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Original article

# Proteomics profiles reveal the potential roles of proteins involved in chicken macrophages stimulated by Lipopolysaccharide

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## Abstract

Lipopolysaccharide (LPS), a core part of gram-negative bacteria, is crucial for inducing an inflammatory response in living things. In the current study, we used LPS from *Salmonella* to stimulate chicken macrophages (HD11). Proteomics was used to investigate immune-related proteins and their roles further. Proteomics investigation revealed 31 differential expression proteins (DEPs) after 4 hours of LPS infection. 24 DEPs expressions were up-regulated, while seven were down-regulated. In this investigation, ten DEPs were mainly enriched in *S. aureus* infection, complement, and coagulation cascades, which were all implicated in the inflammatory response and clearance of foreign pathogens. Notably, complement C3 was shown to be up-regulated in all immune-related pathways, indicating that it is a potential protein in this study. This work contributes to a better understanding and clarification of the processes of *Salmonella* infection in chickens. It might bring up new possibilities for treating and breeding *Salmonella*-infected chickens.

**Keywords:** chicken macrophage (HD11), immune response, lipopolysaccharide (LPS), proteomics

## Introduction

In recent years, chicken consumption has increased, boosting the breeding industry's rapid expansion (Stewardson et al. 2014, Zhang et al. 2021). Nevertheless, the disease's incidence has grown owing to high-density production and the continual extension of the breeding area (Galanos et al. 1985). Gram-negative bacterial infections are the most serious threats in husbandry, because they are pathogens of infectious diseases. The common gram-negative bacteria in live-

stock husbandry are *Salmonella*, *E. coli* and *Pasteurella* (Donnenberg 2000). In severe cases, the bacteria can cause an inflammatory response in animals. It may affect the growth rate of the animal and even result in death, resulting in great losses for the poultry industry (Akira et al. 2006). Public health and safety are also at risk.

According to reports, billions of dollars were lost annually as a result of these losses (Tobias et al. 1986, Wright et al. 1989, Wright et al. 1990). Furthermore, human infections, like *E. coli* and *Salmonella*, kill

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1.7-2.5 million people a year. Currently, although medication and vaccination are practical and easy to administer, they are time-consuming and labor-intensive for large farms. Moreover, prolonged use of drugs or vaccines in poultry breeding can also lead to drug resistance, drug residues, environmental contamination, and other issues (Hanash et al. 2002).

The lipopolysaccharide (LPS), also known as endotoxin, is a highly acylated glycolipid in the cytoderm of gram-negative bacteria and plays a critical part in pathogenesis (Hanash 2003). The classical molecule of LPS is composed of a tripartite structure: Lipid A, the conserved and hydrophobic backbone, which represents the bioactive center and significant toxic effects of gram-negative bacteria (Forsgren and Sjöquist 1966); a core oligosaccharide chain, which is linked to lipid A; and an O side chain or O antigen polysaccharide, which given the abundance of bacterial species (Atkins et al. 2008, Goward et al. 1993, Laarman et al. 2010). Toll-like receptors (TLR), transmembrane proteins on cell surfaces, bind to LPS and trigger inflammatory responses in the host (Liu et al. 2021). In mammalian cells, LPS stimulation was performed through protein interactions (Elzawahry et al. 2014). Endotoxin binding protein (LBP) is a soluble protein that binds to CD14 by LPS, an anchor protein that facilitates the transfer of LPS to TLR4/MD-2 and triggers inflammation (Naor et al. 2009, Chu et al. 2014).

By completing the chicken genome, researchers will be able to analyze the relationship between complex traits and genes at the molecular level. Subsequent studies have shown that lncRNAs regulate gene expression at the transcriptional, post-transcriptional, and epigenetic levels. Because proteins perform the majority of life activities and undergo a series of processing after RNA, proteomics provides a framework based on protein series. The proteome reflects cellular and disease states and phenotypes better than the transcriptome. Protein expression can be accurately described at the whole-cell or tissue level as well as in subcellular structures: protein complexes and body fluids. Proteomics can be applied to better understand disease processes, develop new biomarkers for diagnosis and early detection, and speed up drug development (Abou-Abbass et al. 2016, Fazeli et al. 2017, Mohanta et al. 2021).

In this study, we constructed an LPS infection model by stimulating chicken macrophages (HD11) with *Salmonella* LPS in order to observe protein expression in the response to the infection. The inflammatory response affects protein expression differently. Proteomics also screened proteins associated with the immune system. In chickens, these results provide insight into how proteins might regulate gram-negative

bacterial infections. This would also provide a theoretical basis for poultry anti-bacterial infection studies.

