL21)*, and *secreted phosphoprotein 1* (*SPP1*), have been previously associated with feed efficiency phenotypes in livestock species (Alexandre et al., 2019; Horodyska et al., 2018; Keogh et al., 2017; Lindholm-Perry et al., 2020; Manca et al., 2021; Yurchenko et al., 2019; Zhang et al., 2019b). A recent genome-wide association study in Brown Swiss bulls, identified *IL21* as a candidate gene for residual concentrate intake (RCI; Manca et al.,. 2021). The *CCL20* gene was found to be downregulated in rumen epithelial tissue of Holstein bulls after dietary restriction during compensatory gain (Keogh et al., 2017). Compensatory gain is a phenomenon where animals gain weight at a more rapid rate when fed normally after nutrient restriction, which may be considered an induced form of increased feed efficiency. C—C motif chemokine ligand 20 is induced in response to inflammatory signals, while IL21 is a unique immunomodulatory cytokine that can both promote and inhibit the immune response (Mehta et al., 2004; Williams, 2006). In this study, *CCL20* was expressed in lower abundance and *IL21* was expressed in higher abundance in the efficient steers from the Year 2 study. The *SPP1* gene was identified by Horodyska et al. (2018) as up-regulated in muscle tissues of high feed efficiency pigs. Furthermore, Zhang et al. (2019b) discovered *SPP1* up-regulated in the liver of low RFI lambs. In both studies, the direction of expression in contradictory to this study; however, the role of the SPP1 protein may vary by tissue it has roles in both cell growth and wound healing (Liaw, 1998).

A group of genes with functions in lipid metabolism or oxidative stress (*ALOX12, ALPI, FABP6, FABP7, GSTA2, MS4A1*, and *PDK4*) were also found to be differentially expressed in this study. *Fatty acid binding protein 6* (*FABP6*) and *intestinal alkaline phosphatase* (*ALPI*) and *membrane spanning 4-domains A1* (*MS4A1*) were up-regulated in the efficient animals. *Fatty acid binding proteins are* involved in fatty acid uptake, transport, and metabolism, and FABP6 is also able to bind bile acids (Hotamisligil & Bernlohr, 2015; Lin et al., 2022). The *ALPI* is a digestive brush border enzyme that is also part of the gut defense system that is thought to detoxify the tissue of lipopolysaccharide and prevent gut bacterial translocation (Santos et al., 2022). Up-regulation of these genes to increase fatty acid transport and metabolism and to reduce bacterial lipopolysaccharides would be consistent with animals that are more feed efficient. Moreover, these genes were both identified as upregulated in the jejunum of pigs that were more feed efficient (Wang et al., 2019). The *ALOX12* gene was down-regulated among the more efficient animals. The expression of *ALOX12* in response to oxidative stress in mice has been previously reported (Zheng et al., 2020). Higher amounts of *ALOX12* were identified in the lens epithelium of mice with higher amounts of oxidized glutathione and less reduced glutathione indicating oxidative stress (Gupta et al., 2013). Oxidative stress is a state of imbalance between the production and elimination of reactive oxygen species (ROS), and the intestine is a source of ROS. In addition to *ALOX12, GSTA2* was also in lower abundance in the efficient steers.

Genes *ALOX12, GSTA2, PDK4* and *FABP7* were expressed in lower abundance in the efficient (low RFI) animals. These have all been previously implicated with feed efficiency phenotypes in other studies with beef cattle (Foote et al., 2017; Mukiibi et al., 2019; Zhou et al., 2022), pigs (Do et al., 2014) and chickens (Bottje et al., 2017; Luo et al., 2022; Ma et al., 2021). The identification of these genes in other genome wide association studies (GWAS), targeted gene expression, or transcriptome studies support potential roles for them in feed efficiency in various tissues across species (Bottje et al., 2017; Do et al., 2014; Foote et al., 2017; Mukiibi et al., 2019; Luo et al., 2022; Ma et al., 2021; Zhou et al., 2022).

A limitation of this study was the small sample sizes of 16 animals for RNA-seq and 14 animals for validation. The goal was to select animals with separation in RFI phenotypes from larger numbers of animals from each feeding trial (59 and 75 total animals). Only one year of jejunum tissue was sequenced using RNA-seq, which limited the power to detect gene expression differences; however, adding samples from different studies may also introduce environmental noise. There were also ration differences between the two years of the study, which is due to the availability of feed ingredients. The diets were formulated to balance crude protein and net energy; however, variation in ration ingredients may affect gene expression since the the ingredients may affect nutrients available to the jejunum for uptake. We suggest that the next step to follow up this study would be a meta-analysis using RNA-sequencing data from the jejunum tissue of other populations of animals with variation in RFI (Keel & Lindholm-Perry, 2022).

Conclusions

In our study, 32 genes were identified as being differentially expressed in the jejunum of beef steers with divergent RFI phenotypes, indicating that variation in the transcript abundance of specific genes in the jejunum plays a role in feed efficiency. Several of these genes have previously been identified as being candidate genes for feed efficiency or related traits in beef cattle and other livestock species which supports the robustness of our findings. The pathways were identified in the Year 1 population with RNA-seq data included the CCKR signaling pathway, inflammation and immune response pathways, and the p38 MAP signaling pathway. In addition, genes with lipid metabolism functions were also identified. The Year 2 population was used to validate some of these genes identified in the Year 1 animals including those from the CCKR pathway. The *TFF2* and *MEF2B* displayed the same direction of expression in both groups of animals. The Year 2 population was also tested for a targeted set of cytokine and cytokine receptor genes and revealed several with expression associated with RFI. The use of a second, validation population of animals improves the confidence that the expression of certain genes may have a relationship with a complex phenotype like RFI.

Competing interests

The authors have no competing interests to declare.

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Ethics statement

The University of Wyoming Animal Care and Use Committee approved the ethics and the experiment (Protocol #20140603SL00109–01). The procedures for handling cattle complied with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010).

CRediT authorship contribution statement

Rebecca J. Kern-Lunbery: Writing – review & editing, Writing – original draft, Investigation. **Abigail R. Rathert-Williams:** Writing – review & editing, Investigation. **Andrew P. Foote:** Writing – review & editing, Resources. **Hannah C. Cunningham-Hollinger:** Writing – review & editing, Methodology, Investigation. **Larry A. Kuehn:** Writing – review & editing, Methodology, Formal analysis. **Allison M. Meyer:** Writing – review & editing, Writing – original draft, Resources, Conceptualization. **Amanda K. Lindholm-Perry:** Conceptualization, Formal analysis, Writing – original draft.

Declaration of competing interest

The authors have no competing interests to declare.

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