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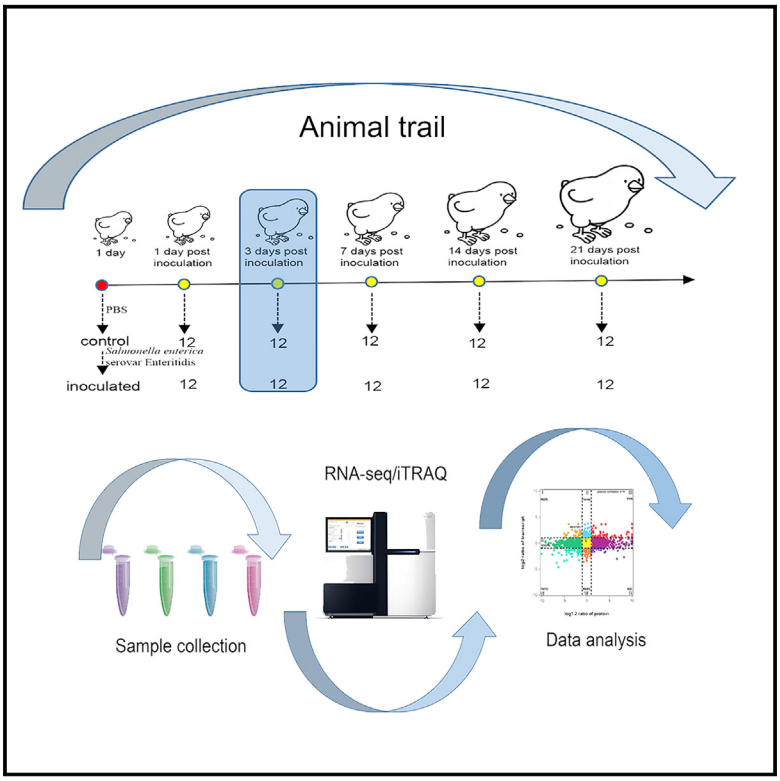
Transcriptomic and proteomic analysis reveals the mechanism of chicken cecum response to *Salmonella enterica* serovar Enteritidis inoculation

Graphical abstract



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In brief

Poultry microbiology; Biochemistry; Molecular biology; Microbiology.

Highlights

- The transcriptome and proteome landscape of chicken cecum following SE inoculation
- SE inoculation induced the chicken immune system, but impaired the metabolic system
- Weak correlation indicates post-transcriptional regulation of protein

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Transcriptomic and proteomic analysis reveals the mechanism of chicken cecum response to *Salmonella enterica* serovar Enteritidis inoculation

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SUMMARY

Salmonella enterica serovar Enteritidis (SE) incurs foodborne illnesses and poses a severe threat to poultry industry and human health. However, the molecular mechanisms underlying chicken responding to SE inoculation remain elusive. Here, we characterized the transcriptome and proteome of chicken cecum 3 days post SE inoculation. Totally, there were 332 differentially expressed genes and 563 differentially expressed protein identified. The upregulated genes were enriched in immune-related processes. The downregulated proteins mainly correlated with metabolic process. The correlation coefficient between the transcriptome and proteome was 0.14. Collectively, we characterized the landscape of mRNAs and proteins in chicken cecum following SE inoculation and found SE inoculation induced chicken immune system at transcriptomic level but impaired the metabolism at protein level. The differences may be caused by complex post-transcriptional regulatory mechanisms or time-dependent delays. Our findings would extend the understanding of the molecular mechanisms underlying chicken responding to SE inoculation.

INTRODUCTION

Salmonella enterica serovar Enteritidis (SE), a major foodborne pathogen, has the capability to disrupt intestinal barrier function, alter the homeostasis of intestinal microbiota, and trigger inflammatory responses in humans.[1] It was estimated that one million illnesses are caused by foodborne salmonellosis, and 400 deaths annually in the United States.[2] SE is one of the dominant pathogens responsible for salmonellosis. In China, there were 26.82% foodborne infections caused by SE.[3] Poultry is considered as a primary source of foodborne diseases caused by SE inoculation.[4] Human are primarily infected with SE through consuming contaminated poultry, meat, or eggs. Egg-associated salmonellosis ranked among the top five causes of outbreak-associated hospitalizations and deaths in the United States in 2017.[5] It is reported that egg-related salmonellosis costs 44 million dollars in Australia each year.[6] SE poses a threat to the poultry industry and human health. Moreover, vaccination and antibiotics have not been efficient to eradicate SE in the poultry industry. Accumulating studies clarified that genetic selection is an efficient method for improving resistance to SE inoculation in chickens.[7,8]

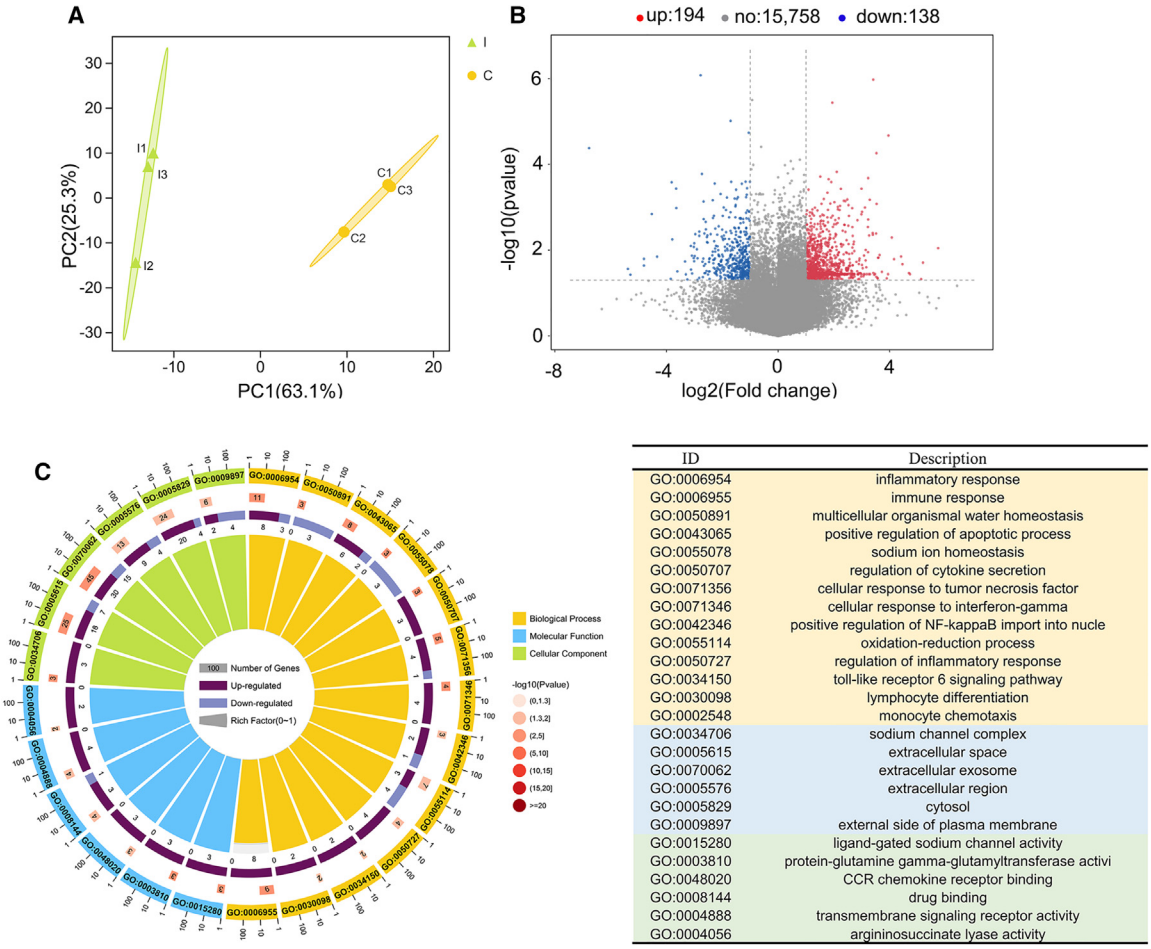
Animals would respond to biotic and abiotic stresses by altering gene or protein expression.[9–11] Toll-like receptors (TLRs) could initiate inflammatory response through detecting SE inoculation in chickens.[12] Following the initial recognition of Salmonella by epithelial cells and resident lymphocytes, pro-inflammatory cytokines and immune related genes including *IL1β*, *IL6*, *IL17*, *IL22*, *IFNγ*, and *iNOS* were induced in chicken cecum.[13] Moreover, multiple metabolic and immune related genes were altered in chicken liver,[14] spleen,[15] and cecum[16,17] following SE infection. Gene functions are ultimately realized in the form of proteins.[18] Proteome analysis can effectively provide accurate quantitative and structural modification information about functional proteins.[19] Polansky et al.[20] found that the decreased proteins in chicken liver were involved in glycolysis, the citrate cycle, oxidative phosphorylation, and fatty acid metabolism following SE infection. The proteins associated with inflammatory diseases, cell differentiation, and transmembrane transport were upregulated in chickens post SE inoculation.[21] However, few studies focused on the genome-wide expression of proteins in chicken cecum following SE infection. Additionally, the correlation between mRNA and protein abundances in cells is notoriously poor.[22] Integrative analysis of mRNA and proteins has the potential to help us better understand gene regulation, genome annotation, and the intricate biological processes underlying disease manifestations. Recent advancements in high-throughput sequencing have facilitated the widespread use of multi-omic