## Materials and Methods

### Cell culture and LPS stimulation

The chicken macrophage-like cell line HD11 was provided by the Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy of Agricultural Sciences, Beijing, China. Cells were cultured in complete RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum, 100mM HEPES (4- (2-hydroxyethyl) piperazine-1-erhanesulfonic acid), 200 U/ml penicillin and streptomycin. The cells were cultured at 37° in 5% CO<sub>2</sub> and passaged twice a week. When growth reached 80%, 100ng/ml of LPS from *Salmonella* was added and total protein was extracted after 4 hours of in vitro stimulation. The two groups were divided: the LPS-stimulated HD11 cell group and the unstimulated HD11 cell group. Five replicate experiments were set up for each group.

### Protein extraction

Samples were sonicated three times on ice with a high-speed ultrasonic processor in lysis buffer (8 M urea, 1 % protease inhibitor cocktail). (Note: for PTM experiments. the inhibitor was added to the lysis buffer). The remaining debris was removed by centrifugation at 12,000 g at 4° for 10 minutes. Finally, the supernatant was collected, and the protein concentration was determined using the BCA kit according to the manufacturer's instructions.

### Trypsin digestion

In order to digest the trypsin, 5 mM dithiothreitol was first added to the solution and stored at 56° for 30 min. After adding 11 mM iodoacetamide, the protein samples were diluted to alkylate at room temperature in the dark for 15 min. Finally, trypsin was added at trypsin to protein ratio of 1:50 and digested overnight. Second digestion at a ratio of 1:100 was then started for 4 h, after which a C18 SPE column was used for desalting.

### TMT labeling

Firstly, the tryptic peptides were digested in 0.5 M TEAB (Triethanolamine buffered saline). The peptides were individually labeled with the respective TMT (Tandem Mass Tag) reagents for each lane and then incubated for 2 h at room temperature. For each sample, 5 µL was collected and then desalted. MS analysis was

