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Original Research Article

# Necrotic enteritis challenge regulates peroxisome proliferator-1 activated receptors signaling and $\beta$ -oxidation pathways in broiler chickens

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# ABSTRACT

Necrotic enteritis (NE) is an important enteric disease in poultry and has become a major concern in poultry production in the post-antibiotic era. The infection with NE can damage the intestinal mucosa of the birds leading to impaired health and, thus, productivity. To gain a better understanding of how NE impacts the gut function of infected broilers, global mRNA sequencing (RNA-seq) was performed in the jejunum tissue of NE challenged and non-challenged broilers to identify the pathways and genes affected by this disease. Briefly, to induce NE, birds in the challenge group were inoculated with 1 mL of Eimeria species on day 9 followed by 1 mL of approximately 10<sup>8</sup> CFU/mL of a NetB producing *Clostridium per*fringens on days 14 and 15. On day 16, 2 birds in each treatment were randomly selected and euthanized and the whole intestinal tract was evaluated for lesion scores. Duodenum tissue samples from one of the euthanized birds of each replicate (n = 4) was used for histology, and the jejunum tissue for RNA extraction. RNA-seq analysis was performed with an Illumina RNA HiSeq 2000 sequencer. The differentially expressed genes (DEG) were identified and functional analysis was performed in DAVID to find protein-protein interactions (PPI). At a false discovery rate threshold <0.05, a total of 377 DEG (207 upregulated and 170 downregulated) DEG were identified. Pathway enrichment analysis revealed that DEG were considerably enriched in peroxisome proliferator-activated receptors (PPAR) signaling (P < 0.01) and  $\beta$ -oxidation pathways (P < 0.05). The DEG were mostly related to fatty acid metabolism and degradation (cluster of differentiation 36 [CD36], acyl-CoA synthetase bubblegum family member-1 [ACSBG1], fatty acid-binding protein-1 and -2 [FABP1] and [FABP2]; and acyl-coenzyme A synthetase-1 [ACSL1]), bile acid production and transportation (acyl-CoA oxidase-2 [ACOX2], apical sodium-bile acid transporter [ASBT]) and essential genes in the immune system (interferon-, [IFN- $\gamma$ ], LCK proto-oncogene, Src family tyrosine kinase [LCK], zeta chain of T cell receptor associated protein kinase 70 kDa [ZAP70], and aconitate decarboxylase 1 [ACOD1]). Our data revealed that pathways related to fatty acid digestion were significantly compromised which thereby could have affected metabolic and immune responses in NE infected birds.

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# 1. Introduction

Necrotic enteritis (NE), is an enteric disease in poultry caused by NetB producing *Clostridium perfringens* strains (Keyburn et al., 2008). The global economic loss of the subclinical and/or clinical form of the disease has been estimated to be 6 billion dollars annually due to weight loss, impaired feed conversion, mortality, and management costs (Wade and Keyburn 2015). For decades, in-

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feed antimicrobials have been used to control the outbreaks of NE in chickens. However, due to the occurrence of antibiotic-resistant bacteria, regulatory and voluntary restrictions on the use of in-feed antibiotics have been set in place, which have negatively impacted poultry production due to the increase of NE outbreaks in flocks (McDevitt et al., 2006). The bacterium *C. perfringens* is naturally present in the intestine of healthy chickens; however, *C. perfringens* strains expressing NetB toxin, along with the action of predisposing factors such as *Eimeria* infection, changes in immunity, mycotoxins, diet composition, and animal protein levels can provide a suitable environment for the proliferation of *C. perfringens* that leads to gastrointestinal epithelium damage and eventually the occurrence of NE in chickens (M'Sadeq et al., 2015; Moore 2016; Van Immerseel et al., 2004).

Deeper understanding of the responses to NE infection in the host cells is essential for developing preventive strategies such as averting bacteria binding with specific cells, producing specific antibodies for disease control, or developing vaccines. Many studies have observed the effects of NE on intestinal microbiota, pH, epithelium structure and immune responses in broilers (Gharib-Naseri et al., 2019a; Golder et al., 2011; Liu et al., 2012). However, global investigation of the gene expression at transcriptomic level in response to the NE challenge has been scarce. Information on differentially expressed genes (DEG) provides a better understanding of the pathogenesis of diseases and identification of genes affected by the infection (Campbell et al., 2010). A powerful tool for the detection of DEG is the sequencing of RNA (RNA-Seq) in a whole-transcriptome, which provides a holistic view of the metabolic pathways and relevant genes in response to these factors (Su et al., 2011). Using such analyses, Kim et al. (2014) and Truong et al. (2017) have reported the activation of many pathways related to immunity responsive to NE infections in broiler chicken intestine.

Intestinal tissue samples used in the present study were obtained from a previous NE challenge experiment conducted by Rodgers et al. (2015) which showed that the NE challenge negatively affected weight gain (WG) and feed conversion ratio (FCR) of the broilers. Stanley et al. (2014) further evaluated the cecal microbiota changes in the challenged birds of the same experiment and reported that the NE challenge significantly altered the gut microbiota and short-chain fatty acids profile compared to the nonchallenged birds. Furthermore, Kitessa et al. (2014) investigated the mucosal mRNA gene expression from same birds used in the Rodgers et al. (2015) study with the candidate gene approach and reported an upregulation in the expression of tumor necrosis factor alpha (TNF- $\alpha$ ) and downregulated mucin2 (MUC2) and cluster of differentiation 36 (CD36) gene in the NE infected birds. To provide further insight into NE pathogenesis, the current study aimed to characterize the effects of NE infection on the transcriptomic profile in the intestine of challenged and non-challenged chickens sampled from the Rodgers et al. (2015) study. We hypothesize that NE infections can alter the expression of intestinal genes involved in the pathways related to digestion and immunity of the chickens. Such gene expression changes may underlie, at least partially, the negative effect of NE on the performance of broiler chickens.

#### 2. Materials and methods

#### 2.1. Animal management and NE challenge

The live phase of the study was approved by and conducted according to the conditions set by the Animal Ethics Committee of the University of New England, Armidale, Australia. Management was conducted in compliance with the Model code of practice for the welfare of animals: domestic poultry (Agriculture and Resource Management Council of Australia and New Zealand, 2002) and the Australian code of practice for the care and use of animals for scientific purposes (Naujoks et al., 2016).

Tissue samples used in this study were obtained from a previous study conducted by Rodgers et al. (2015) which investigated the roles of C. perfringens, Eimeria and fishmeal in broilers in introducing NE challenge. However, only samples from the challenged and nonchallenged birds fed the same basal diet were used from this study. In brief, Rodgers et al. (2015) used 300-day-old Ross 308 male broilers, obtained from Baiada Country Road Hatchery, Tamworth, New South Wales, Australia. The chicks were assigned to 12 pens with 25 birds in each pen (6 pens for each treatment). Birds were raised for 35 days in a temperature-controlled room at Kirby Research Station, the University of New England, Australia. Lighting, humidity and temperature program for the experimental period followed the Ross 308 recommendations (Aviagen 2011). On day 9 birds in the challenged groups were inoculated with 1 mL of phosphate-buffered saline (PBS) suspension containing Eimeria (suspension of 5,000 sporulated oocyst strains of E. acervulina, and Eimeria maxima, and 2,500 sporulated oocysts of E. brunetti from Bioproperties Pty. Ltd., Werribee, VIC, Australia). The nonchallenged groups were gavaged with 1 mL sterile PBS. On days 14 and 15 the birds from the challenged group were inoculated per os with 1 mL of C. perfringens suspension (approximately  $10^8$  to 10<sup>9</sup> CFU/mL), strain EHE-NE18 (Commonwealth Scientific and Industrial Research Organization, Geelong, Australia). Birds had ad libitum access to the same starter (day 0 to 21) and grower (day 22 to 35) diets during the experimental period and the diet compositions and nutrients were described previously (Rodgers et al., 2015).

#### 2.2. Lesion score evaluation

On day 16, 2 birds per pen were randomly chosen and euthanized by cervical dislocation and dissected to determine intestinal NE lesion scores in the whole intestinal tract. The NE lesions were assessed according to Prescott et al. (1978), and the severity of the lesions was scored from 0 to 4. Lesions in the intestinal tract were scored as follows: 0, no lesions; 1, thin-walled or friable small intestine; 2, focal necrosis or ulceration; 3, larger patches of necrosis; 4, severe, extensive necrosis.

# 2.3. Histology

Approximately 2 cm of duodenum tissue was fixed in 10% formalin in 30-mL containers. Fixed samples were then dehydrated, cleared, and embedded in paraffin for sectioning and subsequent histological analysis. Consecutive longitudinal sections (8  $\mu$ m) were placed individually onto Superfrost slides (Thermo Scientific, Rockville, MD) and stained with hematoxylin and eosin. Villus height and crypt depth were measured by the VideoPro 32 program (Leading Edge Pty Ltd, Adelaide, Australia). The height of 20 villi and the depth of 20 crypts were measured from each replicate. The villus height-to-crypt depth (VH:CD) ratio was determined accordingly.

#### 2.4. Intestinal tissue collection and RNA extraction

On day 16, 2 cm of jejunum tissue was separated from one of the euthanized birds, flushed with chilled sterile PBS and collected in 2-mL Eppendorf cap-lock tubes. The samples were immediately snap-frozen in liquid nitrogen and were preserved at -80 °C until RNA extraction. For RNA-seq analysis, the samples from one bird of all 4 replicates of each experimental group (NE challenged and non-challenged) were used. For total RNA extraction, each sample was homogenized with 1 mL of TRIsure (Bioline, Sydney, Australia) using an IKA T10 basic Homogenizer (Wilmington, NC, USA) in accordance with manufacturer's instructions. An RNAeasy mini Kit

(Qiagen, GmbH, Hilden, Germany) was used to further purify the TRIsure extracted RNA. The total RNA purity and quantity were determined using a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and integrity measured by RNA integrity number (RIN) assayed by using an RNA 6000 Nano Kit (Agilent Technologies, Inc., Waldbronn, Germany) with Bio-analyzer (Agilent Technologies, Waldbronn, Germany) following the procedure provided by the manufacturer. The samples were deemed as high-quality if the value of A260/A230 (the ratios of the readings at the 260 nm and 230 nm of the Nanodrop spectrophotometer) was >1.8, A260/A280 value was between 2.0 and 2.2, and RIN > 7.5. Samples with RIN higher than 7.5 were considered high quality. In the present study, RIN values obtained from each sample were above 8.2.

# 2.5. cDNA libraries preparation

RNA samples were submitted to the Australian Genome Research Facility (AGRF) for complementary DNA (cDNA) library preparation and sequencing. To process the RNA samples, Illumina's TruSeq Stranded mRNA Prep Kit was used to purify and fragment the mRNA, and cDNA was synthesized following the manufacturer instructions. For each sample, one library was created (total of 8 libraries) for sequencing. Sequencing of the libraries was performed on an Illumina HiSeq 2000 sequencing system (Illumina, San Diego, CA) using 100 bp paired reads.

# 2.6. Sequencing quality control and read mapping

The quality control (QC) of reads was assessed using the software FASTQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/), whereas trimmomatic v0.36 (Bolger et al., 2014) was used to remove the low-quality reads and adaptors. The cleaned reads were mapped to the chicken reference genome (Gallus gallus) using TopHat v2.1.1 (Trapnell et al., 2009). The gene counts were calculated using the HTSeq v0.6.1 (Anders et al., 2015). Genes with low expression were removed, and the trimmed mean of M-values (TMM) normalization was performed with the edgeR package in R software. A principal component analysis (PCA) was carried out using prcomp R function on the logarithmic copies per million (log<sub>2</sub> CPM) to explore the transcriptomic profile between the control and challenged birds. Differential expression analysis was performed using voom function from the limma R package v 3.32.2 (Ritchie et al., 2015). The DEG between the control and challenge groups were identified with a significant threshold of adjusted *P*-value  $\leq$  0.05 and log<sub>2</sub> fold change  $\geq$ 1).

# 2.7. Pathway enrichment analysis and protein–protein interaction network and heatmap

The chicken Ensembl gene ID were converted to official gene symbols using the DAVID (Database for Annotation, Visualization and Integrated Discovery, version 6.8 https://david.ncifcrf.gov/). The official gene symbols were then used for functional clustering and enrichment relative to the whole genome. Pathway analysis of DEG was carried out using the Kyoto encyclopedia of genes and genomes (KEGG) database (Kanehisa et al., 2006). In addition, the list of DEG was used to identify the interaction network in the jejunum tissue. The PPI interaction network using the STRING v10.5 database (Search Tool for the Retrieval of Interacting Genes/Proteins) was employed to obtain the protein interaction scores where a network of interactions from a variety of sources was generated, including different interaction databases, text mining, genetic interactions, and shared pathway interactions (Franceschini et al., 2012). The software Cytoscape was used to visualize the network. Average gene

counts were considered for the top 100 significant down and upregulated genes when performing the hierarchical clustering. The clustering was performed in gplots (version 3.0.1.1) of R packages (version 3.5.3), and the results are presented as a heat map.

# 2.8. Quantitative PCR validation, primer design, and cDNA preparation

Quantitative PCR (qPCR) analysis was performed to validate the expression of a subgroup of DEG. Twenty genes (Table 1) were selected according to their roles in specific pathways and the foldchange values determined in the RNA-seq analysis. The primers for L-type amino acid transporter-1 (LAT1) (Gilbert et al., 2007), TATAbox binding protein (TBP) were sourced from literature. The primers for all other DEG were designed in this study using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/ primer-blast/). The specificity of primers was then determined using melting curve analysis on a Rotrogene 6000 real-time PCR machine (Corbett, Sydney, Australia) and by the analysis of their amplicons with Agilent 2100 Bioanalyzer DNA 1000 Kit (Agilent Technologies, Inc., Germany) (Fig. 1A and B). Prior to the gene expression evaluation, cDNA was synthesized from RNA through reverse-transcription using the QuantiTect Reverse Transcription Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions, with gDNA eliminated by the addition of DNA. The cDNA was diluted 10 times with nuclease-free water and stored at -20 °C until required.

Ouantitative PCR was performed in duplicates using an SYBR Green kit SensiFAST SYBR No-ROX (Bioline, Sydney, Australia) with a Rotorgene 6000 real-time PCR machine (Corbett Research, Sydney, Australia). The PCR reaction was performed in a volume of 10  $\mu$ L containing 5  $\mu$ L of 2  $\times$  SensiFAST, 400 mmol/L of each primer and 2  $\mu L$  of 10  $\times$  diluted cDNA template. The relative quantity of mRNA of the target genes was calculated by qBase + version 3.0 (Biogazelle, Zwijnbeke, Belgium) software with SDH and TBP as reference genes that were optimized from 10 widely used housekeeping genes prior to the analysis of target genes. The qBase + applied arithmetic mean method to transform the logarithmic cycle threshold (Cq) value to a relative linear quantity using the exponential function for relative quantification of target genes (Hellemans et al., 2007; Vandesompele et al., 2002). The qPCR results were then presented as relative expression levels of the genes in respective treatment groups as means of normalized relative quantities (NRQ). The fold changes for each particular gene analyzed with qPCR were calculated using the mean NRQ values of the NE challenged and non-challenged groups.