Table 1. Summary of RNA sequencing data

Sample	Total reads	Raw data	Mapped reads	Clean data	Q20%	Q30%	GC content (%)
Control1	56,967,996	8.55G	55,565,416	8.33G	99.78	96.02	47
Control2	52,931,950	7.94G	51,568,782	7.74G	99.61	94.87	48
Control3	60,155,278	9.02G	58,708,200	8.81G	99.65	95.14	48
Inoculated1	49,185,124	7.38G	47,981,130	7.20G	99.56	93.91	48
Inoculated2	53,713,626	8.06G	52,277,682	7.84G	99.73	95.55	47
Inoculated3	55,477,488	8.32G	54,132,526	8.12G	99.75	95.85	48

approaches to uncover the mechanisms underlying disease resistance in humans,[23] plants,[24,25] and animals.[26,27] This may provide an effective approach to explore the mechanism of chickens in response to SE infection.

Salmonella mainly colonizes the chicken cecum, invades the intestinal epithelial and dendritic cells, and reaches the submucosal layer.[28] Subsequently, the interaction of Salmonella with chicken macrophages and heterophils triggers an immune



ID	Description
GO:0006954	inflammatory response
GO:0006955	immune response
GO:0050891	multicellular organismal water homeostasis
GO:0043065	positive regulation of apoptotic process
GO:0055078	sodium ion homeostasis
GO:0050707	regulation of cytokine secretion
GO:0071356	cellular response to tumor necrosis factor
GO:0071346	cellular response to interferon-gamma
GO:0042346	positive regulation of NF-kappaB import into nucle
GO:0055114	oxidation-reduction process
GO:0050727	regulation of inflammatory response
GO:0034150	toll-like receptor 6 signaling pathway
GO:0030098	lymphocyte differentiation
GO:0002548	monocyte chemotaxis
GO:0034706	sodium channel complex
GO:0005615	extracellular space
GO:0070062	extracellular exosome
GO:0005576	extracellular region
GO:0005829	cytosol
GO:0009897	external side of plasma membrane
GO:0015280	ligand-gated sodium channel activity
GO:0003810	protein-glutamine gamma-glutamyltransferase activi
GO:0048020	CCR chemokine receptor binding
GO:0008144	drug binding
GO:0004888	transmembrane signaling receptor activity
GO:0004056	argininosuccinate lyase activity

Figure 1. The description of the identified genes

(A) Principal components analysis (PCA) of the identified genes in the C group (control group) and I group (*Salmonella enterica* serovar Enteritidis inoculated group).

(B) The number of the differentially expressed genes (DEGs) between the control and inoculated groups. The red dot represented the upregulated gene, the blue dot represented the downregulated gene.

(C) The enriched Gene Ontology (GO) terms of the DEGs. From outer to inner, the outermost circle represents the IDs of enriched GO terms. The names of GO ID in orange, blue and green represents biological process, molecular function and cell composition, respectively. The second circle represents the GO terms enriched by the shared genes. In the third circle, the piece in dark purple and light purple represents upregulated genes and downregulated genes, respectively. In the innermost circle, each bar represents one GO term, and the size represented the rich factor.