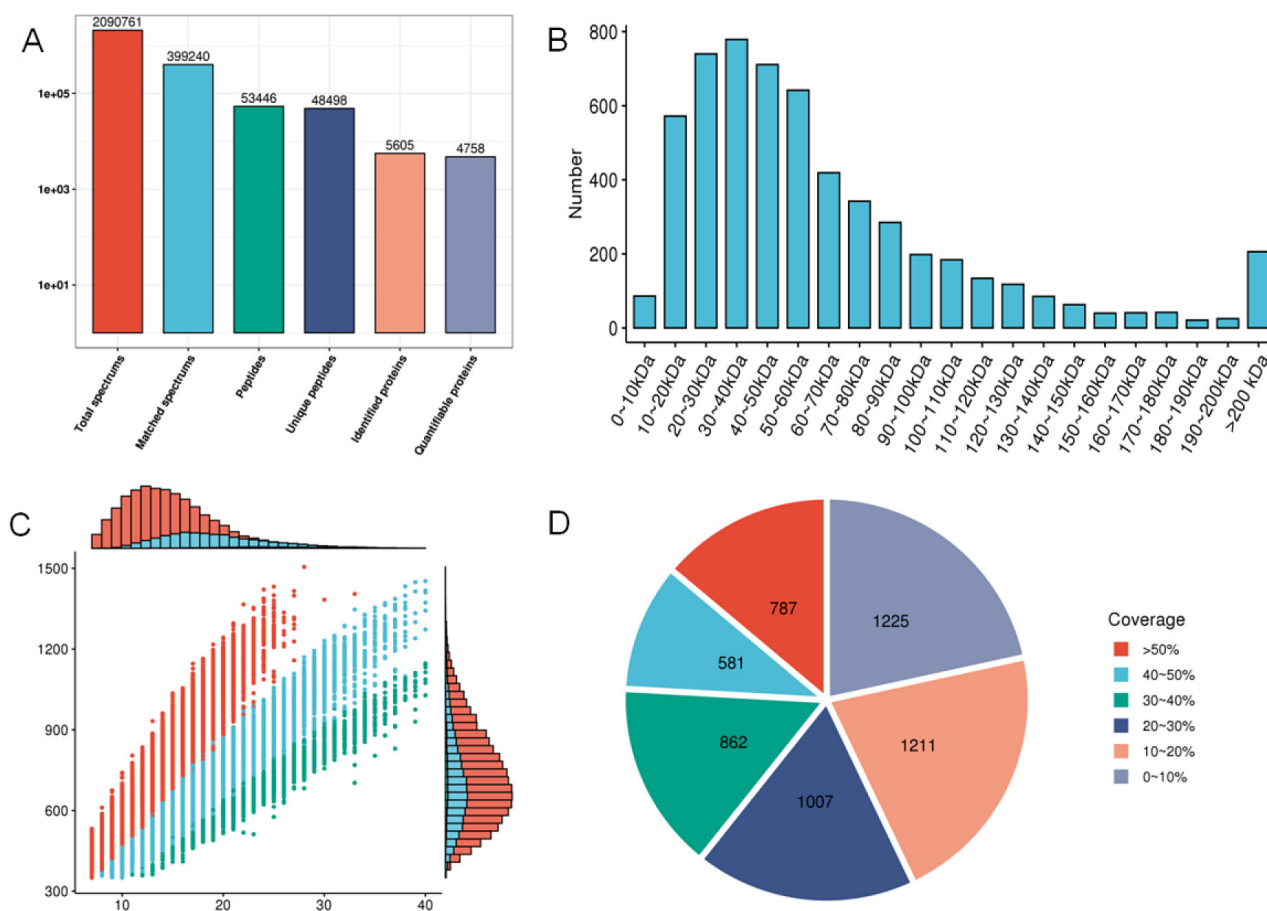


Fig. 1. Characteristics of the identified peptides and proteins. (A) Protein identification overview for the proteome analysis of chicken macrophage infected by LPS. (B) Protein molecular weight distribution. (C) Peptide length distribution. (D) Protein coverage distribution.

performed to check the labeling efficiency, and samples were quenched by adding of 5% hydroxylamine. Samples were desalted using a Strata X C18 SPE column (Phenomenex) and dried through vacuum centrifugation.

### Database search

The secondary mass spectrometry data was retrieved using Maxquant (v1.6.15.0) for this experiment. The search parameters were set: the database was Blast\_Gallus\_gallus\_9031\_PR\_20220314.fasta (27535 sequences), the inverse library was added to calculate the false positive rate (FDR) caused by random matching, and the common contamination library was added to the database to eliminate the effect of contaminated proteins in the identification results; the enzyme cleavage method was set to Trypsin/P; the number of missed cut sites was set to 2; the minimum length of peptide was set to 7 amino acid residues; the maximum number of peptide modifications was set to 5; the mass error tolerance of primary parent ion was set to 20 ppm and 20 ppm for first search and main search respectively and the mass error tolerance of secondary fragment

ion was set to 20 ppm. Carbamidomethyl (C) was set as a fixed modification, and the variable modifications were oxidation of methionine and acetylation of protein N-terminal. The FDR for protein identification and PSM identification were set to 1%.

### Bioinformatics analysis

Clarify the potential functions of obtaining DEPs. DEPs were subjected to Gene Ontology Analysis (GO), including Biology for BP, Cellular Component CC and Molecular Function MF), the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Protein-Protein Interaction Network (PPI) constructed using string databases for analysis. Differentially expressed proteins (DEPs) were identified by  $|\log_2(\text{fold change})| > 0.5$  and the corrected  $p$  ( $\text{padj}$ )  $< 0.05$ . The pathway with a corrected  $p < 0.05$  was considered to be significant.

### Ethics approval

All animal procedures were approved by the Ethical Committee of Bengbu Medical College under Protocol Number 25/2021.