# 2.9. Statistical analysis

Statistical analysis was performed using the SPSS statistics version 24 (IBM SPSS, UK). The student's t-test was applied to determine differences between means of the two groups using SPSS 24 statistics software on the performance parameters and villus height. Intestinal lesion scoring data were analyzed by the non-parametric Kruskal–Wallis test (Kruskal and Wallis, 1952) as the data was not normally distributed.

# 3. Results

# 3.1. Effectiveness of the necrotic enteritis challenge

Table 2 shows the performance (14 to 35 days), jejunal lesion score, and duodenal villus height (16 days) of birds with and without NE challenge. The chickens with NE challenge had lower WG and higher FCR (P < 0.01), higher lesion score (P < 0.05), lower

Table 1	1
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Primers for candidate target and reference genes in expression studies by qPCR in the jejunum.

AIAST5'-aminolevulinate synthase-17060NM_00108012.1P. CACCTCACTCCACACCAAACCAAACCAAACCAAACCAA	Genes	Gene name	Size, pb	Annealing temperature, °C	Accession No.	Sequence (5' to 3')	Reference
MY0M2Myomesin-216760XM_0152848192E: CACTTCCACCACTTCGTATACAC R: ECTONATCCACTTCGTATACACT CACTTGCACACCACCACGGGGCD36CD36 molecule8560XM_0251474491F: CTGAATTCGATACGATCGATCGAT R: CGGAATCGATCGATCGATCGATCGAT CACTTGCAACCACACGATCGATCGATCGATCGAT R: CGGAATCGATCGATCGATCGATCGATCGAT R: CGGAATCGATCGATCGATCGATCGATCGAT R: CGGAATCGACACACGATCGAATCGATCGATCGATCGATCG	ALAS1	5'-aminolevulinate synthase-1	70	60	NM_001018012.1	F: CAGGTGGACAGGAAAGGTAAAGA	This study
CD36CD36 molecule8560SUM 22174931FCTGACATTCACATCACATCGThis study RE CGACATCGATACACTTGACACACATTGATAGCAT RE CGACATCGATCGACACCATTGACACACATTGATGACGATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACACATTGATGATGACACATTGATGATGACACATTGATGATGACACATTGATGATGACACAACATGATGATGATGACATTGACATGACACATTGATGATGACACAACACATGATGATGATGATGACATAGACATAGTGATGATGATGATGATGATGATGATGATGATGATGA	MYOM2	Myomesin-2	167	60	XM_015284819.2	F: CACTTGCCACTGTCGTATGATG	This study
CACCAACTCTCAAACCATTCAAAC CCATTCACACT Member A1CCACACTCCCAACTCCATCACAC This studyABP1Fatty acid binding protein 18660NM_204192.3F: CAAACTCAACTCCAACTCCAACTCAACTCAACT CCACACTCAACTCAACTCAACTCAACTCAACTCAACTC	CD36	CD36 molecule	85	60	XM_025147449.1	R: TCTCTATACCACTCAGCACGAGG F: CTGACATTTGCAGATCCATCTATGG	This study
ALDHIA       Aldehyde dehydrogenase I family, 191       60       XM_015280233.2       F. GCACATTGCATGCAAG, This study         FABPI       Fatty acid binding protein 1       86       60       NM_201492.3       F. CCATGCGACACACACACACGCTT       This study         FABPI       Fatty acid binding protein 2       184       60       NM_001007923.1       F. CCATGCGCCTCAATGCGCT       This study         FABP2       Fatty acid binding protein 2       184       60       NM_00107923.1       F. CCATGCGCCTCAATGCGCT       This study         CCCG       Glucagon       144       60       NM_205260.5       F. CCATGCACACTCCATACCACCCCAAAGC       This study         MEI       Malic enzyme 1       164       60       NM_201303.1       F. CCGCATCCAATGCCAACACTC       This study         MSRB1       Methionine sulfoxide reductase B1       164       60       NM_2013046742.2       F. CCACTGCCAAACCTCCAACTCCAACTC       This study         RCC02       Acyl-CoA oxidase 2       163       60       XM_02149790.1       F. CGACTGCCACACCTCTAACTGCA         R2       CACOX2       Acyl-CoA oxidase 2       163       60       XM_0219190.1       F. GCACTGCCACACACTC       This study         R2       Acoritate decarboxylase 1       146       60       XM_0219190.1       F. CGACTGCCACACACTCAACACACTC <td></td> <td></td> <td></td> <td></td> <td></td> <td>R: CGAGGAACTGTGAAACGATACAGT</td> <td></td>						R: CGAGGAACTGTGAAACGATACAGT	
FABP1Fatty acid binding protein 18660NM_204192.3F. GAAGGCTAAACAAACAACTCGTTCGT F. GAAGGCTAAACAACTAACTAACTAACTAACTAACTAACTA	ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	191	60	XM_015280233.2	F: GCAGATGCAGACTTGGATGAAG R: CCTTGCTGTACACCAGGTAACAG	This study
PBP2Fatty acid binding protein 218460NM_0010079231F: CICATICIALCGTACTGCAThis study R: ACCTGAAACTTCACTTCCACTATGCACCGGlucagon14460NM_0252605.F: CGCATAGCGTCACTACATACTAACTThis study R: CICGTCCAATTGCACTACTAACTAATCACTACTAACTAATCACTACTAACTAATCACTACT	FABP1	Fatty acid binding protein 1	86	60	NM_204192.3	F: GAAGGTAACAACAAACTGGTTGCT	This study
PhB2Padly actio Uniting protein 216460NM_00100/321F: CCARLEGGETCARTERALTills StudyCCGGlucagon14460NM_205260.5F: CCGATCACTCACCTACCTACCTThis studyME1Malic enzyme 116460NM_205260.5F: CCGATCAACCTACCCACGThis studyPEX13Peroxisomal biogenesis factor 1326460NM_001199421.1F: TCGCAGCAACCGCCGATTAGTThis studyMSRB1Methionine sulfoxide reductase B116460NM_001346746.2F: TCGCACCGCGTTCTATAGTThis studyR: CACGCACCCCTATAGTR: CACGCACCCGAACCGCCGATTAGTThis studyR: CTGCCACAGCAACCGCCGATTAGTThis studyRRC2Protein kinase cGMP-dependent 216160XM_025149790.1F: CCGCACCGCAGTAACAGCGCThis studyRCX2Acyl-CoA oxidase 216360XM_001319027.1F: CTGCACCGCGTATAACTCCThis studyAC021Aconitate decarboxylase 114660XM_015275372.2F: ACGCTTACCACCACAGTAGCACCTThis studyR: CACTGTCCCCACGCACCTACTACTAGCTAGCTThis studyR: CACTGTCCCCACCTACACCACGTAGCACCTThis studyR: CACTGTCGCCCTCAGCACCTAC021Aconitate decarboxylase 114660XM_02515972.2F: ACGCATCACCACACACTGC TACTAGCAGCTR: CACTGTCCCCCCACCTACTACTAGCCACCTThis studyR: CACTGTCGCCTCTACCCCAGCThis studyR: CACTGTCCCCCCACCTACCTAGCCACCTThis studyR: CACTGTCGCCTGCCCCCCCCCCCCCCCCCCCCCCCCCCC	<b>F4DD</b> D	Fatty and hinding motoin 2	104	60	NIM 0010070221		This study
GCGGlucagon14460NM_205260.5F: CGCATCACCITAGTCATCCThis study R: CTCCITCAAGTAATCCME1Malic enzyme 116460NM_20430.1F: CGCATCACGTCACGTCATGATCCThis study R: CGATCACGCCGCGTGTGTPEX13Peroxisomal biogenesis factor 1326460NM_001199421.1F: TGCGACAACCGCGGTGTGTThis study R: CAAGCCCGCGTGTGTATGTAMSRB1Methionine sulfoxide reductase B116460NM_001346746.2F: TGCGACAACGCGGATTGCATThis study R: CAAGCCACCGCGTGTGTATGTAPRKG2Protein kinase cGMP-dependent 216160XM_02514979.01F: CGACCAACGCAAAGAAGCTCCThis study R: CTTCCACACCACAAC022Acyl-CoA oxidase 216360XM_00131907.1F: CGGGGTATACTGCThis study R: CACCTGCCACAGCACTTTAAGCACACAC0D1Aconitate decarboxylase 114660XM_0151275372.2F: AGGTTGCACCACACGCTGThis study R: CACTGTACCACACACTGCAC0D1Aconitate decarboxylase 114660XM_025155100.1F: CTGCGGACACCTGThis study R: GAGTTGCGCTACACACAGCGCAC0D1Aconitate decarboxylase 118360XM_015275372.2F: CTGGGACACACTGTThis study R: GAGTTGCGCTACACACAGCAGCACGCDUSP4Dual specificity phosphatase 415560NM_204457.1F: CTGCGACACACCTGTGCTAACTGCThis study R: GCTGTGCTACACACACTGGCTDUSP4Dual specificity phosphatase 415560NM_204457.1F: ATCACACCCCGCGTGCTGACAGCACThis study R: GCTGTGCTCACACACACTGTGTDUSP4Dual specificity phosphatase 4155<	FABP2	Fatty acid binding protein 2	184	60	NM_001007923.1		
Accos       Andagoni       1-44       60       NM_203200-3       F: CORTICACCTCICTANACACC       This study         ME1       Malic enzyme 1       164       60       NM_204303.1       F: CORTICACACACACACACACACACACACACACACACACACACA	CCC	Clucagon	144	60	NM 205260.5		This study
ME1Malic enzyme 116460NM_204303.1F: GCTGCTAAAAGGATATGCATGGT R: CGCACGACCTCATTACGACAGThis study R: CGCACGACCCATTATGCACAGPEX13Peroxisomal biogenesis factor 1326460NM_001199421.1F: TGGCAGCACCGTATTAGT R: CGACGCACCGTATGCATAGTGThis study R: CTAGCGGGTGTGTATGTTAGTMSRB1Methionine sulfoxide reductase B116460NM_001346762F: TGGAGCGGGTGTGTATGTTAGT R: CTTGCACAGGGACCCTTTAGTThis study R: CTTGCACAGGGGTGTGTATGTATGTAPRKG2Protein kinase CGMP-dependent 216160XM_029446473F: CACTGTCCCCAAAGAAGACTCCThis study R: GTTCAACGCCGTTATGTATGTGTAC0X2Acyl-CoA oxidase 216360XM_001319027.1F: GTGGGTTATCACACCTAAGTTAGThis study R: GACTGCACACCTAAGTTAGGTGASBTSolute carrier family 10 member 211960MM_001319027.1F: GTGGGTTATCACACCTAAGTTAGThis study R: GACTGCACACCAAGCTGAC0D1Aconitate decarboxylase 114660XM_02515510.1F: CTGGGACACTCAAAGAGACGCAGTGThis study R: GACTGCACCTAGAGACACACAGTGAC0D1Aconitate decarboxylase 118360XM_02515510.1F: CTGCGACACCCAGTGGAGACACAAGCTThis study R: GACTGCTGGCACTCAAAGACGACACACDUSP4Dual specificity phosphatase 415560MM_204888.1F: ATCACACCCCTGCAGCACCACACACACGACACACACCACCACCACCACCTTATGTCACACGACGACACCACCACCACCACCACCACCACCACCAC	666	Glucagoli	144	00	NIVI_203200.3		This study
MLTIndia Chayine 1IonGoNuc_20400.1I: CONCONTRUCTION CONTROL INTEGRATION CALLPEX13Peroxisomal biogenesis factor 1326460NM_0013467462F: TCGAGCCGCGTTCTATACTGMSRB1Methionine sulfoxide reductase B116460NM_0013467462F: TCGAGCCGCGTTCTATACTGPRKC2Protein kinase cGMP-dependent 216160XM_025149790.1F: CGACCCGGGTCTATACTGPRKC2Protein kinase cGMP-dependent 216160XM_025149790.1F: CGACCCGGGTCTATACTGCACOX2Acyl-CoA oxidase 216360XM_0013467462F: CACTGCCAAAACAACTCCThis studyR: GTTCACCCCCCAAACTAAACTGCR: GATCCCACACTTCCCAAACAAATCGThis studyR: GACTGCCCACACTTACTCCAAACTAACTGCThis studyACOD1Aconitate decarboxylase 114660XM_015275372.2F: AAGCGTCCCACTACACACACTCCCAATGThis studyIL21RInterleukin 21 receptor11160XM_025155100.1F: CTGCGGCACCCACTGCCGTTACCCACACGCGCTThis studyR: GCATCTCGCGCCCCCACTGThis studyR: GCATCTGCGCGCCCACTGCCGTACCCAGGACTThis studyR: GCATCGCGCGCCACTTTCGCCGTGCCCACGCCACTGCCCACGCCCCACGGCCCCTTCCCCCACGCCCCACGCGCCCCCCCC	ME1	Malic enzyme 1	164	60	NM 20/2021		This study
PEX13Peroxisomal biogenesis factor 1326460NM_001199421.1F: TGCGACAACCCGCCATTAGT R: CAGCCACCGCGATTAGT R: CAGCCACCGGCATTAGTAGT R: CATCCCAAAAGCAACCGCGCATTAGT R: CTTGCCAACAGGACCCGCGATTAGTAGT R: CTTGCCAACAGGACCCGCGATTAGTAGT R: CTTGCCAACAGGACCCGTATAGT R: CTTGCCAACAGGACCCGTATAGT R: CTTGCCAACAGGACCCGTATAGT R: CTTGCCAACAGGACCCGTATAGT R: CTTGCCAACAGCACCCGCATTAGCT R: CATCCACCGACTACCACGCA R: CATCACCCATCGCAAAAGAGAATCC R: CATCACCCATCGCAAAAGCAGACCG R: CATCACCCACGGATTACTGCACGA R: CATCACCCACGCATTAGCTGG R: CATCACCCACGCATACATGGTG R: CATCACCATCGCAAAGCAGATCG R: CATCACCACCCAACGTG R: CATCACCACCCAACGTG R: CATCACCACCCAACGTG R: CATCACCACCCAACGTGG R: CATCACCACCCAACGTGG R: CATCACCACCCAACGTG R: CATCACCACCCAACGTG R: CATCACCACCCAACGTG R: CATCACCACCCAACGTG R: CATCACCACCCAACGTG R: CATCACCACCCCAACGTG R: CATCACCACCCCACACGTG R: CATCACCACCCCACACGTG R: CATCACCACCCCAACGTG R: CATCACCACCCCCACACGTG R: CATCACCACCCCCACCACGG CACCACACGCCCCCTTTGCGAATTC CACCACACGCCCCCCTTGCAACGTC CACCACACGCCCCCTTTGCGAATTC CACCACCCCCCCCCCGCG CACCACCCCCCCCCCGCG CACCACCCCCCCCCCGCG CACCACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	IVIL I		104	60	NWI_204505.1		This study
TENDTENDTEND20400INIT_DOTION_21.1.In Countracting the product of the p	DEV13	Perovisornal biogenesis factor 13	264	60	NM 001100/211		This study
MSRB1Methionine sulfoxide reductase B116460NM_0013467462F: TCCAGCCCGCTCTATGTA TCCAGCCCGCGTCTATGTA F: CGCAGCCGCGTCTATGTA F: CGCAGCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCAGCCCGCGTCTATGTA F: CGCGCGCGTATACGCCACCC Fis study R: GTCTCACCCCGCAGCCCA R: CGCCCGCCGTATACATAGCTC F: GTGGGTTATCACACCCTACATGCGTC F: GTGGGTTATCACACCCTACATGCGTC F: GTGGGTTATCACACCCTACATGCGTC F: GTGGGTTATCACACCCTACGTGC F: GTGGGTTATCACACCCTACGTGC F: GTGGGTTATCACCACCACACAGCT F: GTGGGAGACTCGCGCGCTAGCCACGG F: GTGGGCGCCCACGCGCGCGCGCGCGCGCGCGCGCGCGCGC	ILAIJ	refoxisonial biogenesis factor 15	204	60	NWI_001133421.1		This study
MSD1Incluining sinulate relations of the sinulation of the sinulatis of the	MSPR1	Methionine sulfovide reductase B1	164	60	NM 0013467462		This study
PRKG2Protein kinase cGMP-dependent 216160XM_0251497901F: CGACATGCCAAAAGATCC R: GTTCAACCCTTCCGAACCCAThis studyACOX2Acyl-CoA oxidase 216360XM_0049446473F: CGACATGCCAAAAGATCCThis studyASBTSolute carrier family 10 member 211960NM_001319027.1F: GTCGCTTATCAACCTAAGCTGAThis studyACOD1Aconitate decarboxylase 114660XM_025155100.1F: GTCGGACACTCGCACAGCTThis studyR: GACTGCCGCTTACCAACACATCThis studyR: GACTGCCGCTTAGCCAAGTCThis studyR: GACTGCGCGCAGAGCACAGAGCAGAGCAGAGCAGAGCAG	MJKDI	Methonine suitoxide reductase bi	104	60	NWI_001340740.2	R. CTTCCCACACCACACCTTTAAC	This study
ACO22Acyl-CoA oxidase 216360MC_202167601F: GATCACCCTTACATAGTGCThis study R: GTTCACCCTTCCGAACCCAACOX2Acyl-CoA oxidase 216360XM_004944647.3F: CACTCTGCCCAGCCTATAACTGCThis study R: GACCCACCCTTACATAGTGCASBTSolute carrier family 10 member 211960NM_001319027.1F: GTGGGTTATCACACCTACATTAGTGCThis study R: GACTCTGCCCAACGAGTGACOD1Aconitate decarboxylase 114660XM_015275372.2F: AAGGTTCACCCACCAACGATGThis study R: GACTTCGCTCCTTGCGCAAGAGTCAAATCIL21RInterleukin 21 receptor11160XM_025155100.1F: CTGCGGCACTCTGCAACAACTThis study R: GACTTGCTGCTTGCACTGGAATTCGZMAGranzyme A5660NM_204457.1F: CTGCTGGCACTTCATTGCGCTGThis study R: GCTATCCAGGATCCAAGGACADUSP4Dual specificity phosphatase 415560NM_204838.1F: ATCACAGCCCTGGAACACGT R: CAGCACTCTTGCAACGACGTThis study R: CGCACTCGTGGAAACGGTBCL2A1BCL2 related protein A17860NM_204838.1F: ATGCAGCGGTGGAACAGCACThis study R: CGCACCCTGGAAACGGTBCL2A1Neutral amino acid transporter7060RT876067.1F: GCTGGCTGGAACAGGAGAAGAAC R: CAATCGTACTAGTAGTAGGTAGGTAGThis study R: CCCAACCCCATTGTGTAZAP70Zeta chain of T cell receptor associated protein kinase 70 kDa11060NM_001321556.1F: CGTGGCTGACAGGAGGAGAGA R: CAATCGTTAGTAGTAGTGGCGAGTBPTATA-box binding protein14762NM_20510383127F-TAGCCCGGAAGGAGGTG F-TAGCCCGGAGAGGCGTG <td>PRKG2</td> <td>Protein kinase cGMP-dependent 2</td> <td>161</td> <td>60</td> <td>XM 0251497901</td> <td></td> <td>This study</td>	PRKG2	Protein kinase cGMP-dependent 2	161	60	XM 0251497901		This study
AC0X2Acyl-CoA oxidase 216360XM_004944647.3F: CACTCFGCCCAGGTATAACTGCThis study R: CACCCACGCCTTACATAGGTGASBTSolute carrier family 10 member 211960NM_001319027.1F: GACCCACGCCTTACATAGGTGThis study R: CACTGTACCCAACGTAGGTACOD1Aconitate decarboxylase 114660XM_015275372.2F: AAGGTTTCACCCACGCACAGGTGThis study R: GAGTTCCTGCGCTACAGTGIL21RInterleukin 21 receptor11160XM_02515510.1F: CTGGGACACTCACAACAACAGATCAAATC R: GGTGGGGTCTCACTTGGAATTCThis study R: GAGTTGTGGTGCTCACTTGGAATTCGZMAGranzyme A5660NM_204457.1F: CTTCGGAGACTTCAAGAGTCAAAGTC R: GAGTGTGGTGCTCTCTGTGGTGCTThis study R: GAGTGTGGTGCTCTCATGTGGTGCHP71Choline phosphotransferase 118360XM_015285488.2F: GCGACGCGCACGCGTG R: CACCACGCTCTTTGGThis study R: CACCACGCTTTTTGGDUSP4Dual specificity phosphatase 415560NM_204838.1F: ATCACAGCCCTGCGAAAGGT R: CACCACTCTTTCACTGAGGCGACAThis study R: CACCACCTTTTGAACGGGGTGGT R: CATCCCGCGGGAAAACGGTThis study R: CACCACCCTTTGACAGGGCGTGBCL2A1BCL2 related protein A17860NM_204866.1F: GGTGGCTGGGAAAACGGT R: CATGCTGTGAGAGAGAAGACThis study R: CATGCTGTGACTGCAGGTGCGALA71Neutral amino acid transporter7060KT876067.1F: GATGCGACGTGGAGAGAGAGACLA77Vettra amino acid transporter7060NM_20132155.1F: GCTGACCTACAGTTGCAGAGGAGGAGAGAGACTBPTATA-box binding protein14762NM	11002	rioteni kinase comi "dependent 2	101	00	7444_025115750.1	R' CTTCAACCCTTCCGAACCCA	This study
ACODINeuron Ford Watch 210560NM_200151716115R: CARCOCACCICITACATAGETGASBTSolute carrier family 10 member 211960NM_001319027.1F: CTGGGTTACCACACCTAAGTTATG R: CACCTGACACTCTCCCAAGThis study R: CACCGACCCACACACTGThis study R: CACCTGACCCACACCACTGACOD1Aconitate decarboxylase 114660XM_015275372.2F: AAGGATTCACCACCACACACTG R: CAGGTGCTGCGCTTAGCCAAGTGThis study R: CACTGGACACTCAGAAGTCAAATC R: GGTCTGGCTTGCGCTTAGCCAAGTGThis study R: GGTCTGGCTTGGCTTGGAATTC R: GGTCTGGACTCTCAGAGAAATC R: GGTCTGGCTGCTCTCACTTGGAATTC R: GGTCTGGGTGCTACTTCAGTGCCTThis study R: GGTCTGGCTGGCTACTTCAGGACTC R: GGTCTGGCGTGCTACTTCAGTGCCTCHPT1Choline phosphotransferase 118360XM_015285488.2F: CCACCAGGCACCTTTTGG R: CGTATGCCAGGGACAThis study R: GGTGTGCGCACGCGCGCACTTTTGG R: GCTATGCAAGGACACACCTDUSP4Dual specificity phosphatase 415560NM_204838.1F: ATCAAGCCCTGCTGAAACGGAC R: CAGCACTCTTCTCAGAGGACACCTThis study R: CAGCACTCTTCCAGAGGACACCTBCL2A1BCL2 related protein A17860NM_204866.1F: GCTGGCTGGGAAAACGGT R: GCTACTCTGAACAAGGAAACACCLAT1Neutral amino acid transporter7060KT876067.1F: GCTGGACCTACAGTGGAGAATBPTATA-box binding protein14762NM_001321550F: TAGCCCGATGACTACTAGTGGGCGGAATBPTATA-box binding protein14760NM_00122739F-TAGCCGGAGGGGGTG F-ATACGGGGGGGGGTGBarzegar Nafari (2019) R-TGCTGCGGTGAAATGGTGSDHASuccinate dehydrogenase complex74 <td< td=""><td>ACOX2</td><td>Acvl-CoA oxidase 2</td><td>163</td><td>60</td><td>XM 0049446473</td><td>F: CACTGTGCCCAGGTATAACTGC</td><td>This study</td></td<>	ACOX2	Acvl-CoA oxidase 2	163	60	XM 0049446473	F: CACTGTGCCCAGGTATAACTGC	This study