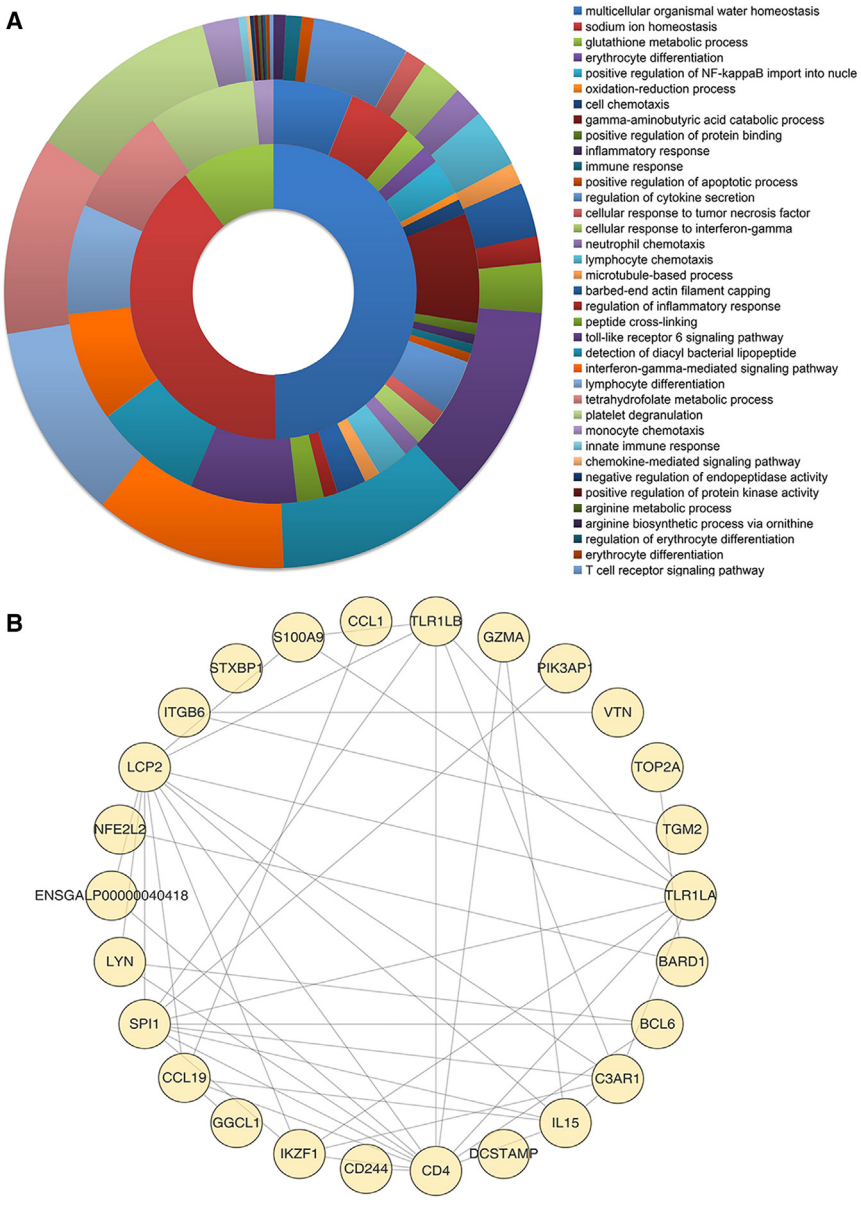


Figure 2. The enriched biological processes (BP) of the differentially expressed genes (DEGs)

(A) From outer to inner, the outermost circle represents the enriched BP terms of the DEGs; the second circle indicates the enriched BP terms of the upregulated genes; the third circle, represents the enriched BP terms of downregulated genes.

(B) The interaction network of immune related DEGs.

response in the chicken.[12] Whereas, the mechanism underlying chickens responding to SE inoculation have not been characterized clearly. In the current study, the landscape for transcriptome and proteome of chicken cecum was characterized following SE infection. These results would provide the comprehensive co-evaluation of transcriptomic and proteomic regulation of chicken cecum and candidate molecules responsible for SE infection.

RESULTS

Characteristics of transcriptome data

After removing low-quality reads, 48.04 Gb of clean data were generated. The average percentage of bases with a Q30 was >93% in each group (Table 1). The number of clean reads was 56,967,996, 52,931,950, and 60,155,278 in the control group and 49,185,124, 53,713,626, and 55,477,488 in the infected group. The average CG content was 47.7%.

The differentially expressed genes between the inoculated and control group

Principal component analysis (PCA) was conducted to visualize differences in gene expression among the groups. The chickens in the control group were clearly distinguished from those in the infected group (Figure 1A). A total of 16,090 genes were identified in chicken cecum (Figure 1B). Of those, 332 differentially expressed genes (DEGs), including 194 upregulated genes and 138 downregulated genes, were identified (Table S1). The heatmap based on the expression of DEGs across the six samples indicated that all DEGs were clustered into two groups (Figure S1). In Group A, expression of genes in the infected group was lower than that in the control group. In Group B, gene expression was higher in the infected group but lower in the control group.

Functional annotation of the differentially expressed genes

Gene Ontology (GO) enrichment results revealed that 332 DEGs were enriched in 46 GO terms ($p < 0.05$) including 28 biological processes (BP), 9 cellular components (CC), and 9 molecular functions (MF) (Figure 1C, Table S2). For MF category, DEGs were associated with CCR chemokine receptor binding activity and transmembrane signaling receptor binding. For CC category, the DEGs were significantly enriched in the sodium channel complex, extracellular space, and extracellular exosomes. In the BP category, the DEGs were primarily involved in inflammatory response, immune response, TLR 6 signaling pathway, and positive regulation of the apoptotic process. Notably, the number of upregulated genes was larger than that of downregulated genes in most GO terms. Regarding the biological process, the DEGs were mainly associated with the TLR 6 signaling pathway, detection of diacyl bacterial lipopeptide, cellular response to

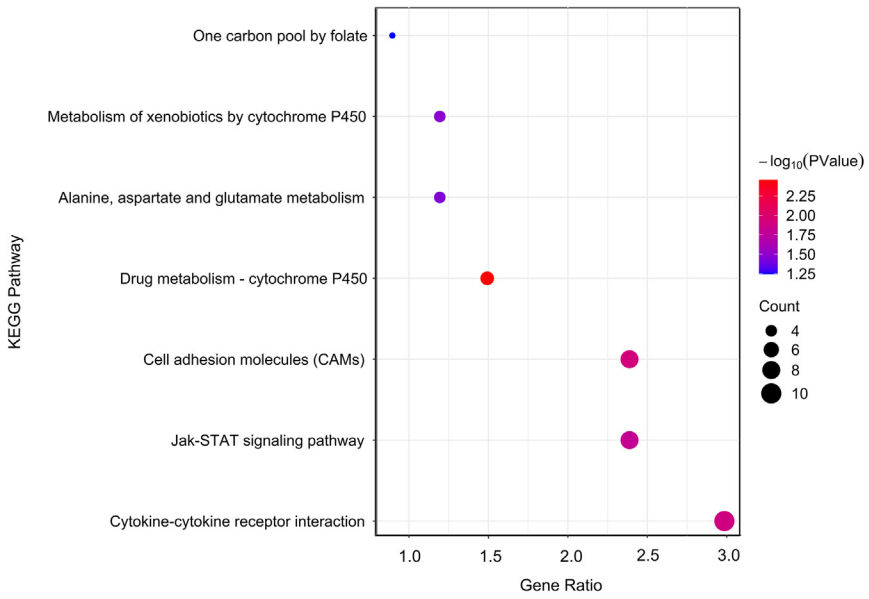


Figure 3. The enriched Kyoto Encyclopedia of Genes and Genomes database (KEGG) pathway of the differentially expressed genes

interferon-gamma, regulation of inflammatory response, cellular response to tumor necrosis factor, inflammatory response, innate immune response, and T cell receptor signaling pathway (Figure 2A), which included 26 upregulated DEGs (Table S3). Among these DEGs, 24 were interactive (Figure 2B). *Toll-like receptor 1 family member A* (*TLR1LA*) and *toll-like receptor 1 family member B* (*TLR1LB*) were significantly enriched with toll-like 6 receptor signaling pathway. The downregulated DEGs were involved in multicellular organism water homeostasis, sodium ion homeostasis, and glutathione metabolic process.