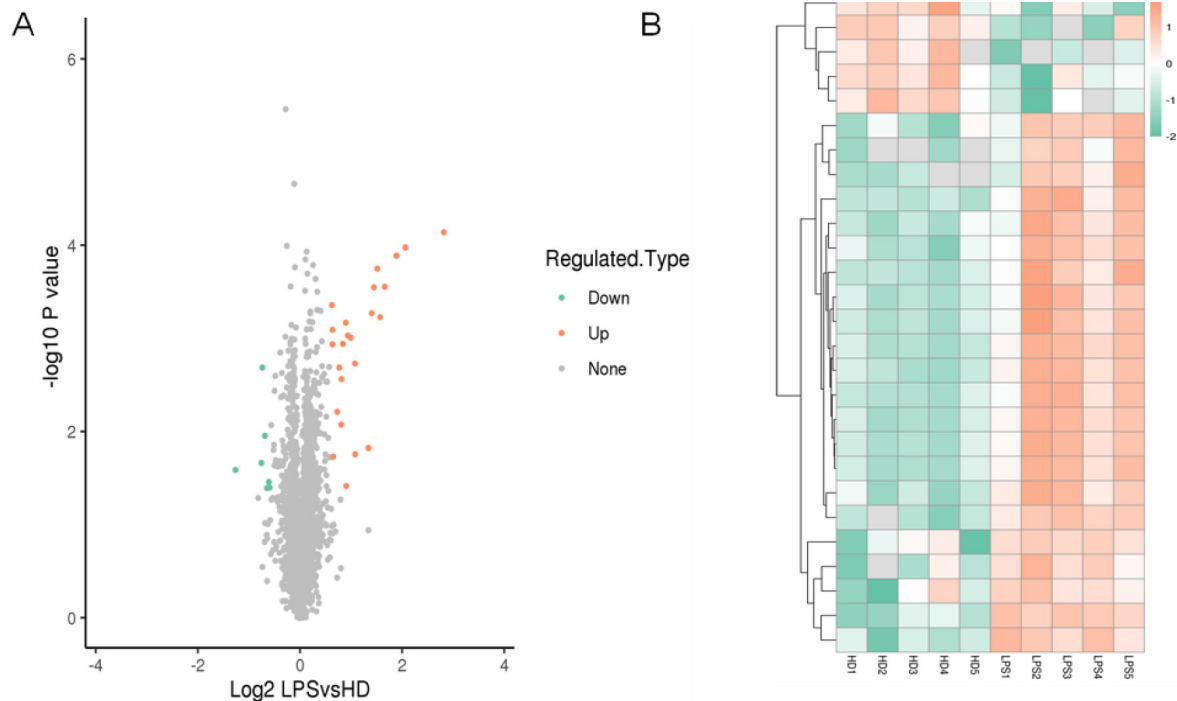


Fig. 2. Functional annotation of DEPs 4 h post-infection. The lipopolysaccharide (LPS) group means HD11 cells subject to LPS stimulation, and the HD group means HD11 cells that were not stimulated. (A) Volcano plot of DEPs. The horizontal coordinate represents the change in the folding of DEPs. The vertical coordinate shows the P-value (logarithmic transformation of 10 at the bottom). Black dots indicate proteins that are not differentially expressed. (B) Heat map diagram of DEPs for the HD group and the LPS-stimulated group. The sample names are given in columns and significant proteins in rows. Proteins are clustered together based on similar expressions. Colours indicate Z score from high (red) to low (green).

## Results

### Proteome identification results

In this study, 2,090,761 spectrums, 53,446 peptides, and 5605 identified proteins were obtained (Fig. 1A). The molecular weight of the proteins showed that most were between 10 and 80 kDa, indicating different stages of molecular weight distribution (Fig. 1B). According to enzymatic digestion and mass spectrometry fragmentation mode, most of the peptides were 7-20 amino acid residues in length. (Fig. 1C). As shown in Fig. 1D, more than half of the sequence coverage (60.7 %) was lower than 30%, suggesting that protein abundance was high.

### Results of DEPs selection

To show significantly Differentially Expressed Proteins (DEPs), the criteria of  $p\text{-value} < 0.05$ , a  $|\log_2 \text{Fold Change}| > 0.5$  was used to screen DEPs. Compared to the HD group (HD group means HD11 cells that were not stimulated), 24 genes were up-regulated, and 7 were down-regulated. The global pattern of proteins with remarkable variation expression can be visualized by volcano maps (Fig. 2A). Samples are treated and classified similarly. This result reflects the reliability of this experiment (Fig. 2B).

### GO analysis and pathway enrichment of DEPs

A full range of functional annotations were performed on the identified proteins to understand their functional properties. Depending on the biological processes, ten proteins were predicted to be involved in immunological processes (Fig. 3A). To discover more about this function, GO enrichment analysis was performed with GO annotation and classification. The results showed that the primary biological processes were enriched in protein-lipid complex remodeling, acute-phase response, regulation of protein activation cascade, regulation of complement activation, and immunoglobulin mediated immune response (GO: 0034368, GO: 0006953, GO: 2000257, GO: 0030449, GO: 0016064). Besides, the down-regulated biological processes were small GTPase mediated signal transduction and negative regulation of cell cycle process (GO: 0010948, GO: 0007264) (Fig. 3B).

In the “Cellular Component” category, proteins were enriched in the lumen region and the secretory granule (Fig. 3C). In the “Molecular Function” category, proteins were mainly enriched in the activity, receptor binding, and ion binding (Fig. 3D).