ASBTSolute carrier family 10 member 211960NM_001319027.1F: GTGGGTTATCACACCTAAGTTATG F: GTGGGTTATCACACCTAAGTTATG TAGCAACGTAGCTACCAACAGTGThis study This study R: CACTGTACCACACACGTG R: GACTTGCTCCGGTTACCCACACAGTGACOD1Aconitate decarboxylase 114660XM_015275372.2F: AAGGTTTCACCCACAACGTG R: GACTTGCTCGCGTTACCCACACAGTGThis study This study R: GACTTGCTCGGTTACCCACACAGTGIL21RInterleukin 21 receptor11160XM_025155100.1F: CTGGGAGACTCAGAAGATCAAATC R: GACTGTGGTCACTCAAGTTGCGTGThis study R: GACTGTGGTCACTCAATGCGTGGZMAGranzyme A5660NM_204457.1F: CTTCCTGGAGATTTGGCGTG R: GACTGTGGTCACTCAATGCGTGThis study R: GACTGTGGTCACTCAATGCGTGCHPT1Choline phosphotransferase 118360XM_015285488.2F: CGAGCAGCCACCTTTTTGG R: GCTGACTCAGAGGACAThis study R: GACTGTCGTCAACGAGAGACADUSP4Dual specificity phosphatase 415560NM_204888.1F: ATCACAGCCCTGCTGAACGGT R: CAGCACTCTTTCACTGAGAGCAThis study R: TGGTGACTCTCTGCAAAGGAAAGACCLAT1Neutral amino acid transporter7060KT876067.1F: GCTGGACTACAGTGAGAGAGAAGACATBPTATA-box binding protein14762NM_20132155.1F: CTGCGCAAAGCGCTG R: GTTGCCGGAAAGGGCTTGThis study R: CGCCGAGAGGGGTGSDHASuccinate dehydrogenase complex7460NM_00127739F-ATACCGGGAGAGGGGTG R-GTTCCTGGGGTGAAATGGTGSDHASuccinate dehydrogenase complex7460NM_00127739F-ATACCGGGAGAGGGGTG R-GTTCGCGGGGTGAAATGGTG<	nconz	heyr con onduse 2	105	00	MM_001511017.5	R' CACCCACGCCTTACATAGGTG	This study
ACOD1       Aconitate decarboxylase 1       146       60       XM_015275372.2       F: AAGGTTTCACCACACACGTG       This study         R: CACTGTACGACATCT CCTCCCAAG       111       60       XM_025155100.1       F: CTGGGACACCACACACGTG       This study         R: GACTGTACGACAATC       This study       R: GACTGTACGACAACATCT       This study       R: GACTGTACGACAACACCAGTG       This study         GZMA       Granzyme A       56       60       NM_020457.1       F: CTTCCTGGAGATTTGTGCGTG       This study         CHPT1       Choline phosphotransferase 1       183       60       XM_015285488.2       F: CGACGACGCACCTTTTTGG       This study         DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: ATCACAGCCCTGCTGAACGT       This study         R:       CACTGTACGACAACGGAAACGGT       This study       R: GCTATCCAAGGAAACGGT       This study         BCL21       BCL2 related protein A1       78       60       NM_204866.1       F: GATGCAACAGGAAACAGC       Gibbert et al. (2007)         R:       CACTGTACGCACTTTGGAAAACGGAAACA       70       60       KT876067.1       F: GATGCAACTGCAAGAACAGAAAC         LA71       Neutral amino acid transporter       70       60       KT876067.1       F: GATGCAACTGCAGAGAACAGGAA         R:       CC	ASRT	Solute carrier family 10 member 2	119	60	NM 0013190271	F. GTGGGTTATCACACCTAAGTTATG	This study
ACOD1       Aconitate decarboxylase 1       146       60       XM_015275372.2       F: AAGGTTTCACCCACCAACAGTG       This study         IL21R       Interleukin 21 receptor       111       60       XM_025155100.1       F: CTGGGAGACTCAGAAGATCAAATC       This study         GZMA       Granzyme A       56       60       NM_204457.1       F: CTGGCGGCCTCACTTGGAATTC       This study         CHP71       Choline phosphotransferase 1       183       60       XM_015285488.2       F: CGACCAGCCACCTTTTTGG       This study         DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: AGGCATCCAGAAACGCCT       This study         BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGCGCGGAAAACGGCT       This study         LA71       Neutral amino acid transporter       70       60       KT876067.1       F: GGTGCACGCAGCAAGAACA       Gilbert et al. (2007)         R: CAATGCTAACTGTGTAGTAGCTAGCTAGCTAGGAAGAAC       70       60       NM_001321556.1       F: GCTGGACCACGCAGAGAGAAGAAC         IBP       TATA-box binding protein       147       62       NM_001321556.1       F: GCTGGCAGGAGAGAGAGAGAGAGAGA         TBP       TATA-box binding protein       147       62       NM_00127739       F-TAGCGCGAGAGAGAGGCGTG       <	1001	Solute currier lunning to member 2	115	00	1111_001313027.1	R' CACTGTACGACATCTGCTCCAAG	This study
InterferenceIL21RInterference	ACOD1	Aconitate decarboxylase 1	146	60	XM 015275372.2	F <sup>•</sup> AAGGTTTCACCCACCAACAGTG	This study
IL21R       Interleukin 21 receptor       111       60       XM_025155100.1       F: CTGGGGAGACTCAGAGATCAACC       This study         GZMA       Granzyme A       56       60       NM_204457.1       F: CTGCGGAGACTCAGAGATCAACC       This study         CHPT1       Choline phosphotransferase 1       183       60       XM_015285488.2       F: CGAGCAGCCAGACCTCAAGGACA         DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: ATCACAGCCCTGCTGAACGT       This study         BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGGGAAAACCGT       This study         R: CCCCACACCCACTITTGTT       70       60       NM_001321556.1       F: GCTGGACTCACAGTGAGAGACA       Gibert et al. (2007)         R: CCCCAACCCACTITGTTGT       110       60       NM_001321556.1       F: GCTGGACTACAGTAGGAGAGA       This study         IZ17       Veutral amino acid transporter       70       60       NM_001321556.1       F: GCTGGACCTACAGTTGGGAAGACAC         IZ18       TATA-box binding protein       147       62       NM_001321556.1       F: GCTGGACTACAGTGGGAGAGA       This study         R: CCCCCAGCCGCAGAGAGA       147       62       NM_001321757.6       F: ATACCGCGAGGAGAGGGGGTG       Atis et al. (2005)         <		neonnaite accarbonynase i	110		1002/00/202	R' GAGTTGCTGCGTTAGCCAGTG	inio otady
ALTIIntercention of the optical of the op	II.21R	Interleukin 21 receptor	111	60	XM 0251551001		This study
GZMA       Granzyme A       56       60       NM_204457.1       F: CTTCCTGGAGATTTGTGCGTG       This study         CHPT1       Choline phosphotransferase 1       183       60       XM_015285488.2       F: CGAGCAGGCACCTTTTGG       This study         DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: ATCACAGCCCTGCTGAACGT       This study         BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGCACTGCAAGGAACGT       This study         LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GATTGCAACGGGGGAAAAGAGAC         ZAP70       Zeta chain of T cell receptor associated protein kinase 70 kDa       110       60       NM_20510383127       F: AGCTGGATGTGGAGGGGGGGGGGGGGGGGGGGGGGGGGG	122111	interreduur 21 receptor			102010010011	R <sup>·</sup> GGTCTGGCTCTCACTTGGAATTC	inio otady
CHPT1       Choine phosphotransferase 1       183       60       XM_015285488.2       F: CGAGCAGGCACCTTTCATGTCCT       This study         DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: ATCACAGCCCTGCTGAACGT       This study         BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGCTACTCTGATGGAGGAACGAGGAGGAGGAGGAGGAGGAGGGGGGGAAAACGAGGGGGG	GZMA	Granzyme A	56	60	NM 204457.1	F: CTTCCTGGAGATTTGTGCGTG	This study
CHPT1       Choline phosphotransferase 1       183       60       XM_015285488.2       F: CGAGCAGCACCTITITIG C       This study         DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: ATCACAGCCCTGCTGAACGT       This study         BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGGGAAAACGGT       This study         LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GCTGGCACCTACAGTGGAAGA       Gilbert et al. (2007)         ZAP70       Zeta chain of T cell receptor associated protein kinase 70 kDa       110       60       NM_20510383127       F: ATGCCGGTGATGCGGGAAA       This study         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGATGCCGTAT       Li et al. (2005)         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGAAGAAAGGGGTTG       Barzegar Nafari (2019)         R-TGCTGGGGTGGAAATGGTG       Sarzegar Nafari (2019)       R-TGCTGGGGTGAAATGGTG       Barzegar Nafari (2019)       R-TGCTGGGGTGAAATGGTG	02.0.1	Standynie II	20			R' GAGTGTGGTGCTACTTCATGTCCT	inio otady
DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: ATCACAGCCCTGCTGAACGT       This study R: CAGCACTCTTTCACTGAGTCGATG         BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGGGGAAAACGGT       This study R: TGGTACTCTCTGAACAGGAAGAACCGT         LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GATTGCAACGGGTGAGAGA       Gilbert et al. (2007) R: CCCCAACCGACTTTTGTTT         ZAP70       Zeta chain of T cell receptor associated protein kinase 70 kDa       110       60       NM_20510383127       F: TAGCCGATGATGGGGAAGA       This study R: CAATGCTGTAGTAGTAGGTGCGGAA         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGATGCCGTAT R- GTTCCTGTGGCGTTGC       Li et al. (2005) R- GTTCCTGTGGCGTGG         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGGAAGGAAATGGTG       Barzegar Nafari (2019) R-TGCTGGGGTGGAAATGGTG	CHPT1	Choline phosphotransferase 1	183	60	XM 015285488.2	F: CGAGCAGGCACCTTTTTGG	This study
DUSP4       Dual specificity phosphatase 4       155       60       NM_204838.1       F: ATCACAGCCCTGCTGAACGT R: CAGCACTCTTTCACTGAGTCGATG       This study         BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGGGAAACGGT R: GGTACTCTTGAACAAGGAAGAAC       This study         LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GATGCAACGGGTGGAAGAGAAC       Gilbert et al. (2007) R: CCCCAACCCACTTTGTGT         ZAP70       Zeta chain of T cell receptor associated protein kinase 70 kDa       110       60       NM_201321556.1       F: GCTGGACTACAGTGGGAAGAGAAGACAC       This study         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGATGCCGTAT R- GTTCCTGTGCGCTGC       Li et al. (2005) R- GTTCCTGTGCGCTGG       Barzegar Nafari (2019) R-TGCTGGGGGTGGTAAATGGTG         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGGAAGGAAATGGTG       Barzegar Nafari (2019) R-TGCTGGGGTGAAATGGTG						R: GCTATGCAGGATCCAAGGACA	····· ·····
BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGGGAAAACGGT       This study R: TGGTACTCTCTGAACAAGGAAAGAAC         LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GATTGCAACGGGTGATGTGA       Gilbert et al. (2007) R: CCCCACACCCACTTTTGTTT         ZAP70       Zeta chain of T cell receptor       110       60       NM_001321556.1       F: GCTGGACCTACAGTTGGGAAGAA       This study R: CAATGCTGTAGTAGTAGGGGCGGA         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGATGCCGTAT R- GTTCCCTGTGTCGCCTTGC       Li et al. (2005) R- GTTCCCTGTGTCGCTTGC         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGAAGGAAGGGGTTG       Barzegar Nafari (2019) R-TGCTGGGCTGGTAAATGCTG	DUSP4	Dual specificity phosphatase 4	155	60	NM 204838.1	F: ATCACAGCCCTGCTGAACGT	This study
BCL2A1       BCL2 related protein A1       78       60       NM_204866.1       F: GGTGGCTGGGAAAACGGT       This study R: TGGTACTCTCTGAACAAGGAAAGAAC         LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GATTGCAACGGGTGATGTGA R: CCCCACACCCACTTTTGTT       Gilbert et al. (2007) R: CCCCACACCCACTTTGTTT         ZAP70       Zeta chain of T cell receptor associated protein kinase 70 kDa       110       60       NM_001321556.1       F: GCTGGACCTACAGTGGGAAGAA R: CAATGCTGTAGTAGTAGGTGGCGGA       This study This study R: datact and the study         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGGTGCGCTAT R- GTTCCCTGTGTCGCCTTGC       Li et al. (2005) R- GTTCCCTGTGTCGCTTGC         SDHA       Succinate dehydrogenase complex flavoprotein subunit A       74       60       NM_00127739       F-ATACGGAAAGGAAGGGGTTG R- GTTCCTGGGCTGGTAAATGGTG       Barzegar Nafari (2019) R-TGCTGGGGTGGTAAATGGTG					_	R: CAGCACTCTTTCACTGAGTCGATG	, <b>,</b>
LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GATTGCAACGGGTGATGTGA       Gilbert et al. (2007)         ZAP70       Zeta chain of T cell receptor       110       60       NM_001321556.1       F: GCTGGACCTACAGTTGGGAAGA       This study         R:       CAATGCTGTAGTAGTAGGTGCGGA       R: CAATGCTGTAGTAGTAGGTGCGGAA       This study         BP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGATGCCGTAT       Li et al. (2005)         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGAAAGGAAGGGGTTG       Barzegar Nafari (2019)         R-TGCTGGGGTGGTAAATGGTG       R-TGCTGGGGTGGTAAATGGTG       R-TGCTGGGGTGGTAAATGGTG       Barzegar Nafari (2019)	BCL2A1	BCL2 related protein A1	78	60	NM 204866.1	F: GGTGGCTGGGAAAACGGT	This study
LAT1       Neutral amino acid transporter       70       60       KT876067.1       F: GATTGCAACGGGTGATGTGA       Gilbert et al. (2007)         ZAP70       Zeta chain of T cell receptor       110       60       NM_001321556.1       F: GCTGGACCTACAGTTGGGAAGA       This study         associated protein kinase 70 kDa       110       60       NM_001321556.1       F: GCTGGACCTACAGTTGGGAAGA       This study         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGGTGCCGTAT       Li et al. (2005)         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGGAAGGAAGTGGGTG       Barzegar Nafari (2019)         R-TGCTGGGGTGGTAAATGGTG       R-TGCTGGGGTGGTAAATGGTG       R-TGCTGGGGTGGTAAATGGTG       Barzegar Nafari (2019)		F				R: TGGTACTCTCTGAACAAGGAAAGAAC	····· · ·····
ZAP70       Zeta chain of T cell receptor       110       60       NM_001321556.1       F: GCTGGACCTACAGTTGGGAAGA R: CAATGCTGTGGGAAGA       This study         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGATGCCGTAT Li et al. (2005)       Li et al. (2005)         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGGAAGGAAGTGGTGG       Barzegar Nafari (2019)         R-TGCTGGGGTGGAAAGTGGTG       R-TGCTGGGGTGGAAATGGTG       R-TGCTGGGGTGGTAAATGGTG       Barzegar Nafari (2019)	LAT1	Neutral amino acid transporter	70	60	KT876067.1	F: GATTGCAACGGGTGATGTGA	Gilbert et al. (2007)
ZAP70       Zeta chain of T cell receptor       110       60       NM_001321556.1       F: GCTGGACCTACACTTGGGAAGA       This study         associated protein kinase 70 kDa       R: CAATGCTGTAGTAGTAGGTGGGGAA       This study       R: CAATGCTGTAGTAGTAGGTGGGGAAGA       This study         TBP       TATA-box binding protein       147       62       NM_20510383127       F-TAGCCCGATGATGCCGTAT       Li et al. (2005)         SDHA       Succinate dehydrogenase complex       74       60       NM_00127739       F-ATACGGGAAGGAAGGGGTTG       Barzegar Nafari (2019)         R-TGCTGGGGTGGTAAATGGTG       R-TGCTGGGGTGGTAAATGGTG       R-TGCTGGGGTGGTAAATGGTG       Barzegar Nafari (2019)		······				R: CCCCACACCCACTTTTGTTT	
associated protein kinase 70 kDa R: CAATGCTGTAGTAGTAGGTGCGGA TBP TATA-box binding protein 147 62 NM_20510383127 F-TAGCCCGATGATGCCGTAT Li et al. (2005) R- GTTCCCTGTGTCGCTTGC SDHA Succinate dehydrogenase complex 74 60 NM_00127739 F-ATACGGGAAGGAAGGAGGGGTTG Barzegar Nafari (2019) flavoprotein subunit A R-TGCTGGGGTGGTAAATGGTG	ZAP70	Zeta chain of T cell receptor	110	60	NM 001321556.1	F: GCTGGACCTACAGTTGGGAAGA	This study
TBP     TATA-box binding protein     147     62     NM_20510383127     F-TAGCCCGATGATGCCGTAT     Li et al. (2005)       SDHA     Succinate dehydrogenase complex     74     60     NM_00127739     F-ATACGCGAAGGAAGGAGGGGTTG     Barzegar Nafari (2019)       flavoprotein subunit A     R-GTTCCTGGTGGCGTGGTAAATGGTG     R-TGCTGGGGTGGTAAATGGTG		associated protein kinase 70 kDa				R: CAATGCTGTAGTAGTAGGTGCGGA	· ·····
SDHA     Succinate dehydrogenase complex     74     60     NM_00127739     F-ATACGGGAAGGAAGGGGTTG     Barzegar Nafari (2019)       flavoprotein subunit A     R-GTCCTGGGTGGTAAATGGTG	TBP	TATA-box binding protein	147	62	NM 20510383127	F-TAGCCCGATGATGCCGTAT	Li et al. (2005)
SDHA     Succinate dehydrogenase complex     74     60     NM_00127739     F-ATACGGGAAGGAAGGGGTTG     Barzegar Nafari (2019)       flavoprotein subunit A     R-TGCTGGGGTGGTAAATGGTG     R-TGCTGGGGTGGTAAATGGTG				-		R- GTTCCCTGTGTCGCTTGC	()
flavoprotein subunit A R-TGCTGGGGTGGTAAATGGTG	SDHA	Succinate dehydrogenase complex	74	60	NM 00127739	F-ATACGGGAAGGAAGGGGTTG	Barzegar Nafari (2019)
		flavoprotein subunit A				R-TGCTGGGGTGGTAAATGGTG	0 ··· ··· ·· ()