Kyoto Encyclopedia of Genes and Genomes (KEGG) database analysis revealed that DEGs were significantly enriched in six pathways ($p < 0.05$): drug metabolism-cytochrome P450, cell adhesion molecules (CAMs), cytokine-cytokine receptor interaction, JAK-STAT signaling pathway, and metabolism of xenobiotics by cytochrome P450 and alanine, aspartate, and glutamate metabolism (Figure 3). Eight DEGs, including *oncostatin M receptor* (*OSMR*), *LIF receptor subunit alpha* (*LIFR*), *cytokine inducible SH2 containing protein* (*CISH*), *signal transducer and activator of transcription 1* (*STAT1*), *interleukin-2 receptor subunit gamma* (*IL2RG*), *interleukin 15* (*IL15*), *interleukin-22 receptor subunit alpha 2* (*IL22RA2*) and *interleukin 13 receptor subunit alpha 2* (*IL13RA2*), were involved in the JAK-STAT signaling pathway (Table S4).

Identification of the differentially expressed proteins

A total of 22,638 peptides were identified (Table S5), with the majority length range of 7–27 amino acids (Figure 4A). A total of 3,473 proteins were characterized in the control and infected groups. With fold change (FC) > 1.5 and $p < 0.05$, 563 differentially expressed proteins (DEPs) were identified, including 225 upregulated proteins and 338 downregulated proteins (Figure 4B).

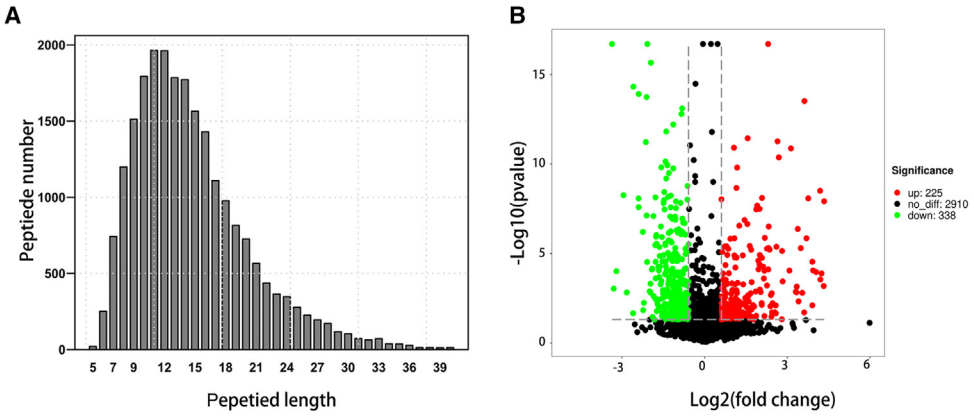
Function enrichment of the differentially expressed proteins

The DEPs were significantly enriched in 132 GO terms (56 BP, 44 CC, and 32 MF) (Table S6). For BP category, the DEPs were primarily clustered into three categories: (1) metabolic-related GO terms, such as tricarboxylic acid cycle, oxidation-reduction process, fatty acid beta-oxidation, mitochondrial electron transport, cytochrome *c* to oxygen and fatty acid metabolic process; (2) immune-related GO terms, such as defense response to bacterium, acute-phase response, and positive regulation of protein secretion; and (3) other GO terms like epithelial cell differentiation, cell-cell adhesion, and cell redox homeostasis regulation of cell shape (Figure 5). For CC category, the DEPs were mainly associated with three categories: (1) mitochondrion-related terms such as mitochondrial inner membrane, mitochondrial nucleoid, and mitochondrial respiratory chain complex I; (2) extracellular region-related GO terms such as extracellular exosome, extracellular matrix, and extracellular space; and (3) other GO terms such as focal adhesion and immunological synapse. In the MF category, the DEPs were associated with ATPase activity, ATPase activity, NAD binding, electron carrier activity, fatty-acyl-CoA binding, and poly(A) RNA binding. Notably, most DEPs in the enriched GO terms were downregulated. Given the importance of the oxidation-reduction process, we identified 20 DEPs involved in this process (Table S7). In particularly, *peroxiredoxin 6 (PRDX6)* and *peroxiredoxin 1 (PRDX1)* were involved in various metabolic pathway (Figure 6).

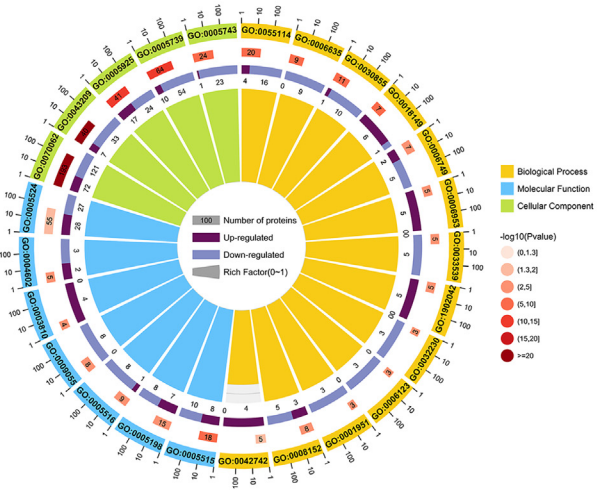
The DEPs were significantly enriched in 28 pathways ($p < 0.05$), such as metabolism-related pathways, including the biosynthesis of antibiotics, carbon metabolism, PPAR signaling pathway, tricarboxylic acid cycle, fatty acid degradation, and oxidative phosphorylation (Figure 6A). Eighteen DEPs in the gene families of UQCRC, COX, ATP, and NDUF, were enriched in the oxidative phosphorylation pathway (Table S8). Moreover, the number of downregulated proteins was higher than that of upregulated proteins. The downregulated proteins were mainly associated with metabolic pathways. The upregulated proteins were enriched only in three pathways: RNA transport, ribosome, and protein processing in the endoplasmic reticulum (Figure 6B).

Relationship between transcriptome and proteome data

The Spearman correlation coefficient between protein and corresponding mRNAs expression was 0.14. In this study, genes were highly enriched in the fifth quadrant, followed by the fourth and



C



ID	Description
GO:0055114	oxidation-reduction process
GO:0006635	fatty acid beta-oxidation
GO:0030855	epithelial cell differentiation
GO:0018149	peptide cross-linking
GO:0006749	glutathione metabolic process
GO:0006953	acute-phase response
GO:0033539	fatty acid beta-oxidation using acyl-CoA dehydrogenase
GO:1902042	negative regulation of extrinsic apoptotic signaling pathway via death domain receptors
GO:0032230	positive regulation of synaptic transmission, GABAergic
GO:0006123	mitochondrial electron transport, cytochrome c to oxygen
GO:0001951	intestinal D-glucose absorption
GO:0008152	metabolic process
GO:0042742	defense response to bacterium
GO:0070062	extracellular exosome
GO:0043209	myelin sheath
GO:0005925	focal adhesion
GO:0005739	mitochondrion
GO:0005743	mitochondrial inner membrane
GO:0005515	protein binding
GO:0005198	structural molecule activity
GO:0005516	calmodulin binding
GO:0009055	electron carrier activity
GO:0003810	protein-glutamine gamma-glutamyltransferase activity
GO:0004602	glutathione peroxidase activity
GO:0005524	ATP binding

Figure 4. The identified peptides and proteins

(A) The length of identified peptides.

(B) The identified proteins in the control and inoculated group. The red dot represented upregulated proteins in the inoculated group, and the green dot represented downregulated proteins in the inoculated group.