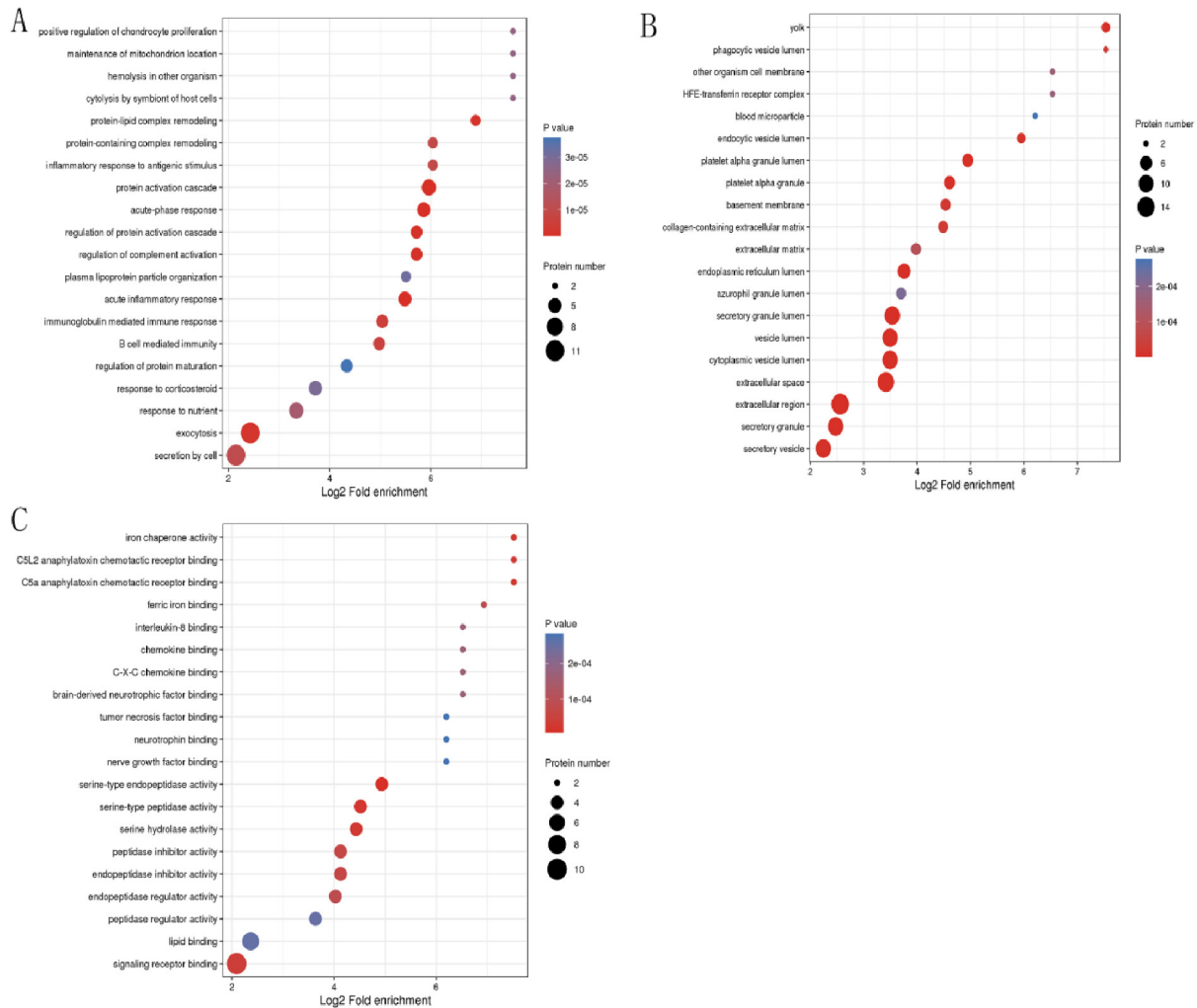


Fig. 3. GO analysis and GO functional enrichment of DEPs with 4 h post-infection. (A) GO enrichment analysis of DEPs in 'Biological Process'. (B) GO enrichment analysis of DEPs in 'Cellular Component'. (C) GO enrichment analysis of DEPs in 'Molecular Function'.

### KEGG analysis of proteins

Analysis of the KEGG pathway proteins revealed that the DEPs were related to the immune response following LPS infection. They were involved in the following signaling pathways, complement and coagulation cascades, *S. aureus* infection, primary immunodeficiency, systemic lupus erythematosus, african trypanosomiasis and leishmaniasis. The mapping of these response pathways was relative primarily to other pathways and maybe a relevant pathway for this study. As shown in Fig. 4, after LPS infection, several DEPs were nearly associated with immune function and inflammatory response, such as up-regulated expression of C3, A2M, BCR, and Immunoglobulin. We hypothesize that the HD11 may be involved in the development of *Salmonella* infection through activation of the mentioned pathways in the target cells.

### Cluster analysis of protein

As a result of demonstrating the main biological information functions of DEPs, a clustering analysis was performed in order to analyze the sample proteins in greater depth. By grouping and categorizing DEPs, we can separate sets of DEPs from collections of proteins, where proteins with similar expressions may function similarly. The DEPs folds were divided into four groups from Q1-Q4 to clarify the correlation between protein functions (Fig. 5A). The difference multiples in the groups Q3 and Q4 are better clustered than in the groups Q1 and Q2. The differential multiple of DEPs screened in this experiment was the Q3 group, and the target proteins screened were more reasonable, with similarly coloured proteins with similar expression patterns. According to the GO enrichment and KEGG enrichment analysis, most of the relevant pathways and enriched fractions are in the Q3 group. Therefore, we think these up-regulated DEPs play a vital role in this.