duodenal villus height (P < 0.05) and VH:CD ratio (P < 0.01) compared to the non-challenged birds. However, feed intake was not affected by the NE challenge during 14 and 35 days (P > 0.05).

# 3.2. Quality of RNA-seq reads and deferentially expressed genes

On average, 21, 543, 431 reads with a length of 100 bp were generated from the 8 cDNA libraries. Table 3 presents a summary for read features, including the alignment rates for both groups, which all exceed 80% and were mapped successfully to the chicken reference genome. The average number of sequence reads was 22.3 for non-challenged, and 20.7 million for the NE challenged birds. On average, more than 97% of the reads passed the quality control and cleaning process (Table 3).

The principal component analysis (PCA) plot showed the transcriptomic profile in the jejunum tissue with a clear separation of samples by the principal component 1 (PC1) which explained over 36% of the variance and principal component 2 (PC2) which accounted for 16% of the total variance between the samples (Fig. 2).

Differential gene expression (DEG) analysis showed 377 DEG (log2 fold change > 1; false discovery rate < 0.05) differentially expressed genes between the two groups. Among the DEG, 207

were significantly up-regulated, and 170 genes were significantly down-regulated by NE challenge (Appendix Table 1). The top three up and down-regulated genes in the challenged birds were myosin binding protein C-1 (*MYBPC1*), apical sodium—bile acid transporter (*ASBT*) and aconitate decarboxylase-1 (*ACOD1*), and phosphoenol-pyruvate carboxykinase-1 (*PCK1*), myomesin-2 (*MYOM2*) and protein kinase cGMP-dependent 2 (*PRKG2*), respectively. A full list of the top 35 significantly up- and down-regulated genes are shown in Tables 4 and 5, respectively. Table 6 illustrates the effect of NE challenge on fold change of genes related to immunity compared to the non-challenged birds.

#### 3.3. Pathway enrichment, protein-protein interaction

In total, 2 KEGG pathways were significantly enriched, including the peroxisome proliferator-activated receptors (PPAR) signaling pathways and fatty acid degradation pathway. Table 7 shows the significant KEGG pathways related to DEG. It was shown that all genes enriched in both pathways were down-regulated by the NE challenge.