(C) Significant Gene Ontology (GO) terms of differentially expressed proteins. From outer to inner, the outermost circle represents the IDs of enriched GO terms. The names of GO ID in orange, blue and green represent biological process, molecular function and cell composition respectively. The second circle indicates all differentially expressed proteins enriched in GO terms. In the third circle, the piece in dark purple and light purple represents upregulated and downregulated proteins, respectively. In the innermost circle, each bar represents one GO term, and the size represents the rich factor.

sixth quadrants (Figure 7). Proteins enriched in the first, second, and fourth quadrants showed lower abundances than the related RNA, but showed higher abundances than related RNA in the sixth, eighth, and ninth quadrants. Moreover, we found that the expression of genes was consistent with that of the proteins in quadrants III and VII. The expression of genes and proteins in quadrants I and IX showed opposite trends. The genes and proteins in quadrants I and IV were mainly associated with immunological synapses, negative regulation of cell adhesion, and positive regulation of protein secretion (Table S9). However, most genes and proteins in quadrants III and IX were involved in interleukin-27-mediated signaling pathway, ATPase activity, peroxiredoxin activity, thioredoxin peroxidase activity, and NAD+ binding (Table S10). The proteins in the fourth and sixth quadrants were mainly involved in metabolic pathways and oxidative reduction processes (Table S11 and 12). In these two-quadrants the proteins were differentially expressed, but the related genes showed no difference.

RT-qPCR and western blotting

The expression of randomly selected genes was determined using RT-qPCR with the primers listed in Table 2. Significant difference for the expression of PRDX6, CCL4, TLR1B, and TLR1A between the control group and the inoculated group was observed, which were consistent with those of RNA-seq, indicating that the RNA-seq data were reliable (Figure 8A). The abundance of *PRDX6* and *β-actin* was analyzed by western blotting. *PRDX6* was more abundant in the control group (Figure 8B), which was consistent with the proteome data.

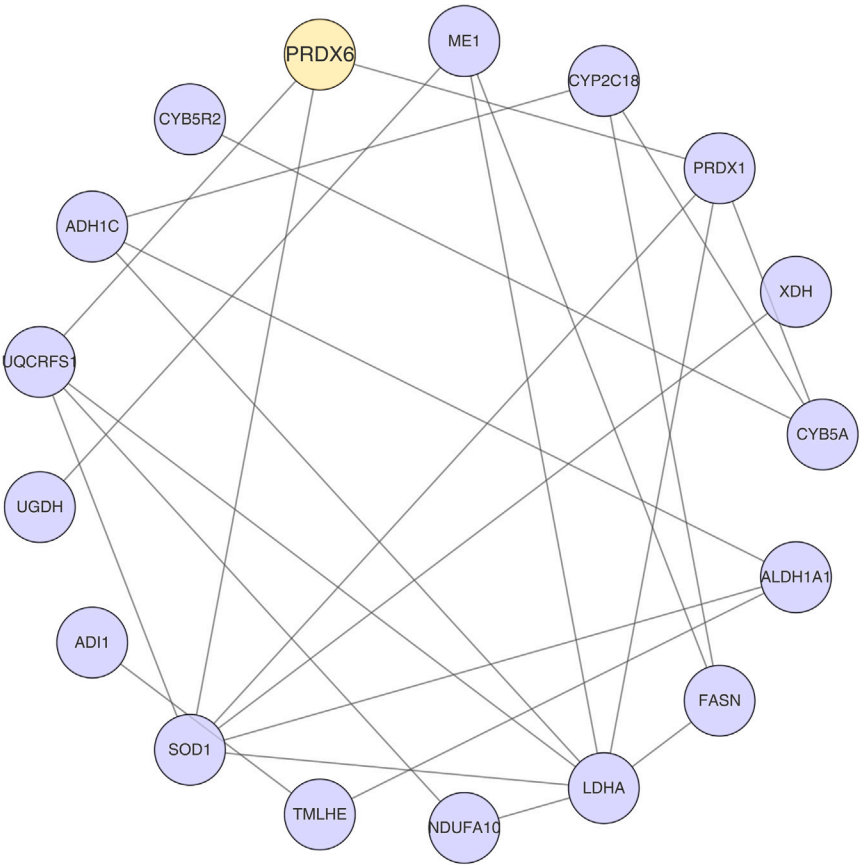


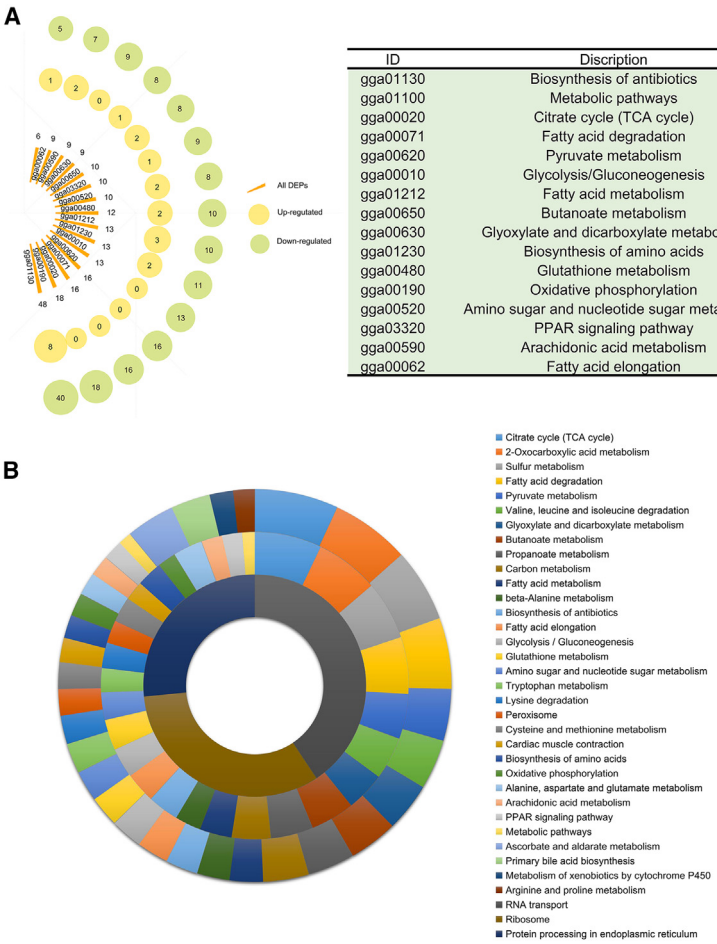
Figure 5. The protein-protein interaction network of the differentially expressed proteins in metabolism-related pathways

DISCUSSION

SE, one of the most common Salmonella serotypes reported worldwide, is the primary source of human intestinal inoculation.[29] Previous studies have identified several genes and proteins involved in SE inoculation in chickens.[8] Moreover, changes in the host transcriptome and proteome following pathogen inoculation may contribute to uncovering the mechanisms of pathogenesis.[24] We combined RNA-seq and iTRAQ profiling of chicken cecum to explore the molecular mechanisms underlying the response to SE infection.