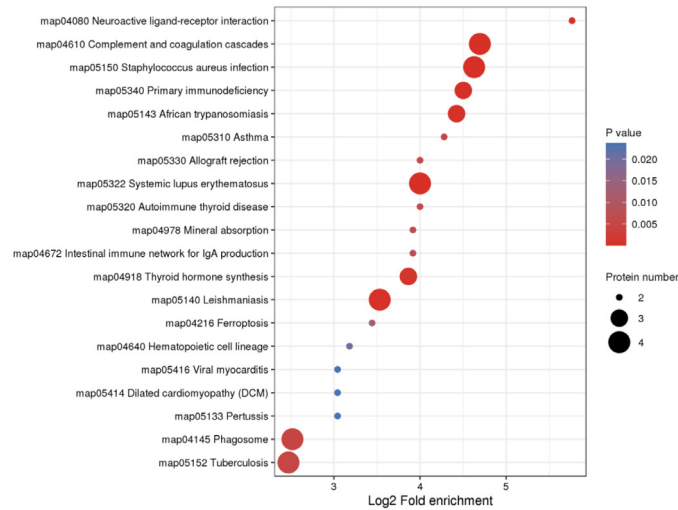


Fig. 4. Target proteins KEGG pathway analysis. The vertical axis corresponds to the various pathways, and the horizontal one corresponds to the enrichment multiple. The circle size describes the quantity of protein enriched in the respective pathway. The larger the size of dots, the more proteins enriched in that pathway. The colour of the dots represents the range of the q-value.