Hierarchical clustering analysis was performed using the top 100 most extreme DEG in the 2 groups of birds. The pattern of Α



**Fig. 1.** TDNA gen electrophoresis of the qPCR products showing that the primers were specific in amplification. (A) Ladder: DNA ladder; MYOM2, 167 bp; *ALAS1*, 70 bp; CD36, 85 bp; *ALDH1A1*, 191 bp; *ASBT*, 119 bp; *FABP1*, 86 bp; *FABP2*, 184 bp; *GCG*, 144 bp; *ME1*, 164 bp; *PEX13*, 264 bp; *PRKG2*, 161 bp; *ACOX2*, 163 bp. (B) Ladder: DNA Ladder; *IL21R*, 111; *GZMA*, 56 bp; *DUSP4*, 155 bp; *SDHA*, 74 bp; *BCL2A1*, 78 bp; *ZAP70*, 110 bp; *ACOD1*, 146 bp; *CHPT1*, 183 bp; *LAT1*, 70 bp; *TBP*, 147 bp. *MYOM2* = myomesin-2; *ALAS1* = 5'-aminolevulinate synthase-1; *CD36* = cluster of differentiation 36; *ALDH1A1* = aldehyde dehydrogenase-1 family member A1; *ASBT* = apical sodium–bile acid transporter; *FABP1* and *FABP2* = fatty acid-binding protein-1 and -2; *GCG* = glucagon; *ME1* = malic enzyme; *PEX13* = peroxisomal biogenesis factor-13; *PRKG2* = protein kinase cGMP-dependent 2; *ACOX2* = acyl-CoA oxidase-2; *IL21R* = interleukin-21 receptor; *GZMA* = granzyme A; *DUSP4* = dual-specificity phosphatase-4; *SDHA* = succinate dehydrogenase 1; *CHPT1* = choline phosphotrase-1; *LAT1* = L-type amino acid transporter-1; TBP = TATA-box binding protein.

expression for the 100 genes is presented in the heat map as shown in Fig. 3. The list of genes of the heat map is provided in the supplementary file (Appendix Table 2).

To further explore the interactions between the DEG identified using the edgeR/cuffdiff, these DEG were subjected them to STRING analysis for PPI network analysis. The nodes in these networks are genes, and the links between them (edges) represent co-regulation (Fig. 4). The size of the nodes indicates the number of interactions of each gene with other DEG genes, therefore, any changes in gene expression of proteins with high interactions in a network may result in deep dysfunction of the interactome system (Zamanian-Azodi et al., 2016). Genes such as aldehyde dehydrogenase-2

Effect of NE challenged on WG, FI, FCR of birds	(day 14 to 35), and	duodenum morphology and jejunum	lesion scores on day 16
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Treatments	Performance			Histology, μm			Lesion score <sup>1</sup>
	WG, g/bird	FI, g/bird	FCR	VH	CD	VH-to-CD ratio	
Non-challenged NE challenged P-value	1,708 <sup>a</sup> 1,437 <sup>b</sup> 0.005	2,911 2,762 0.239	1.710 <sup>b</sup> 1.899 <sup>a</sup> 0.045	1,420 <sup>a</sup> 1,011 <sup>b</sup> 0.021	179 193 0.457	7.96 <sup>a</sup> 5.27 <sup>b</sup> 0.005	0.00 <sup>b</sup> 0.50 <sup>a</sup> 0.011

NE = necrotic enteritis, WG = weight gain; FI = feed intake; FCR = feed conversion ratio, VH = villus height, CD = crypt depth.

<sup>a, b</sup> Means sharing the same superscripts are not significantly different from each other at P < 0.05.

<sup>1</sup> Jejunum lesion score day 16.

#### Table 3

Number of reads obtained from the RNA sequencing and the mapped reads to the reference genome (Gallus gallus) in the non-challenged and necrotic enteritis (NE) challenged broiler groups.

No.	Group	Raw reads	Cleaned reads	Mapped	Mapping rate, %
1	Non-challenged	23,735,190	22,326,785	18,833,668	84.9
2	Non-challenged	23,102,585	21,879,408	18,468,203	84.4
3	Non-challenged	20,558,626	19,502,432	16,538,279	84.8
4	Non-challenged	21,960,653	20,844,944	17,795,298	85.4
5	NE challenged	20,853,776	19,737,566	16,031,560	81.2
6	NE challenged	20,710,878	19,665,303	16,028,299	81.5
7	NE challenged	20,396,891	19,046,762	15,581,264	81.8
8	NE challenged	21,028,852	19,662,408	15,893,653	80.8



**Fig. 2.** Principal component analysis plot of differentially abundant transcripts. The plot shows log<sub>2</sub> counts per million mapped reads (CPM) for both groups. The horizontal and vertical axes show 2 principal components that respectively explain variation between different groups. Control, non-challenged birds (black); NE, necrotic enteritis challenged birds (red); PC1, the axis showing the first principal direction along which the samples show the largest variation; PC2, the axis showing the second most important direction which is orthogonal to the PC1 axis.

(*ALDH2*), aldehyde dehydrogenase-1 family member A1 (*ALDH1A1*), enoyl-CoA delta isomerase-2 (*ECI2*), acyl-CoA synthetase-1 (*ACSL1*), enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (*EHHADH*), acyl-CoA synthetase bubblegum family member-1 (*ACSBG1*), and acyl-CoA oxidase-2 (*ACOX2*) show the highest interaction with other DEG, and could indicate their importance related to the negative responses observed in the infected chickens. Aldehyde dehydrogenase-2 and *ALDH1A1* are among the genes with the highest number of interactions.

#### 3.4. Validation of differential gene expression data by qPCR

To validate the DEG identified by sequencing analysis, a qPCR analysis was performed for 20 genes including 9 down-regulated genes, i.e., *ACOD1*, *ASBT*, *BCL2* related protein A1 (*BCL2A1*), choline phosphotransferase-1 (*CHPT1*), dual-specificity phosphatase-4 (DUSP4), granzyme A (*GZMA*), interleukin-21 receptor (*IL21R*), *LAT1*,

zeta-chain (TCR) associated protein kinase 70 kDa (*ZAP70*) and 11 upregulated genes: *ACOX2*, 5'-aminolevulinate synthase-1 (*ALAS1*), *ALDH1A1*, *CD36*, glucagon (*GCG*), fatty acid-binding protein-1 (*FABP1*), fatty acid-binding protein-2 (*FABP2*), malic enzyme 1 (*ME1*), *MYOM2*, peroxisomal biogenesis factor-13 (*PEX13*) and *PRKG2*. All the 20 selected genes for validation showed consistent up or down-regulation in response to the NE challenge with a strong positive correlation ( $R^2 = 0.813$ ; P < 0.001) in the fold change between qPCR and the RNA-seq data (Table 8). The qPCR results confirm the reliability of the differential expression analysis using RNA-Seq data.

# 4. Discussion

The current study identified pathways and corresponding genes in the jejunum of broilers that are responsive to NE infection. Two main metabolic pathways that were responsive to the challenge were involved in fatty acid production and utilization. Most downregulated genes, affected by the NE infection, were related to enzymes active in oxidation and fatty acid transportation. The NE challenge also up-regulated genes related to immunity. The results confirmed our hypothesis that NE infections can alter the expression of intestinal genes involved in the pathways related to digestion and immunity of the chickens, possibly in a negative way, which leads to negative changes in the performance of the challenged birds.

## 4.1. Fatty acid related genes

It is well known that the small intestine is an important organ for digestion, absorption, and transportation of dietary nutrients including lipid. Lipids play a key role in many biological processes such as energy sourcing and storage and inflammatory responses, and they participate in overall homeostasis as signaling molecules (Grygiel-Górniak 2014; Robinson and Mazurak 2013). Nine genes from the PPAR pathway and six genes in the  $\beta$ -oxidation pathway were suppressed in the NE challenged birds, indicating reduced lipid metabolism and fatty acid utilization in the challenged birds. The *PPAR* pathway (categorized as  $\alpha$ ,  $\beta/\sigma$ , and  $\gamma$  types) is closely associated with lipid and carbohydrate metabolism, energy

Тор З	5 upregulated	DEG in NE challenged	birds relative to non-	-challenged birds.
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Gene symbol	Gene name	Fold change	<i>P</i> -value	FDR
MYBPC1	Myosin binding protein C, slow type	+3.616	0.0006	0.0128
SLC10A2, ASBT	Solute carrier family 10 member 2	+3.254	0.0005	0.0115
ACOD1	Aconitate decarboxylase 1	+2.652	0.0002	0.0064
ANKF1	Ankyrin repeat and fibronectin type III domain containing 1	+2.600	0.0000	0.0026
IFN-γ	Interferon gamma	+2.590	0.0043	0.0352
WASF1/wave 1	WAS protein family member 1	+2.199	0.0000	0.0027
BEND4/ccdc4	BEN domain containing 4	+2.197	0.0003	0.0086
TM4SF19	Transmembrane 4 L six family member 19	+2.110	0.0001	0.0056
RNF208	Ring finger protein 208	+1.998	0.0000	0.0012
KRT19	Keratin 19	+1.989	0.0053	0.0400
LNX1	Ligand of numb-protein X 1	+1.932	0.0064	0.0449
WNT5B	Wnt family member 5B	+1.914	0.0068	0.0463
SLC7A5	Solute carrier family 7 member 5	+1.893	0.0000	0.0040
SCN3B	Sodium voltage-gated channel beta subunit 3	+1.888	0.0028	0.0276
FRMD3	FERM domain containing 3	+1.874	0.0001	0.0054
NEURL1B	Neuralized E3 ubiquitin protein ligase 1B	+1.804	0.0003	0.0084
PLCD4	Phospholipase C delta 4	+1.717	0.0021	0.0235
DUSP4	Dual specificity phosphatase 4	+1.659	0.0000	0.0039
GZMA	Granzyme A	+1.655	0.0003	0.0082
CEBPB	CCAAT enhancer binding protein beta	+1.634	0.0009	0.0152
ASS1	Argininosuccinate synthase 1	+1.631	0.0001	0.0051
HYAL3	Hyaluronoglucosaminidase 3	+1.551	0.0018	0.0221
BCL2A1	BCL2 related protein A1	+1.545	0.0039	0.0334
KCNJ5	Potassium voltage-gated channel subfamily J member 5	+1.516	0.0024	0.0254
EOMES	Eomesodermin	+1.506	0.0020	0.0230
THEMIS2	Thymocyte selection associated family member 2	+1.486	0.0000	0.0038
SLC11A1	Solute carrier family 11 member 1	+1.486	0.0009	0.0154
DCT	Dopachrome tautomerase	+1.479	0.0024	0.0253
NRG1	Neuregulin 1	+1.474	0.0002	0.0076
PROKR2	Prokineticin receptor 2	+1.473	0.0019	0.0223
SCL6A7/LAT1	Solute carrier family 6 member 7	+1.458	0.0032	0.0300
INSRR	Insulin receptor related receptor	+1.453	0.0000	0.0026
ART1	ADP-ribosyltransferase 1	+1.429	0.0011	0.0168
IL21R	Interleukin 21 receptor	+1.414	0.0018	0.0217
NEXMIF	Neurite extension and migration factor	+1.389	0.0034	0.0305

DEG = differentially expressed genes; NE = necrotic enteritis; FDR = false discovery rate.

balance, intestinal inflammation and hemostasis and ameliorating insulin sensitivity (Dupont et al., 2012; Ricote et al., 2004; Varga et al., 2011; Zhou et al., 2016). The NE infected broilers have previously shown to have dysregulated lipid metabolism in the liver through the reduced expression of *PPAR-* $\gamma$  and *PPAR-* $\alpha$  genes (Qing et al., 2017).

Additionally, the  $\beta$ -oxidation pathway, the main metabolic pathway for energy supply from fatty acids in cells (Bartlett and Eaton 2004), was also suppressed by the NE challenge. Among the DEG in the PPAR pathways, FABP1, FABP2, FAT/CD36, ACOX2, and PCK1 were significantly down-regulated in our study. The FABP1 and FABP2 genes are involved in the uptake of long-chain fatty acids (Pelsers et al., 2005) and can also modulate critical lipid-sensitive pathwavs in adipocytes and macrophages in humans that are important upon the occurrence of intestinal inflammation (Levy et al., 2001). Downregulation of FABP1 and FABP2 in NE infected birds is in agreement with previous reports where same observation was described (Guo et al., 2013). In the current study, the intestinal histology results confirmed significant villus damage in the challenged birds. It is suggested that down-regulation of FABP1 and FABP2 in the jejunum of challenged birds could be attributed to structural damage and intestinal epithelium loss (Chen et al., 2015). The gene CD36 is also involved in the regulation of fatty acid uptake, and is a necessary compound for transporting cholesterol and triglyceride from the intestine to the liver (Abumrad and Davidson 2012). Down-regulation of CD36 has resulted in subclinical inflammation and impaired barrier integrity in mice (Cifarelli et al., 2017). Altogether, the down-regulation of genes such as FABP1, FABP2 and CD36 can reduce fatty acid utilization and suppress immunity in the intestinal tissue.

In the current study, three DEG were common in both PPAR and β-oxidation pathways which are ACSBG1, ACSL1 and EHHADH. The ACSBG1 and ACSL1 genes are a strong activator for long fatty acid substrates (Pei et al., 2003). It has been reported that fruit flies with an inoperative ACSBG1 gene showed an accumulation of saturated very-long-chain fatty acids in their body (Pei et al., 2006), and the down-regulation of this gene has been previously reported in NE infected chickens (Qing et al., 2018) indicating the possible role of the gene in the fatty acid metabolic pathway. Lower expression of ACSL1 is known to reduce triglyceride synthesis and fatty acids in the cells and lead to inflammation in the tissue (Yan et al., 2015). Furthermore, EHHADH is mostly known for its important role in the peroxisomal  $\beta$ -oxidation pathway (Houten et al., 2012). Therefore, the down-regulations of these genes may indicate reduced fatty acid metabolism and energy expenditure and activated immunity reactions. The PPI analysis showed that ALDH2 and ALDH1A1 are among the genes with the highest number of interactions. These two genes are part of the aldehyde dehydrogenases (ALDH) family which are involved in a variety of biological processes such as inflammation, mitochondrial respiration, and xenobiotic metabolism (Chen et al. 2009, 2016). Down-regulation of the gene can induce inflammation, apoptosis and necrosis by the activation of ROS pathway (Fang et al., 2018; Liang et al., 2017).

# 4.2. Glycogenesis related genes

The strongest down-regulated gene found in the challenged group compared to non-challenged birds is *PCK1*. The PCK1 protein plays a key role in glutamine uptake, the main precursor of gluconeogenesis in the intestine, and thus the production of

Top 35 downregulated DEG in NE challenged birds relative to non-challenged birds.