In the current study, a total of 332 DEGs, including 194 upregulated genes and 138 downregulated genes, were identified following SE infection. Moreover, the upregulated DEGs were assigned to immune-related GO processes, such as the TLR 6 signaling pathway, regulation of inflammatory response, innate immune response, and JAK-STAT signaling pathway, which was consistent with previous studies focused on chicken spleen, liver, and cecum in response to SE inoculation.[14,21,30] These results indicate that chickens respond to SE inoculation by altering their immune system.[31–33] Immune responses are highly energy-dependent processes, and energy metabolism is involved in immune networking for self-defense and against pathophysiology.[34] Several recent reviews[35–38] demonstrated that metabolism plays a critical role in controlling immunity and inflammation and, in turn, immunity has a profound impact on metabolism at the cellular, tissue, and organismal levels. Li et al.[15] found that the interaction between the immune system and metabolism contributes to the response of laying hens to SE inoculation at the onset of laying. Here, we found that the downregulated DEPs were mainly associated with metabolic related pathways such as oxidation-reduction process, mitochondrial electron transport, fatty acid degradation, and oxidative phosphorylation. Previous studies have also found that DEPs in chicken macrophages, heterophils, liver, and blood serum respond to SE inoculation by regulating metabolic processes.[20,39] The response of chickens to SE infection is a dynamic process that involves both immune and metabolic processes.[40] Notably, gene expression at the transcriptomic and translational level is not always consistent. Joint analysis of transcriptome and proteome data can provide more comprehensive gene expression information.[41] Our integrated analysis showed a weak correlation between proteome and transcriptome. Similar results were characterized in transcriptome-proteome comparison studies focusing on the growth and development of *Camellia oleifera*,[42] annelid *Platynereis dumerilii*,[43] and goats.[44] These studies suggest that the differences in transcriptome and proteome may be caused by complex post-transcriptional regulatory mechanisms or time-dependent delays following SE inoculation in chickens.

TLRs are responsible for detecting microbial pathogens by identifying evolutionarily conserved molecular motifs of infectious microbes and activating signaling pathways that result in an immune response against microbial inoculation.[45–47] Here, the toll-like 6 signaling pathway was enriched by the TLR family, including *TLR1LA* and *TLR1LB*. Young chickens would respond to SE inoculation through altering the expression of *TLR1LA*, *TLR1LB*, *TLR2*, and *TLR4*.[17,48,49] Moreover, TLRs would trigger innate immune responses mainly by regulating the nuclear factor-κB (NF-κB)-dependent signaling pathway.[50] Accordingly, the positive regulation of NF-κB import into the nucleus was also significantly enriched by the upregulated genes. Therefore, we inferred that SE may be initially identified in the chicken cecum by *TLR1A* and *TLR1B*. Following SE recognition, innate immune responses are activated, mainly by regulating the nuclear NF-κB-dependent signaling pathway. We found that the inflammatory response was significantly enriched by *C-C motif chemokine ligand 1* (*CCL1*), *C-C motif chemokine ligand 4*



ID	Discription
gga01130	Biosynthesis of antibiotics
gga01100	Metabolic pathways
gga00020	Citrate cycle (TCA cycle)
gga00071	Fatty acid degradation
gga00620	Pyruvate metabolism
gga00010	Glycolysis/Gluconeogenesis
gga01212	Fatty acid metabolism
gga00650	Butanoate metabolism
gga00630	Glyoxylate and dicarboxylate metabolism
gga01230	Biosynthesis of amino acids
gga00480	Glutathione metabolism
gga00190	Oxidative phosphorylation
gga00520	Amino sugar and nucleotide sugar metabolism
gga03320	PPAR signaling pathway
gga00590	Arachidonic acid metabolism
gga00062	Fatty acid elongation

Figure 6. Kyoto Encyclopedia of Genes and Genomes database (KEGG) pathway annotation of the differentially expressed proteins (DEPs)

(A) The enriched pathways of DEPs.

(B) The enriched KEGG pathways of the DEPs. From outer to inner, the outermost circle represents the enriched pathway of DEPs; the second circle indicates the enriched pathways of the downregulated proteins; the third circle represents the enriched pathways of the upregulated proteins.

(*CCL4*), *C-C motif chemokine ligand 19* (*CCL19*), *B-cell CLL/lymphoma 6* (*BCL6*), *TLR1A* and *TLR1B*, which were upregulated in the chicken cecum. These genes are involved in the immune response by regulating the migration of immature lymphoid progenitor cells, recirculation of mature naive T cells and lymphocytes, and inhibiting the proliferation of various bacteria or viruses.[51,52] Therefore, the identification of SE inoculation by TLRs may trigger the rapid activation of innate immunity by inducing production of pro-inflammatory molecules and costimulatory molecules in the chicken cecum.

The JAK-STAT pathway is the principal signaling mechanism for multiple cytokines and growth factors, and provides a direct mechanism for translating extracellular signals into transcriptional responses. Activation of this pathway stimulates cell proliferation, differentiation, migration, growth, survival, apoptosis, and pathogen resistance in silkworms infected with *Beauveria bassiana*.[53] However, the suppression of JAK-STAT pathway would result in dysfunction of B cells and T cells, and caused severe immune-deficiency in humans.[54] In the current study, JAK-STAT signaling pathway was significantly enriched by DEGs including *IL2RG*, *IL15*, *IL13RA2*, *IL22RA2*, *OSMR*, *LIFR*, *CISH*, and *STAT1*. The overexpression of pro-inflammatory cytokines such as *IL2RG*, *IL13RA2*, *IL15*, and *IL22RA2* could drive immune activation through regulating cell growth, cell activation, differentiation, and homing of the immune cells to the sites of infection to control and eradicate the intracellular pathogens in testis and cecum.[55–57] These genes were significantly enriched in JAK-STAT signaling pathway, suggesting that they may act upstream factors to affect the downstream genes involved in the response to SE infection. Accordingly, *STAT1* and *OSMR* play key roles in the immunoglobulin class-switch recombination through controlling the differentiation and maturation of T-cells.[58,59] The upregulation of *STAT1* and *OSMR* elicits an immune response via the interferon pathway following pathogen infection in mice and chickens.[60,61] In our study, these genes were upregulated, indicating that the JAK-STAT signaling pathway was induced in chickens following SE infection.[62,63]

The immune response is a highly energy dependent process, and energy metabolism is also involved in the immune network for self-defense and against pathogenesis.[34] T cells rely on oxidative phosphorylation to sustain energy demands[64] and oxidative phosphorylation provides more than 95% of one cell's energy in the form of ATP that organisms use to support life and maintain metabolic homeostasis.[65] In this study, the DEPs were involved in oxidative phosphorylation pathway. Polansky et al.[20] reported that the decreased proteins in chicken liver were involved in oxidative phosphorylation following SE infection, which was consistent with our current study. Therefore, we speculated oxidative phosphorylation pathway may be key maker for chicken responding to SE infection. However, Sekelova et al.[66] found SE infection would induce oxidative phosphorylation in chicken cecum at 4 days post inoculation (dpi). The oxidative phosphorylation was the key signature in chicken cecum infected with SE.[67] Hence, the proteins involved in oxidative phosphorylation may be essential maker in detecting the SE infection. Further experiments should be performed to explore these molecular makers.