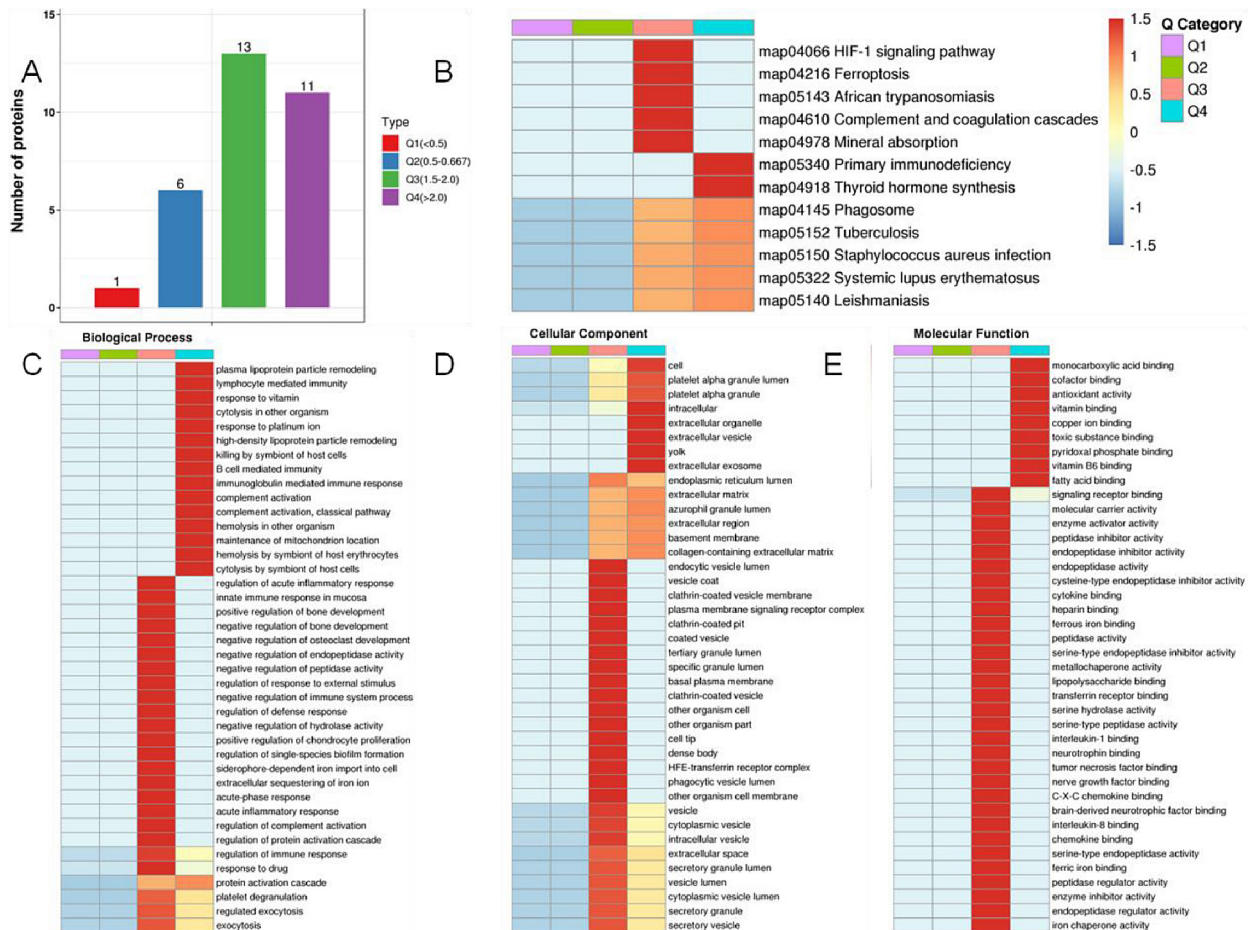


Fig. 5. Heat map showing the clustering of DEPs in samples. Splitting DEPs multiply into four parts Q1-Q4. Multiples of variance <0.5 for Q1, 0.5-0.667 for Q2, 1.5-2.0 for Q3 and >2.0 for Q4. The relevant functions in different Q-groups were clustered together using a hierarchical clustering method based on the Fisher's exact test P-value obtained from the enrichment analysis and plotted as a heat map. (A) The number of proteins per group and classification criteria. (B) KEGG clustering heat map. The heat map is depicted horizontally of the different Q-groups and vertically for the DEPs enriched to the relevant functional KEGG pathway. The colour blocks corresponding to the functional descriptions of the different Q-groups of DEPs enrichment indicate the strength of the enrichment. Red indicates strong enrichment, and blue indicates weak enrichment. (C) The heat map is depicted horizontally for the different Q groups and vertically for the biological process that the DEPs are enriched. (D) The heat map is depicted horizontally for the different Q groups and vertically for the Cellular Component that the DEPs are enriched. (E) The heat map is depicted horizontally for the different Q groups and vertically for the Molecular Function that the DEPs are enriched.

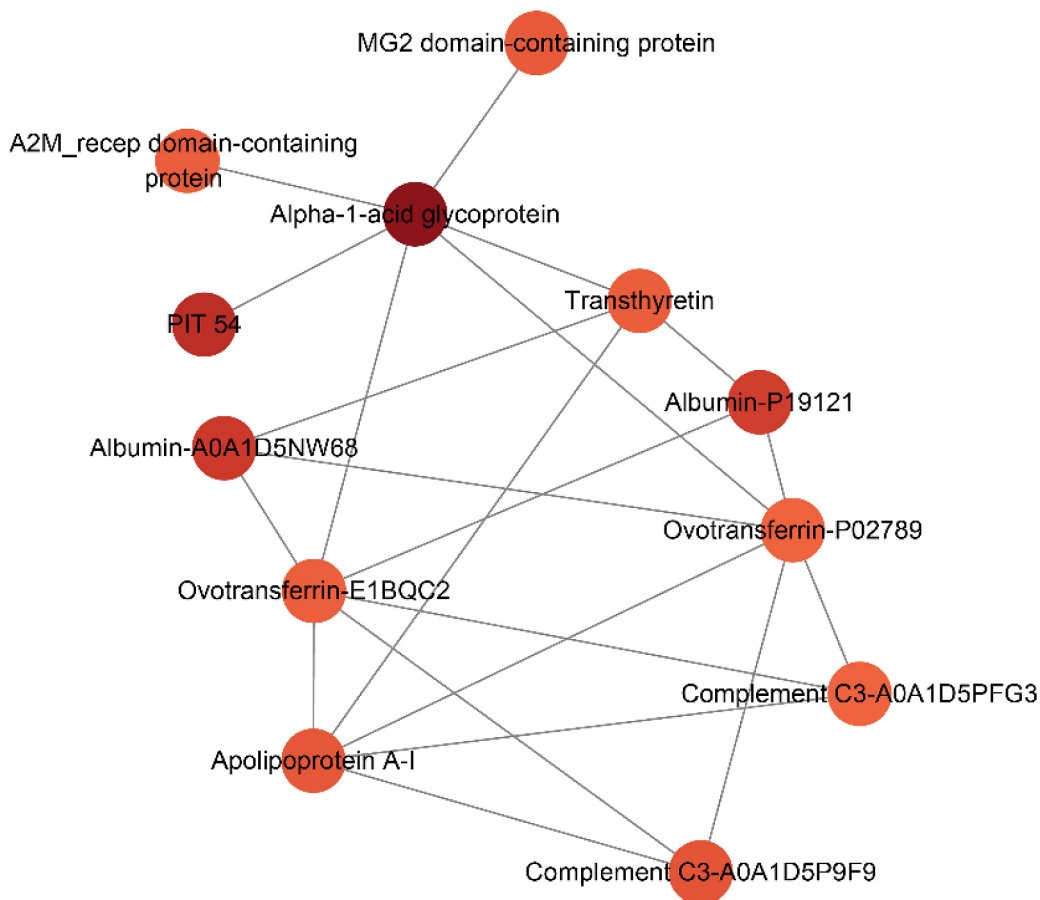


Fig. 6. Interactions are between DEPs and DEPs. DEPs interactions were extracted according to a confidence score  $> 0.7$  (high confidence). The circles in the diagram indicate DEPs, with various colours representing differential protein expression (green for down-regulated proteins, red for up-regulated proteins); the darker the colour, the greater the differential multiple.