PKt1         Phospheenolpyruvate carboxykinase 1         -3.052         5.88F-05         0.0043           MYOM2         Myomesin 2         -2.935         0.000558         0.0120           PKRC2         Protein kinase cGMP-dependent 2         -2.929         0.000144         0.0063           PAXDC2         Fatty acid hydroxylase domain containing 2         -2.676         0.001366         0.0189           ADAC         Arylacetamide deacetylase         -2.633         4.066-07         0.0011           CYP2ACI         Cytochrome P450, family 2, subfamily AC, polypeptide 1         -2.520         0.000651         0.0129           DDO         p-aspartate oxidase         -2.244         0.000659         0.0130           DBM4         Pyruvate dehydrogenase kinase 4         -2.040         0.000659         0.0130           DDX         p-aspartate oxidase         -1.979         0.005847         0.0425           ENP7         Ectonucleotide pyrophosphatase/phosphodiesterase 7         -1.952         0.000643         0.0128           CYP4V2         Cytochrome P450 family 4 subfamily V member 2         -1.904         0.000259         0.0002           ADDIL1A1         Aldehydre dehydrogenase 1 family member A1         -1.738         0.00028         0.00021           D1HA1	Gene symbol	Gene name	Fold change	P-value	FDR
MYONZ         Myomesin 2         -2.935         0.000558         0.0120           PRKG2         Protein kinase CdMP-dependent 2         -2.929         0.000144         0.0063           AADAC         Anylacetamide descriptase         -2.676         0.001366         0.0189           AADAC         Anylacetamide descriptase         -2.633         4.06E-07         0.0011           CYP2AC1         Cytochrome P450, family 2, subfamily AC, polypeptide 1         -2.582         1.85E-05         0.0029           AGMO         Alkylgiverol monooxygenase         -2.2448         0.006693         0.0130           DDO         p-aspartate oxidiase         -2.448         0.000659         0.0130           BRCB11         ATP binding cassette subfamily B member 11         -1.979         0.005847         0.0425           ENPP7         Ectonucleotide pyrophosphatase/phosphodiesterase 7         -1.952         0.000643         0.0128           CVP4V         Cytochrome P450 family 4 subfamily M member 2         -1.994         0.000539         0.00312           ALDH1A1         Aldelyde dehydrogenase 1 family member 11         -1.898         5.93E-05         0.0021           CVP4V         Cytochrome P450 family 4 subfamily M member 2         -1.738         0.000259         0.00312	PCK1	Phosphoenolpyruvate carboxykinase 1	-3.052	5.89E-05	0.0043
PRKC2         Protein kinase cGMF-dependent 2         -2.929         0.000144         0.0063           FAXDC2         Fatty aci hydroxylase domain containing 2         -2.675         0.001366         0.0189           ADAC         Anylacetamide deacetylase         -2.633         4.06E-07         0.0011           CYP2ACI         Cytochrome P450, family 2, subfamily AC, polypeptide 1         -2.582         1.85E-05         0.0029           DDO         p-aspartate oxidase         -2.2448         0.00659         0.0130           DDO         p-aspartate oxidase         -2.448         0.00059         0.0034           DRIM         Pyruvate dehydrogenase kinase 4         -2.040         0.00059         0.0082           CPIV42         Cytochrome P450 family 4 subfamily V member 2         -1.954         0.000643         0.0128           CPIV42         Cytochrome P450 family 4 subfamily V member 2         -1.964         0.000259         0.0082           ALDH1A1         Aldehydrogenase I family member A1         -1.898         5.93E-05         0.00027           DETV         Extoucleotide pyrophosybatase/phosphateiserase 7         -1.738         0.00028         0.00028           DDD         DP36         Darbiny 4 subfamily 7 member A1         -1.898         5.93E-05         0.00027	MYOM2	Myomesin 2	-2.935	0.000558	0.0120
FAXDC2         Fatty acid hydroxylase domain containing 2         -2.676         0.001366         0.0189           MDAC         Arylacetamide deacetylase         -2.633         4.06E-07         0.0011           CYP2AC1         Cytochrome P450, family 2, subfamily AC, polypeptide 1         -2.582         1.85E-05         0.0029           AGMO         Alkylglycerol monoxygenase         -2.520         0.0006803         0.0463           DDO         0-aspartate oxidase         -2.448         0.0006803         0.0128           DDA         Provate dehydrogenase kinase 4         -2.040         0.000597         0.0128           BABCB 11         ATP binding cassetts subfamily 8 member 11         -1.979         0.000547         0.0425           ENPP7         Ectonucleotide pyrophosphatase/phosphodiesterase 7         -1.954         0.000259         0.00024           CY44/2         Cytochrome P450 family 4 subfamily W member 2         -1.804         0.000259         0.0027           DEME1         Malic enzyme 1         -1.779         1.26E-05         0.0027           FETUB         Fetuin B         -1.733         0.00629         0.0454           DNM1         Dynamin 1         -1.733         0.00629         0.0037           GSTT1         Glutathione S-transferase t	PRKG2	Protein kinase cGMP-dependent 2	-2.929	0.000144	0.0063
ADAC         Ayfacetamide de acceptase         -2.633         4.06E-07         0.0011           CYP2AC1         Cytochrome P450, family 2, subfamily AC, polypeptide 1         -2.582         1.85E-05         0.0029           AGMO         Alkylgityceri olmonoxygenase         -2.520         0.000651         0.0129           DDO         p-aspartate oxidase         -2.448         0.006653         0.0130           ABCB11         ATP binding cassette subfamily B member 11         -1.979         0.00543         0.0128           PDV4         Pytruvate dehydrogenase kinase 4         -2.040         0.000659         0.0082           ABCB11         ATP binding cassette subfamily B member 11         -1.979         0.00543         0.0128           CYP4V2         Cytochrome P450 family 4 subfamily V member 2         -1.904         0.000259         0.0082           ALDH1A1         Aldehyde dehydrogenase 1 family member A1         -1.881         0.003339         0.0312           ME1         Malic enzyme 1         -1.779         1.26E-05         0.0027           PHTB         Pehydrogenase/reductase 7         -1.738         0.00028         0.0043           DHK7         Dehydrogenase/reductase 7         -1.738         0.00021         0.0074           FTETUB         Fettu	FAXDC2	Fatty acid hydroxylase domain containing 2	-2.676	0.001366	0.0189
CYP2ACI         Cyňachrome P450, family 2, subfamily AC, polypeptide 1         -2.520         1.85E-05         0.0029           AGMO         Alkylglycerol monooxygenase         -2.520         0.000651         0.0129           DDO         p-aspartate oxidase         -2.448         0.006803         0.0463           PDK4         Prytwate dehydrogenase kinase 4         -2.040         0.000559         0.0130           BCB11         ATP binding cassette subfamily B member 11         -1.979         0.005847         0.0425           ENPP7         Ectonucleotide prophosphatase/phosphodiesterase 7         -1.952         0.000643         0.00128           CYP4V2         Cytochrome P450 family 4 subfamily M ember 2         -1.904         0.000259         0.0042           CD36         CD36 molecule         -1.881         0.003539         0.0312           DE1         Malic enzyme 1         -1.738         0.00028         0.0004           DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0003           DNM1         Dynamin 1         -1.737         8.97E-05         0.0031           GSTT1         Clutathione S-transferase theta 1         -1.738         0.00022         0.0043           AC012         Acyl-CoA thioesterase 12         <	AADAC	Arylacetamide deacetylase	-2.633	4.06E-07	0.0011
ACMO         Åkylglycerol monoxygenase         -2.520         0.000651         0.0129           DDO         p-aspartate oxidase         -2.448         0.000639         0.0463           DDK4         Pyruvate dehydrogenase kinase 4         -2.040         0.000659         0.0130           ABCB11         ATP binding cassette subfamily B member 11         -1.979         0.005847         0.0425           ENPP7         Ectonucledice pyrophosphotiesterase 7         -1.952         0.000643         0.0128           CYP4V2         Cytochrome P450 family 4 subfamily V member 2         -1.904         0.000259         0.0082           ALDH1A1         Aldehyde dehydrogenase 1 family member A1         -1.898         5.93E-05         0.0043           CD36         CD36 molecule         -1.779         1.26E-05         0.0027           FETUB         Fetuin B         -1.779         1.26E-05         0.00045           DNM1         Dynamin 1         -1.737         8.97E-05         0.0051           CSTT1         Glutathione S-transferase theta 1         -1.713         0.006629         0.0043           CO12         Acyl-CoA thioesterase 12         -1.619         0.00121         0.0074           FCDD         Formimidoyltransferase cyclodeaminase         -1.652	CYP2AC1	Cytochrome P450, family 2, subfamily AC, polypeptide 1	-2.582	1.85E-05	0.0029
DD0         b-spartate oxidase         -2.448         0.006803         0.0463           PDK4         Pyruvate dehydrogenase kinase 4         -2.040         0.00059         0.0130           ABCB 11         ATP binding cassette subfamily B member 11         -1.979         0.005847         0.0425           ENP7         Ectonucleotide pyrophosphatase/phosphodiesterase 7         -1.952         0.000643         0.0128           CYP4V2         Cytochrome P450 family 4 subfamily V member 2         -1.904         0.000259         0.0082           ALDH1A1         Aldehyde dehydrogenase 1 family member A1         -1.881         0.003539         0.0312           CD36         CD36 molecule         -1.779         1.26E-05         0.0040           DHK1         Malic enzyme 1         -1.779         1.26E-05         0.0005           FETUB         Fetuin B         -1.773         8.97E-05         0.0005           DNM1         Dynamin 1         -1.738         0.000629         0.0454           BOX1         Gamma-butyrobetaine hydroxylase 1         -1.738         0.00021         0.0074           CPZ4         Gatuathione S-ransferase teta 1         -1.713         0.006629         0.0454           BOX1         Gamma-butyrobetaine hydroxylase 1         -1.713	AGMO	Alkylglycerol monooxygenase	-2.520	0.000651	0.0129
PDK4         Pyruvate dehydrogenase kinase 4         -2.040         0.000659         0.0130           ABCB11         ATP binding cassette subfamily B member 11         -1.979         0.005847         0.0425           ENPP7         Ectonucleotide pyrophosphatase/phosphodiesterase 7         -1.952         0.000643         0.0128           CYF4V2         Cytochrome P450 family 4 subfamily V member 2         -1.904         0.000259         0.0083           CD36         CD36 molecule         -1.851         0.003539         0.0312           ME1         Malic enzyme 1         -1.779         1.26E-05         0.0004           DHKS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0055           DNM1         Dynamin 1         -1.778         8.97E-05         0.0037           CSTT1         Glutathione 5-transferase theta 1         -1.713         0.006629         0.0454           BBOX1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0073           CPX4         Glutathione peroxidase 4         -1.624         5.68E-05         0.0043           ACO12         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           SUSD2         Subhi domain containing 1         -1.572	DDO	D-aspartate oxidase	-2.448	0.006803	0.0463
ABCB11         A <sup>T</sup> P binding casette subfamily B member 11         -1.979         0.005847         0.0425           ENPP7         Ectonucleotide pyrophosphatase/phosphodiesterase 7         -1.952         0.000643         0.0128           CYP4V2         Cytochrome P450 family 4 subfamily V member 2         -1.994         0.000259         0.0032           ALDH1A1         Aldehyde dehydrogenase 1 family member A1         -1.898         5.93E-05         0.00312           CD36         CD36 molecule         -1.779         1.26E-05         0.0027           FETUB         Fetuin B         -1.779         1.26E-05         0.00043           DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0065           DNM1         Dynamin 1         -1.737         8.97E-05         0.0071           GIUtathione S-transferase theta 1         -1.713         0.006629         0.0454           BDX1         Guman-butyrobetiane hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Forminidoyltransferase cyclodeaminase         -1.690         3.1E-05         0.0037           GPX4         Glutathione proxidase 4         -1.624         5.68E-05         0.0041           ACD12         Acyl-CoA thioesterase 12         -1.619	PDK4	Pyruvate dehydrogenase kinase 4	-2.040	0.000659	0.0130
ENPP7         Ectonucleotide pyrophosphatase/phosphodiesterase 7         -1.952         0.000643         0.0128           CYP4V2         Cytochrome P450 family 4 subfamily V member 2         -1.904         0.000259         0.0083           CD36         CD36 molecule         -1.888         5.93E-05         0.0043           CD36         CD36 molecule         -1.851         0.003539         0.0312           ME1         Malic enzyme 1         -1.779         1.26E-05         0.00045           DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0085           DNM1         Dynamin 1         -1.737         8.97E-05         0.0017           GGSTT1         Glutathione S-transferase theta 1         -1.713         0.006629         0.0454           BB0X1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Forminidoyltransferase cyclodeaminase         -1.619         0.000127         0.0058           AC012         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           FABP2         Fatty acid binding protein 2         -1.572         4.67E-05         0.0049           ACMSD         Aminocarboxymuconate semialdehyde decarboxylase         -1.561 </td <td>ABCB11</td> <td>ATP binding cassette subfamily B member 11</td> <td>-1.979</td> <td>0.005847</td> <td>0.0425</td>	ABCB11	ATP binding cassette subfamily B member 11	-1.979	0.005847	0.0425
CYP4/2         Cytochrome P450 family 4 subfamily V member 2         -1.904         0.000259         0.0082           ALDH1A1         Aldehyde dehydrogenase 1 family member A1         -1.898         5.93E-05         0.0043           CD36         CD36 molecule         -1.871         0.003539         0.0312           ME1         Malic enzyme 1         -1.779         1.26E-05         0.00040           DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0085           DNM1         Dynamin 1         -1.737         8.97E-05         0.0045           DSX11         Glutathione S-transferase theta 1         -1.713         0.006629         0.0454           BB0X1         Guutathione peroxidase 4         -1.624         5.68E-05         0.0037           CPX4         Glutathione peroxidase 4         -1.624         5.68E-05         0.0028           AC012         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           SUSD2         Sushi domain containing 1         -1.579         1.6E-05         0.0028           SUSD2         Sushi domain containing 2         -1.571         0.00163         0.0066           ANO5         Anoctamin 5         -1.551         0.00392         0.0333     <	ENPP7	Ectonucleotide pyrophosphatase/phosphodiesterase 7	-1.952	0.000643	0.0128
ALDH1A1       Aldehyde dehydrogenase 1 family member A1       -1.898       5.93E-05       0.0043         CD36       CD36 molecule       -1.851       0.00339       0.0312         CD36       CD36 molecule       -1.779       1.26E-05       0.0027         FETUB       Fetuin B       -1.778       0.0028       0.0040         DHRS7       Dehydrogenase/reductase 7       -1.738       0.0028       0.0051         GSTT1       Glutathione S-transferase theta 1       -1.713       0.006629       0.0454         BB0X1       Gamma-buttyrobetaine hydroxylase 1       -1.708       0.00021       0.0077         FTCD       Forminidoyltransferase cyclodeaminase       -1.624       5.68E-05       0.0043         AC012       Acyl-CoA thioesterase 12       -1.619       0.000127       0.0058         FABP2       Fatty acid binding protein 2       -1.579       1.6E-05       0.0028         SUSD2       Sushi domain containing 1       -1.571       0.00127       0.0031         ACMSD       Anectamin 5       -1.551       0.0032       0.0333         SUSD2       Sushi domain containing 1       -1.572       4.67E-05       0.0024         ACMSD       Anectamin 5       -1.551       0.00163       0.00	CYP4V2	Cytochrome P450 family 4 subfamily V member 2	-1.904	0.000259	0.0082
CD36         CD36 molecule         -1.851         0.003539         0.0312           ME1         Malic enzyme 1         -1.779         1.26E-05         0.0027           DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0085           DNM1         Dynamin 1         -1.737         8.97E-05         0.0051           GSTT1         Glutathione S-transferase theta 1         -1.738         0.00028         0.0074           BB0X1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Formimidoyltransferase cyclodeaminase         -1.690         3.1E-05         0.0037           GRX4         Glutathione peroxidase 4         -1.619         0.00127         0.0058           FABP2         Fatty acid binding protein 2         -1.619         0.00127         0.0011           PDXDC1         PX domain containing 1         -1.579         1.6E-05         0.0033           SUSD2         Sushi domain containing 2         -1.561         2.03E-06         0.0016           ANO5         Anniocarboxymuconate semialdehyde decarboxylase         -1.561         2.03E-06         0.0016           ANO5         Ancetamin 5         -1.551         0.00392         0.0333	ALDH1A1	Aldehyde dehydrogenase 1 family member A1	-1.898	5.93E-05	0.0043
ME1         Malic enzyme 1         -1.779         1.26E-05         0.0027           FETUB         Fetuin B         -1.760         4.63E-05         0.0048           DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0051           DNM1         Dynamin 1         -1.737         8.97E-05         0.0051           CST1         Glutathione S-transferase theta 1         -1.713         0.006629         0.0454           BB0X1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Formimidoyltransferase cyclodeaminase         -1.659         3.1E-05         0.0037           CPX4         Glutathione peroxidase 4         -1.624         5.68E-05         0.0043           AC012         Acyl-CA thioesterase 12         -1.606         5.01E-07         0.0058           SUSD2         Sushi domain containing 1         -1.572         4.67E-05         0.0028           SUSD2         Sushi domain containing 2         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.561         0.000163         0.0066           ANDO	CD36	CD36 molecule	-1.851	0.003539	0.0312