In summary, the response of chickens to SE inoculation is a complex and dynamic process. SE inoculation induced an immune response at the transcriptomic level but impaired metabolic processes in the chicken cecum. The immune system processes and the TLR 6 signaling pathway were induced, whereas the metabolic processes like oxidative phosphorylation, oxidation-reduction process were reduced. *TLR1A* and *TLR1B*

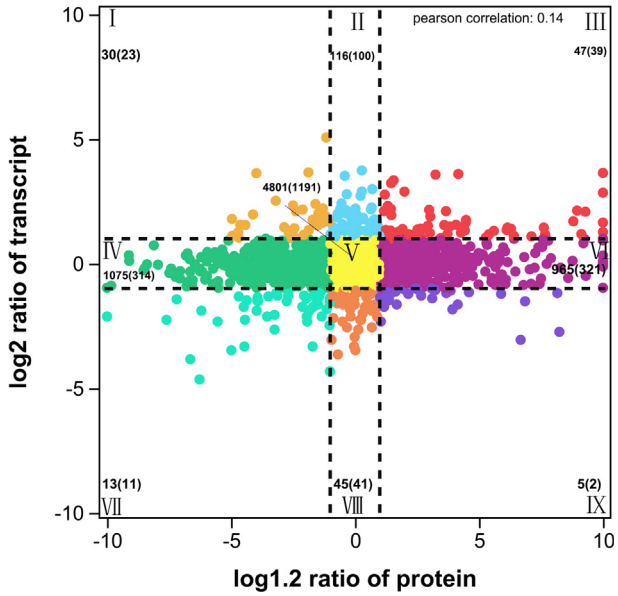


Figure 7. Nine-quadrant associate analysis

Scatterplot of 9-quadrant associated analyses of mRNA and proteins from log2 FC and log1.2 FC. Number I-IX, quadrant NO. The number mRNAs and proteins were shown in each quadrant (in parentheses).

play critical roles in driving the response of chickens to SE infection. Our findings will provide novel insights into the molecular mechanisms underlying the chickens' response to SE infection.

Limitations of the study

Limitations of our study include that the SE model mainly focused on chickens; we should check the response of different type cells to SE inoculation. Moreover, we plan to conduct further experiments on revealing the molecular mechanism contributing to the difference between the transcriptome and proteome in chickens following SE inoculation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Xianyao Li (xyli@sdau.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The transcriptome data are available in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) at NCBI, with the SRA Accession Number: SRR17670781-SRR17670786. The proteome data have been deposited in iProX/ProteomeXchange under accession number: IPX0005204001.

This paper does not report the original code.

Any additional information required to analyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

X.L. and L.L. conceived and designed the experiments and revised the manuscript. Y.W. and Y.Z. performed the experiments. Y.W. analyzed the data and wrote the manuscript. X.M., Y.D., Y.R., and L.L. were involved in sample collection. X.L. participated in the revision of manuscript.

DECLARATION OF INTERESTS

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

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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Table 2. Primer sequences and product size

Gene symbol	Ensemble ID	Sequence (5′-3′)	Product size
beta-actin	ENSGALG00000009621	F: TGCTGTGTTCCCATCTATCG R: TTGGTGACAATACCGTGTTCA	150 bp
CCL4	ENSGALG00000034478	F: CCTCGCTGTCCTCCTCATT R: CACTGGCTGTTGGTCTCGT	147 bp
TLR1A	ENSGALG00000017485	F: ATGACCAGCCGTATGAAATC R: TGCGTTCCGCTCAAGTC	261 bp
TLR1B	ENSGALG00000027093	F: AAACTCCCTCCTCCACCTTG R: ACATCCGTCTTTGTTCTAATC	312 bp
PRDX6	ENSGALG00000003053	F: TCCGCTTCCACGACTTCCT R: CGTCCTTGTCCCGCTCAT	152 bp

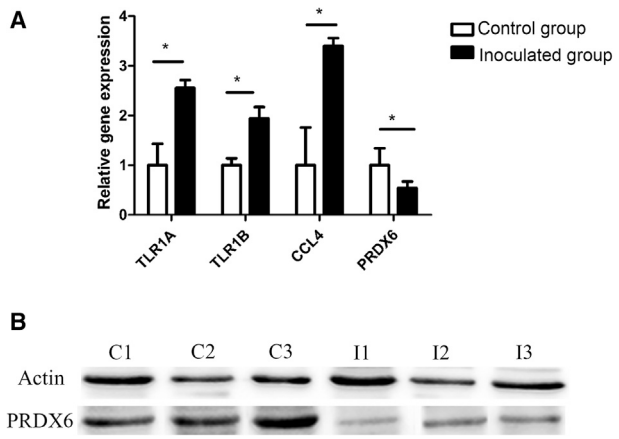

Figure 8. The results of RT-qPCR and western blotting

(A) The fold change of the expression for the differentially expressed genes tested with qRT-PCR and RNA-seq. Data were represented as mean ± SD.
(B) The expression of PRDX6 in the Control (C) and Inoculated (I) group tested with western blot.

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Peroxiredoxin-6 Rabbit pAb	Beijing Bioss biotechnology company	Cat# bs-23508R
Goat Anti-Rabbit IgG H&L, HRP conjugated	Beijing Bioss biotechnology company	Cat# bs-0295G-HRP
Beta-Actin Rabbit pAb	Beijing Bioss biotechnology company	Cat# bs-0061R
Bacterial and virus strains		
Salmonella enterica serovar Enteritidis	The China Veterinary Culture Collection Center	CVCC3377
Biological samples		
Jining Bairi chicken	Shandong Bairi Chicken Breeding Co., Ltd (Salmonella enterica serovar Enteritidis)	
Deposited data		
Transcriptome data	This paper	Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) at NCBI with the SRA Accession Number: SRR17670781-SRR17670786.
Proteome data	This paper	iProX:IPX0005204001
Software and algorithms		
HISAT 2.0	Kim et al.[68]	https://daehwankimlab.github.io/hisat2/
StringTie	Pertea et al.[69]	https://github.com/gpertea/stringtie
R packge Ballgown	Frazee et al.[70]	https://bioconductor.org/packages/release/bioc/html/ballgown.html
ProteinPilotTM V4.5	Open source	https://sciex.com/products/software/proteinpilot-software
DAVID 6.8	Sherman et al.[71]	https://david.ncifcrf.gov/
STRING 11.5	Szklarczyk et al.[72]	https://cn.string-db.org/
GraphPad 6.0	GraphPad Software	https://www.graphpad.com/scientific-software/prism/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal trail and sample collection

Jining Bairi chicken, a dual-purpose indigenous breed, was provided by Shandong Bairi Chicken Breeding Co., Ltd (Jining, Shandong, China). *Salmonella enterica* serovar Enteritidis (CVCC3377), purchased from the China Veterinary Culture Collection Center (http://cvcc.ivdc.org.cn/), was enriched in LB broth at 37°C for 16 h, pelleted at 4000rpm for 5 min, and diluted with sterilized PBS to prepare the inoculant. The concentration of the SE inoculant was measured using a plating method. Animal trials were conducted following previously described methods.[73] In detail, 168 two-day-old SE-negative chickens were randomly clustered into two groups (84 chickens in each group). Chickens in the inoculated group were orally inoculated with 0.3 mL 10^9 colony-forming units (cfu)/mL SE inoculant. In the control group, chickens were inoculated with the same volume of sterile PBS. The chickens were raised in two separate isolators with free access to feed and water. At 1, 3, 7, 14, 21, 28, and 35 dpi, 24 chickens (12 in the control group and 12 in the infected group) were euthanized by cervical dislocation for cecum collection. These tissues were immediately frozen in liquid nitrogen and stored in −80°C for further experiments.