FETUB         Fetuin B         -1.760         4.63E-05         0.0040           DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.0085           DNM1         Dynamin 1         -1.737         8.97E-05         0.0051           CSTT1         Glutathione S-transferase theta 1         -1.713         0.006629         0.0454           B80X1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Formimidoyltransferase cyclodeaminase         -1.690         3.1E-05         0.0037           CPX4         Glutathione peroxidase 4         -1.624         5.68E-05         0.0043           AC012         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           FABP2         Fatty acid binding protein 2         -1.579         1.6E-05         0.0024           SUSD2         Sush idomain containing 1         -1.572         4.67E-05         0.0046           ACMSD         Aminocarboxymuconate semialdehyde decarboxylase         -1.561         2.03E-06         0.0016           ANOS         Anotamin 5         -1.551         0.000163         0.00666           HESX1         HESX homeobox 1         -1.541         0.001476         0.0197	ME1	Malic enzyme 1	-1.779	1.26E-05	0.0027
DHRS7         Dehydrogenase/reductase 7         -1.738         0.00028         0.00051           DNM1         Dynamin 1         -1.737         8.97E-05         0.0051           GSTT1         Glutathione S-transferase theta 1         -1.713         0.006629         0.0454           BB0X1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Forminidoyltransferase cyclodeaminase         -1.624         5.68E-05         0.0043           AC012         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           FABP2         Fatty acid binding protein 2         -1.606         5.01E-07         0.0012           DVSDC1         PX domain containing 1         -1.579         1.6E-05         0.0028           SUSD2         Sushi domain containing 2         -1.561         2.03E-06         0.0016           ACMSD         Aminocarboxymuconate semialdehyde decarboxylase         -1.561         2.03E-06         0.0016           AN05         Anotamin 5         -1.551         0.000163         0.0066           HESX1         HESX homeobox 1         -1.541         0.001476         0.0012           PDE6C         Phosphodiesterase 6C         -1.509         0.005998	FETUB	Fetuin B	-1.760	4.63E-05	0.0040
DNM1         Dynamin 1         -1.737         8.97E-05         0.0051           GSTT1         Glutathione S-transferase theta 1         -1.713         0.006629         0.0454           BB0X1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Formimidoyltransferase cyclodeaminase         -1.690         3.1E-05         0.0037           GPX4         Glutathione peroxidase 4         -1.624         5.68E-05         0.0043           AC012         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           FABP2         Fatty acid binding protein 2         -1.606         5.01E-07         0.0011           DVXDC1         PX domain containing 1         -1.579         1.6E-05         0.0028           SUSD2         Sushi domain containing 2         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.551         0.00163         0.0066           HESX1         HESX homeobox 1         -1.541         0.00176         0.0177           PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431      A	DHRS7	Dehydrogenase/reductase 7	-1.738	0.00028	0.0085
GSTT1         Glutathione S-transferase theta 1         -1.713         0.006629         0.0454           BBOX1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Forminidoyltransferase cyclodeaminase         -1.690         3.1E-05         0.0037           GPX4         Glutathione peroxidase 4         -1.624         5.68E-05         0.0043           AC012         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           FABP2         Fatty acid binding protein 2         -1.606         5.01E-07         0.0011           PDXDC1         PX domain containing 1         -1.579         1.6E-05         0.0043           SUSD2         Sushi domain containing 2         -1.567         0.0033         0.0066           ANO5         Annocarboxymuconate semialdehyde decarboxylase         -1.561         2.03E-06         0.0016           ANO5         Anoctamin 5         -1.551         0.000163         0.0066           HESX 1         HESX homeobox 1         -1.473         0.001476         0.017           PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431           FABP1         Fatty acid binding protein 1         -1.476         0.002718 <td>DNM1</td> <td>Dynamin 1</td> <td>-1.737</td> <td>8.97E-05</td> <td>0.0051</td>	DNM1	Dynamin 1	-1.737	8.97E-05	0.0051
BBOX1         Gamma-butyrobetaine hydroxylase 1         -1.708         0.00021         0.0074           FTCD         Formimidoyltransferase cyclodeaminase         -1.690         3.1E-05         0.0037           GPX4         Glutathione peroxidase 4         -1.624         5.68E-05         0.0043           AC012         Acyl-CoA thioesterase 12         -1.619         0.000127         0.0058           FABP2         Fatty acid binding protein 2         -1.606         5.01E-07         0.0011           PDXDC1         PX domain containing 1         -1.579         1.6E-05         0.0028           SUSD2         Sushi domain containing 2         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.561         2.03E-06         0.0016           AN05         Anoctamin 5         -1.591         0.000147         0.0016           AVS1         HESX homeobox 1         -1.541         0.00163         0.0066           AVS1         HESX homeobox 1         -1.509         0.005998         0.0431           ACSL1         Acyl-CoA synthetase long chain family member 1         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.476         0.0002718         0.0272 </td <td>GSTT1</td> <td>Glutathione S-transferase theta 1</td> <td>-1.713</td> <td>0.006629</td> <td>0.0454</td>	GSTT1	Glutathione S-transferase theta 1	-1.713	0.006629	0.0454
FTCD       Formimidoyltransferase cyclodeaminase       -1.690       3.1E-05       0.0037         GPX4       Glutathione peroxidase 4       -1.624       5.68E-05       0.0043         AC012       Acyl-CoA thioesterase 12       -1.619       0.000127       0.0058         FABP2       Fatty acid binding protein 2       -1.606       5.01E-07       0.0011         DXDC1       PX domain containing 1       -1.579       1.6E-05       0.0028         SUSD2       Sushi domain containing 2       -1.561       2.03E-06       0.0016         ACMSD       Aminocarboxymuconate semialdehyde decarboxylase       -1.561       2.03E-06       0.0016         ANO5       Anoctamin 5       -1.551       0.000163       0.0066         HESX1       HESX homeobox 1       -1.541       0.001476       0.0112         PDE6C       Phosphodiesterase 6C       -1.509       0.0025988       0.0431         ACSL1       Acyl-CoA synthetase long chain family member 1       -1.473       0.0272       0.0052         FAM83B       Family with sequence similarity 83 member B       -1.476       0.002718       0.0272         FABP1       Fatty acid binding protein 1       -1.473       9.25E-05       0.0052         HSD17B4       Hydroxysteroid	BBOX1	Gamma-butyrobetaine hydroxylase 1	-1.708	0.00021	0.0074
GPX4       Glutathione peroxidase 4       -1.624       5.68E-05       0.0043         AC012       Acyl-CoA thioesterase 12       -1.619       0.000127       0.0058         FABP2       Fatty acid binding protein 2       -1.606       5.01E-07       0.0011         PDXDC1       PX domain containing 1       -1.579       1.6E-05       0.0028         SUSD2       Sushi domain containing 2       -1.572       4.67E-05       0.0040         ACMSD       Aminocarboxymuconate semialdehyde decarboxylase       -1.561       2.03E-06       0.00163         CDA       Cytidine deaminase       -1.551       0.00163       0.0066         ANO5       Anoctamin 5       -1.551       0.001476       0.017         PDE6C       Phosphodiesterase 6C       -1.509       0.005998       0.0431         ACSL1       Acyl-CoA synthetase long chain family member 1       -1.499       1.04E-06       0.0012         FAM83B       Family with sequence similarity 83 member B       -1.476       0.002718       0.0272         FABP1       Fatty acid binding protein 1       -1.473       9.25E-05       0.0052         HSD17B4       Hydroxysteroid 17-beta dehydrogenase 4       -1.469       0.00012       0.0057         HIKL       Kitotho	FTCD	Formimidoyltransferase cyclodeaminase	-1.690	3.1E-05	0.0037
AC012       Acyl-CoA thioesterase 12       -1.619       0.00127       0.0058         FABP2       Fatty acid binding protein 2       -1.606       5.01E-07       0.0011         PDXDC1       PX domain containing 1       -1.579       1.6E-05       0.0028         SUSD2       Sushi domain containing 2       -1.572       4.67E-05       0.0040         ACMSD       Aminocarboxymuconate semialdehyde decarboxylase       -1.561       2.03E-06       0.0016         CDA       Cytidine deaminase       -1.561       2.03E-06       0.0016         ANO5       Anoctamin 5       -1.551       0.001476       0.0197         PDE6C       Phosphodiesterase 6C       -1.509       0.005998       0.0431         ACSL1       Acyl-CoA synthetase long chain family member 1       -1.476       0.002718       0.0272         FABP1       Fatty acid binding protein 1       -1.473       9.25E-05       0.0052         FAMP3B       Family with sequence similarity 83 member B       -1.476       0.00012       0.0052         FABP1       Fatty acid binding protein 1       -1.473       9.25E-05       0.0052         HSD17B4       Hydroxysteroid 17-beta dehydrogenase 4       -1.469       0.00012       0.00274         KL       Klotho <td>GPX4</td> <td>Glutathione peroxidase 4</td> <td>-1.624</td> <td>5.68E-05</td> <td>0.0043</td>	GPX4	Glutathione peroxidase 4	-1.624	5.68E-05	0.0043
FABP2       Fatty acid binding protein 2       -1.606       5.01E-07       0.0011         PDXDC1       PX domain containing 1       -1.579       1.6E-05       0.0028         SUSD2       Sush domain containing 2       -1.572       4.67E-05       0.0040         ACMSD       Aminocarboxymuconate semialdehyde decarboxylase       -1.567       0.00392       0.0333         CDA       Cytidine deaminase       -1.561       2.03E-06       0.00163         ANO5       Anoctamin 5       -1.551       0.001476       0.0197         PDE6C       Phosphodiesterase 6C       -1.509       0.005998       0.0431         ACSL1       Acyl-CoA synthetase long chain family member 1       -1.476       0.002718       0.0272         FAM83B       Family with sequence similarity 83 member B       -1.476       0.002718       0.0272         FAMP1       Fatty acid binding protein 1       -1.473       9.25E-05       0.0052         HSD17B4       Hydroxysteroid 17-beta dehydrogenase 4       -1.469       0.00012       0.0057         MLKL       Kited lineage kinase domain like pseudokinase       -1.438       0.000212       0.0074	ACO12	Acyl-CoA thioesterase 12	-1.619	0.000127	0.0058
PDXDC1         PX domain containing 1         -1.579         1.6E-05         0.0028           SUSD2         Sushi domain containing 2         -1.572         4.67E-05         0.0040           ACMSD         Aminocarboxymuconate semialdehyde decarboxylase         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.561         2.03E-06         0.0016           ANO5         Anoctamin 5         -1.551         0.000163         0.0066           HESX 1         HESX homeobox 1         -1.541         0.001476         0.0197           PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431           ACSL1         Acyl-CoA synthetase long chain family member 1         -1.476         0.002718         0.0272           FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0052           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0057           MLKL         Mixed lineage kinase domain like pseudokinase         -1.456         0.002283         0.0274           KL         Klotho         -1.438	FABP2	Fatty acid binding protein 2	-1.606	5.01E-07	0.0011
SUSD2         Sushi domain containing 2         -1.572         4.67E-05         0.0040           ACMSD         Aminocarboxymuconate semialdehyde decarboxylase         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.561         2.03E-06         0.0016           ANO5         Anoctamin 5         -1.551         0.000163         0.0066           ANO5         Anoctamin 5         -1.541         0.001476         0.0197           PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431           ACSL1         Acyl-CoA synthetase long chain family member 1         -1.476         0.002718         0.0272           FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0052           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0054           MLKL         Kitotho         -1.456         0.002283         0.0247	PDXDC1	PX domain containing 1	-1.579	1.6E-05	0.0028
ACMSD         Aminocarboxymuconate semialdehyde decarboxylase         -1.567         0.00392         0.0333           CDA         Cytidine deaminase         -1.561         2.03E-06         0.0016           ANO5         Anoctamin 5         -1.551         0.001473         0.0017           HESX         MESX homeobox 1         -1.541         0.005998         0.0431           PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431           ACSL1         Acyl-CoA synthetase long chain family member 1         -1.479         1.04E-06         0.0012           FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0052           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0054           MLKL         Klotho         -1.456         0.002283         0.0227	SUSD2	Sushi domain containing 2	-1.572	4.67E-05	0.0040
CDA         Cytidine deaminase         -1.561         2.03E-06         0.00163           ANO5         Anoctamin 5         -1.551         0.000163         0.0066           HESX1         HESX homeobox 1         -1.541         0.001476         0.0197           PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431           ACSL1         Acyl-CoA synthetase long chain family member 1         -1.499         1.04E-06         0.0012           FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0228           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0059           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0056           MLKL         Mixed lineage domain like pseudokinase         -1.436         0.000212         0.0074	ACMSD	Aminocarboxymuconate semialdehyde decarboxylase	-1.567	0.00392	0.0333
AN05         Anoctamin 5         -1.551         0.000163         0.0066           HESX1         HESX homeobox 1         -1.541         0.001476         0.0197           PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431           ACSL1         Acyl-CoA synthetase long chain family member 1         -1.499         1.04E-06         0.0012           FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0059           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0057           MLKL         Mixed lineage domain like pseudokinase         -1.436         0.000212         0.0074	CDA	Cytidine deaminase	-1.561	2.03E-06	0.0016
HESX1       HESX homeobox 1       -1.541       0.001476       0.0197         PDE6C       Phosphodiesterase 6C       -1.509       0.005998       0.0431         ACSL1       Acyl-CoA synthetase long chain family member 1       -1.499       1.04E-06       0.0012         FAM83B       Family with sequence similarity 83 member B       -1.476       0.002718       0.0272         FABP1       Fatty acid binding protein 1       -1.473       9.25E-05       0.0052         HSD17B4       Hydroxysteroid 17-beta dehydrogenase 4       -1.469       0.00012       0.0054         MLKL       Mixed lineage kinase domain like pseudokinase       -1.438       0.000212       0.0074	ANO5	Anoctamin 5	-1.551	0.000163	0.0066
PDE6C         Phosphodiesterase 6C         -1.509         0.005998         0.0431           ACSL1         Acyl-CoA synthetase long chain family member 1         -1.499         1.04E-06         0.0012           FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0059           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0057           MLKL         Mixed lineage kinase domain like pseudokinase         -1.438         0.000212         0.0074	HESX1	HESX homeobox 1	-1.541	0.001476	0.0197
ACSL1         Acyl-CoA synthetase long chain family member 1         -1.499         1.04E-06         0.0012           FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0052           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0056           MLKL         Mixed lineage kinase domain like pseudokinase         -1.456         0.002283         0.0247           KL         Klotho         -1.438         0.00012         0.0071	PDE6C	Phosphodiesterase 6C	-1.509	0.005998	0.0431
FAM83B         Family with sequence similarity 83 member B         -1.476         0.002718         0.0272           FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0052           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0056           MLKL         Mixed lineage kinase domain like pseudokinase         -1.438         0.00212         0.0074	ACSL1	Acyl-CoA synthetase long chain family member 1	-1.499	1.04E-06	0.0012
FABP1         Fatty acid binding protein 1         -1.473         9.25E-05         0.0052           HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0056           MLKL         Mixed lineage kinase domain like pseudokinase         -1.456         0.002283         0.0247           KL         Klotho         -1.438         0.000212         0.0074	FAM83B	Family with sequence similarity 83 member B	-1.476	0.002718	0.0272
HSD17B4         Hydroxysteroid 17-beta dehydrogenase 4         -1.469         0.00012         0.0056           MLKL         Mixed lineage kinase domain like pseudokinase         -1.456         0.002283         0.0247           KL         Klotho         -1.438         0.000212         0.0074	FABP1	Fatty acid binding protein 1	-1.473	9.25E-05	0.0052
MLKL         Mixed lineage kinase domain like pseudokinase         -1.456         0.002283         0.0247           KL         Klotho         -1.438         0.000212         0.0074	HSD17B4	Hydroxysteroid 17-beta dehydrogenase 4	-1.469	0.00012	0.0056
<i>KL</i> Klotho –1.438 0.00212 0.0074	MLKL	Mixed lineage kinase domain like pseudokinase	-1.456	0.002283	0.0247
	KL	Klotho	-1.438	0.000212	0.0074

DEG = differentially expressed genes; NE = necrotic enteritis; FDR = false discovery rate.

# Table 6

Several immune-related genes significantly affected by the necrotic enteritis challenge.

Gene	Gene name	Gene function	log <sub>2</sub> FC
MSRB1	Methionine sulfoxide reductase B1	Innate immune response	-0.718
TLR3	Toll-like receptor 3	Immune system	-0.578
PEX13	Peroxisomal biogenesis factor 13	Fatty acid alpha-oxidation	-0.480
PDK4	Pyruvate dehydrogenase kinase 4	ATP binding, kinase activity	-1.894
INF-γ	Interferon-gamma	Macrophage activation	+2.600
LCK	LCK proto-oncogene, Src family tyrosine kinase	Regulation of T-cell activation	+1.115
TMEM173	Transmembrane protein 173	Innate immune response	+1.217
IL21R	Interleukin 21 receptor	Protein binding	+1.415
ZAP70	Zeta-chain T cell receptor associated protein kinase 70 kDa	Regulation of T-cell differentiation	+1.179
PTPN22	Protein tyrosine phosphatase non-receptor type 22	T-cell differentiation	+1.039

FC = fold change.

# Table 7

Metabolic pathways affected by the necrotic enteritis challenge.

Pathway	<i>P</i> -value	Benjamini	DEG
PPAR signaling Fatty acid degradation	0.00015 0.0017	0.008 0.035	CD36, ACOX2, ACSBG1, ACSL1, EHHADH, FABP1, FABP2, ACAA1, PCK1 ACAA1, ACSL1, EHHADH ACSBG1, ECI2, ALDH2

DEG = differentially expressed genes; PPAR = peroxisome proliferator-activated receptors; CD36 = cluster of differentiation 36; ACOX2 = acyl-CoA oxidase-2; ACSBG1 = acyl-CoA synthetase bubblegum family member-1; ACSL1 = acyl-coenzyme A synthetase-1; EHHADH = enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; FABP1 and FABP2 = fatty acid-binding protein-1 and -2; ACAA1 = acetyl-CoA acyltransferase 1, PCK1 = phosphoenolpyruvate carboxykinase 1; ECl2 = enoyl-CoA delta isomerase-2, ALDH2 = aldehyde dehydrogenase-2.



Fig. 3. Heat map showing the top 100 differentially expressed genes (DEG). Each horizontal row represents a single gene, and each column represents 1 of the 2 experimental groups. Colors indicate a positive (blue) or negative (red) fold-change. The log2 counts per million mapped reads (CPM) values were used to cluster all the presented differentially expressed genes in Java TreeView by hierarchical clustering using Euclidean distance and pairwise average linkage methods.

glucose (Croset et al., 2001; Rajas et al., 2000). Tissue inflammation is an energy-consuming process that increases the energy metabolic rate (Martin et al., 2003), and the fuel needed for such increased demand is glutamine (Wilmore 2001). It is also important to note that the primary source of energy for T-cells is glucose and glutamine (Bental and Deutsch 1993); thus, the downregulation of PCK1 may lead to lower T-cell activity in the body (Ho et al., 2015) resulting in compromised immunity. Another strongly down-regulated gene in the current results is Pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4). Inactivation of PDK4 plays an essential role in cellular energy metabolism (Sugden and Holness 2006) and can also regulate the response of macrophages in immunity activated situations (Min et al., 2019). Park et al. (2018) reported that deficiency of PDK4 in vitro and in vivo can reduce the expression of gluconeogenic genes which can subsequently diminish glucose production. These reports support the finding in the present study that the reduction of PDK4 was associated with the reduced expression of glucagon (GCG), a key hormone stimulating gluconeogenesis and the uptake of glucose. Down-regulation of GCG is also observed in inflammatory bowel disease and the secretion of inflammatory cytokines (Gu et al., 2018). Thus, GCG down-regulation observed in the current study may be an indication of inflammation and a disease condition in general.

# 4.3. Bile acid production and uptake related genes

Among the highest up-regulated genes in the NE challenged birds, was the *ASBT*. This protein is a primary intestinal bile salt transporter, and is a critical component of the enterohepatic circulation of bile acids (Hagenbuch and Dawson 2004). *C. perfringens* is known to have bile acid hydrolyze activity, which can hydrolyze the amide bond of conjugated bile salts and produce free bile salts. Free bile salts that have lower detergent properties for emulsifying fat (Cole and Fuller 1984; Hill 1995), thus, lead to lower fat absorption. On the other hand, the down-regulation of two genes essential in cholesterol and bile acid production, *ACOX2* and *ME1*,

suggest a lower production of cholesterol (Vilarinho et al., 2016). Furthermore, the ACOX2 protein, is believed to mediate the first step in  $\beta$ -oxidation and act as a bile acid intermediate (Baumgart et al., 1996). It should be noted that a portion of the bile salts secreted by hepatocytes are not newly synthesized and have already undergone enterohepatic recycling. Due to the hydrolyses effects of *C. perfringens* on the produced bile acids and the reduced synthesized amount of these substances in the liver, the increased expression of *ASBT* could be a response to enable the body to maximize the reabsorption of bile acids and enable the body to compensate for the possible deficiency of this product.

# 4.4. Immunity related genes

The NE infection significantly up-regulated several immunityrelated genes such as ACOD1, INF- $\gamma$ , Src family tyrosine kinase (LCK) and ZAP70. Among the highly up-regulated genes, ACOD1 is identified as a liposaccharide-inducible gene within macrophages that suggests an important role of this gene in the immune system (Lee et al., 1995). Macrophages form a heterogeneous population of immune cells that can respond efficiently to environmental and microbial signals. Up-regulation of interferon- $\gamma$  (*IFN*- $\gamma$ ) has been previously observed under NE infection (Park et al., 2008; Wu et al., 2018) which is in agreement with the observation in the current study. The INF- $\gamma$  protein capacitates macrophages to release a microbicidal product hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Nathan et al., 1983) which is an effective tool against bacteria, fungi and parasites (Liew et al., 1990). The ACOD1 gene has recently been shown to produce enzymes that act as an antimicrobial peptide and have bacteriostatic effects on some harmful bacteria (Michelucci et al., 2013). It is believed that the expression of this gene is largely dependent on the functional IFN signaling (Naujoks et al., 2016). Furthermore, LCK-null mice have shown blocked T-cell development and it seems that the expression of this gene is the initial event in the activation of T-cell antigen receptor complex (van Oers et al., 1996). The expression of LAT1 has shown to be increased in NE challenged broilers (Gharib-Naseri et al., 2019b), which is also



**Fig. 4.** Visualization for the predicted results of protein–protein interactions of DEG in the jejunum of NE challenged and non-challenged broilers. Each node represents a protein, and each line refers to an interaction. Line thickness reflects the strength of PPI. Node colors represent fold change, and node font sizes and circle sizes of the corresponding genes indicate the number of PPI. The largest cluster mostly represents genes related to the PPAR and  $\beta$ -oxidation pathways. The network is generated using the Cytoscape tool. DEG = differentially expressed genes; PPI = protein–protein interactions; PPAR = peroxisome proliferator-activated receptors.

observed in the current study. Hayashi et al. (2013) reported that *LAT1* is a major transporter for essential amino acid uptake in activated T-cells. Altogether, it seems that the NE infection has upregulated many genes related to T-cells activation. The upregulation of some nutrient transporter genes could be due to the increasing demand for the essential amino acids from the T-cell function.

# 4.5. Skeletal muscle related genes

Negative effects of NE infections are primarily observed in the lower body weight which is related to reduced meat yield compared to the non-infected birds (Xue et al., 2017). Current results have shown that a number of genes related to skeletal muscle were differentially expressed between challenged and non-challenged birds. The genes

Com	parison of	the	gene ex	pression	data	between	RNA-seq	and c	IPCR <sup>1</sup>
			0						

Gene	Fold change		
	qPCR	RNA-seq	
ACOD1	+13.5	+2.65	
ASBT	+58.6	+3.25	
BCL2A1	+5.02	+1.54	
CHPT1	+10.9	+1.32	
DUSP4	+7.14	+1.66	
GZMA	+11.3	+1.65	
IL21R	+7.92	+1.41	
LAT1	+6.18	+1.46	
ZAP70	+8.02	+1.06	
ACOX2	-5.95	-1.04	
ALAS1	-2.75	-1.10	
ALDH1A1	-4.35	-1.90	
CD36	-14.0	-1.85	
GCG	-2.86	-1.12	
FABP1	-2.52	-1.48	
FABP2	-5.16	-1.61	
ME1	-3.80	-1.78	
MYOM2	-11.2	-2.93	
PEX13	-4.20	-1.32	
PRKG2	-11.2	-2.93	

qPCR = quantitative PCR ; ACOD1 = aconitate decarboxylase 1; ASBT = apical sodium—bile acid transporter; BCL2A1 = BCL2 related protein A1; CHPT1 = choline phosphotransferase-1; DUSP4 = dual-specificity phosphatase-4; GZMA = granzyme A; IL21R = interleukin-21 receptor; LAT1 = L-type amino acid transporter-1; ZAP70 = zeta chain of T cell receptor associated protein kinase 70 kDa; ACOX2 = acyl-CoA oxidase-2; ALAS1 = 5'-aminolevulinate synthase-1; ALDH1A1 = aldehyde dehydrogenase-1 family member A1; CD36 = cluster of differentiation 36; GCG = glucagon; FABP1 and FABP2 = fatty acid-binding protein-1 and -2; ME1 = malic enzyme; PEX13 = peroxisomal biogenesis factor-13; PRKG2 = protein kinase cGMP-dependent 2.

<sup>1</sup> For the qPCR gene expression normalization, *SDH* and *TBP* genes were used as reference genes. Positive signs show upregulation, and negative signs show downregulation of genes in necrotic enteritis challenged birds.

PRKG2 and MYOM2 were among the strongly down-regulated genes, while MYBPC1 was strongly up-regulated in the NE challenged birds. The PRKG2 encodes a protein connected to organized columnar growth plates, and the knockout of this gene can leading to dwarfism in both mice and cattle (Koltes et al., 2009; Pfeifer et al., 1996). MYOM2 is a myosin-binding protein expressed in muscle sarcomeres (Van Der Ven et al., 1996), and has a critical role in muscle development in broilers (Liu et al., 2016). However, MYOM2 is also known to be associated with the immune system because it is an intracellular member of the immunoglobulin superfamily (Van Der Ven et al., 1996). Liu et al. reported that blocking *TNF*- $\alpha$  activity up-regulated *MYOM2* expression. Furthermore, MYBPC1 belongs to the myosin-binding protein C family of proteins that are expressed in straight muscles (Ackermann et al., 2013) and plays an important structural and regulatory role in muscle function (James and Robbins 2011). It has been shown that the expression of *MYBPC1* is counteracted by *TNF-* $\alpha$  in skeletal muscles (Meyer et al., 2015). The altered expression of these genes could have also affected the muscle growth and could be a reason for poor growth in the challenged birds. However, because RNA samples were obtained from the intestinal tissue, these changes are only the reflection of intestinal muscle development, and in some genes related to muscles, the changes can be due to the tissue damage caused by the infection. Further investigation is needed to identify the exact role of these genes and detect if expression changes of these genes are also observed in the skeletal muscles of challenged birds or not.

# 5. Conclusion

In conclusion, the transcriptomic analysis on the intestinal tissue of NE challenged birds showed significant changes in the expression of genes involved in their respective metabolic pathways that cause distinct responses to the disease. The analysis also revealed that fatty acid metabolism and absorption are widely affected by the infection. Immunity genes, especially those related to macrophage activity, were activated in infected birds. This study has taken a further step in identifying the responses of NE infected broilers at transcriptomic level, which can help develop therapeutic treatments by targeting the relevant pathways in the host and thereby aiding poultry producers to control this disease in the postantibiotic era. There are many remaining questions concerning the regulation of lipid metabolism and the immune system, and thus further investigations are warranted for a better understanding of how the predisposing factors and NetB-producing *C. perfringens* modulate the expression of the genes and respective pathways and their functions.

# **Author contributions**

Kosar Gharib-Naseri: Data curation, Formal analysis, Investigation, Writing- Original draft preparation; Sara de Las Heras-Saldana: Data curation, Formal analysis, Methodology, Investigation, Reviewing and Editing; Sarbast Kheravii: Methodology, Investigation, Data curation, Reviewing and Editing. Lihong Qin: Investigation, Data curation, Reviewing and Editing. Jingxue Wang: Investigation, Data curation, Reviewing and Editing. Shu-Biao Wu: Conceptualization, Data curation, Investigation, Project administration. Reviewing and Editing.

# **Conflict of interest**

We declare that we have no financial or personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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# Appendix supplementary data

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