Ethical permission for these studies was obtained from the Laboratory Animal Management and Use Committee of Shandong Agricultural University (SDAUA-2017-041). Written formal informed consent was obtained from all participants or their next of kin. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this work is consistent with those guidelines.

METHOD DETAILS

RNA extraction and RNA-Seq

At 3 dpi, three individual ceca from each group were randomly selected for RNA isolation. Total RNA was extracted from each cecum using the TRIzol reagent (Invitrogen, CA, USA). RNA concentration and quality were measured using a DS-11 Spectrophotometer (DeNovix, DE, USA) and 1% agarose gel electrophoresis, respectively. Finally, six RNA sequencing libraries were constructed using the qualified samples. Finally, ten micrograms of total RNA from each individual were subjected to Poly(A) mRNA purification using poly T oligo-attached magnetic beads (Invitrogen, CA, USA). Following the purification, mRNA was broken into small fragments and reverse-transcribed to cDNA for library construction following the manufacturer's instructions (Illumina, San Diego, USA). Paired-end sequencing was performed using the HiSeq 4000 platform (LC Sciences, Hangzhou, China).

Protein extraction, digestion and iTRAQ labeling

The proteins were isolated from the cecum used for RNA-seq using the method described in a previous study.[74] In detail, the cecum was ground in liquid nitrogen, dissolved in lysis buffer with 150 μL of radioimmunoprecipitation assay (RIPA) buffer (CST, USA), and then subjected to sonication for 5min. Following cellular debris removal, the protein concentration was quantified using a BCA kit (Beyotime, Shanghai, China), and the qualified protein was detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Total of 20 μg qualified protein of each individual was reduced using with 1 mM Tris-(2-carboxyethyl) phosphine for 1 h at 60°C, and alkylated with 1 mM methylmethanethiosulfonate for 10 min at room temperature. Then, the protein was digested into peptides using trypsin Gold (Promega, Madison, WI, USA) with protein: trypsin ratio of 30:1 at 37°C for 16h. The peptides were desalted on a Strata X C18 SPE column (Phenomenex, CA, USA), vacuum-dried, resuspended in 0.5M TEAB (Applied Biosystems, Foster City, USA). Each peptide mixture was then labeled using the 8-plex iTRAQ reagent kit (Applied Biosystems, Foster City, USA) and loaded onto a MacroSpin VydacC18 reverse-phase minicolumn (Nestgroup Inc., Southborough, MA, USA). Following elution, the samples were dried using SpeedVac (Thermo Fisher, San Jose, CA, USA), and separated using a strong cation exchange (SCX) column. Each SCX fraction was dissolved in a solvent [5% (v/v) acetonitrile, 0.1% (v/v) acetic acid] and 0.01% trifluoroacetic acid. Peptides were separated and eluted on a C18 column at a flow rate of 200 nL/min using a Proxeon EASY-nLC system (Odense, Denmark). The elution was conducted using a gradient including various percentages of solvent B. Subsequently, the eluted peptides were analyzed using tandem mass spectrometry (MS/MS) in a TripleTOF 5600 system (AB SCIEX, Framingham, MA, USA) in positive ion mode.

Real time quantitative PCR and western blotting

One microgram of total RNA was reverse-transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Takara, Dalian, China). The specific primer for real time quantitative PCR (RT-qPCR) were designed using Primer Premier5.0 (Table 2). RT-qPCR was performed according to the ABI 7500 guidelines. The RT-qPCR reaction mixture (20 μL) consisted of cDNA 2 μL, forward primer 0.5 μL, reverse primer 0.5 μL, SYBR Green Master 10 μL, and ddH_2O 7 μL. The amplification parameters were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and a single melting cycle at 95°C for 15 s, and 65°C for 1 min. Triplicate was performed for each sample using PrimeScript One Step RT-PCR Kit (TaKaRa, Shiga, Japan). The relative gene expression in different sample was calculate using the $2^{-\Delta\Delta Ct}$ method.

The proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF, 0.22 μm, Millipore, USA) membrane at 200 mA for 90 min. The membranes were incubated with blocking buffer (Beyotime Biotechnology Inc. Shanghai, China) for 1.5 h at room temperature. The membranes were then incubated with primary antibodies against *peroxiredoxin 6 (PRDX6)* and *anti-beta actin* at 4°C overnight, washed thrice with TBST buffer (Solarbio, Beijing, China), and incubated with HRP peroxidase-conjugated secondary antibody (Bioss, Beijing, China) at 4°C for 4 h. Proteins were visualized using BeyoECL Plus (Beyotime Biotechnology Inc. Shanghai, China), and then quantified using a Fusion FX imaging system and the Fusion Capt Advance FX7 software (Vilber Lourmat, Paris, France).

QUANTIFICATION AND STATISTICAL ANALYSIS

Bioinformatics analysis of RNA-seq data

Clean data was obtained by filtering out low-quality reads, adapters, and poly-N reads, and aligned with the chicken reference genome (http://ftp.ensembl.org/pub/release-105/fasta/gallus_gallus/) using the HISAT 2.0.[68] StringTie (v1.3.0)[69] was applied to the uniquely reads mapping, assembling, quantifing, and estimate the gene expression by calculating Fragments Per Kilobase of exon model per million mapped reads (FPKM). The DEGs were identified with Fold Change (FC) > 2 and p value <0.05 using R packge Ballgown.[70]

Proteomics normalization and filtering

Tandem mass spectra were searched against the chicken protein database (http://www.uniprot.org/proteomes/UP000000539) using ProteinPilot V4.5 for computational analysis. Peptides with a Mascot probability analysis confidence interval more than 95% were counted as identified peptides. The identified protein contained at least two unique peptides with an FDR <0.01. ProteinPilot software

was used to quantify the peak areas of the iTRAQ reporters. Finally, proteins with FC > 1.5 and $p < 0.05$ were considered as DEPs. The Spearman correlation between the proteins and corresponding co-expressed transcripts was analyzed using omicshare platform (https://www.omicshare.com/).

Functional annotation and enrichment analysis

GO and KEGG pathway enrichment analysis for DEGs and DEPs were performed with DAVID 6.8[71] (https://david.ncifcrf.gov/). The default parameter was applied, and $p < 0.05$ was considered significant. The protein functional network was constructed using STRING 11.5[72] (https://cn.string-db.org/).

The calculation for the expression of differentially expressed genes

Data are presented as mean ± SD. One-way ANOVA was applied to detect significant differences the expression of each gene between the inoculated group and control group using Prism 6 (GraphPad). p value < 0.05 was considered as significant.