### Protein-protein interaction networks analysis

A PPI analysis was performed to see if there was a correlation with LPS-induced chicken macrophage-associated proteins (Fig. 6). The top 50 proteins with the tightest interactions were screened, and protein interaction networks were mapped to clearly demonstrate protein-protein interactions. Among them, the most up-regulated were Alpha-1-acid glycoprotein, Pralbumin, AlbuminP19121, and AlbuminA0A1D5NW68, C3 and ovotransferrin.

### Discussion

In this study, we infected chicken macrophages (HD11) with *Salmonella* LPS. Proteins play a big role in host response to *Salmonella* after chicken infection. Proteome studies of LPS infection in chickens are rare. To better understand LPS pathogenesis, we did a proteomics analysis of HD11 treated with LPS.

We discovered that the majority of the DEPs were greatly abundant in immune-related pathways, such as complement and coagulation cascades, *S. aureus* infec-

tion and SLE. The *S. aureus* infection signaling pathway is associated with several pathways and the B cell receptor signaling pathway. It regulated the inflammatory response and the clearance of foreign pathogens. Staphylococcal protein A (SpA) binds to the IgG region, preventing phagocytosis, while Second Immunoglobulin-binding protein (Sbi) binds directly to IgG (Sarma and Ward 2011, Gonzalez et al. 2015, McGuinness et al. 2016, Nygaard et al. 2016). Sbi also binds to complement C1q, thereby affecting the classical pathway of the complement cascade (Keene et al. 2011, McGonigal et al. 2016). In addition, this SpA can also link to the BCR of the B cell receptor signaling pathway (Shi et al. 2021). Another toxic factor on the surface of *S. aureus* staphylococcal superantigen-like protein 7 (SSL7) also combines immunoglobulin A (IgA) with complement C5 (van den Berg et al. 2011). Under normal conditions, C5 is cleaved into C5a, a potent chemoattractant, and C5b, which forms the membrane attack complex formation: C5b-9 (Ko et al. 2013, Jongerius et al. 2019, Ben-Yosef et al. 2021). SSL7 effectively inhibits phagocytosis and inhibits the membrane attack complex from being formed in the

(West and Damania 2010). These results suggest that most signaling pathways show a crosstalk and cascade effect on LPS resistance.

After screening the proteomics, we found 31 DEPs, 24 of which were up-regulated, and 7 were down-regulated. A total of 10 DEPs were involved in immune-related activities. When LPS stimulated HD11, all immune-related DEPs were up-regulated such as C3, IgG, IgA, and BCR. C3 was the most up-regulated protein in terms of differential expression, which confirms its importance. Innate immunity relies on the complement system. Complement activation is triggered by three different pathways: classical, lectin, and alternative, with convertases playing a major role in complementation (Ricklin 2012, Zipfel et al. 2021, Cheng et al. 2022). All three pathways require C3, the most central component of the complement system. There are three ways pathogens can invade an organism: 1. Complement labels them with C3b and makes them phagocytosed (Katsafadou et al. 2019, Mohanty et al. 2019). 2. A chemoattractant molecule like C5a activates chemotaxis, which recruits macrophages and neutrophils. When these phagocytes get to the injury site, they will try to engulf and dissolve the invaders (Noris and Remuzzi 2013, Mateu-Borrás et al. 2022). 3. Attacks membrane complexes directly to lyse invaders (Boes et al. 1998).

To reveal the relationship with LPS-inducible chicken macrophages related proteins. We built the DEPs-DEPs interaction network, and several DEPs are prominent. This analysis exposed multiple interconnected proteins that link inflammation and infection responses which all are produced by the liver. These proteins can be divided into three categories. The first protein, acute response protein (APRP), is made when the body responds to an acute situation (Li and Chen 2020). These include Proalbumin, Transthyretin, AlbuminP19121 and AlbuminA0A1D5NW68, which are all upregulated. The second is Ovotransferrin, which is a macromolecular component of the cuticle. It's what makes antimicrobials work. It's crucial for egg-laying species to reproduce smoothly (Kulshreshtha et al. 2022). Microorganisms compete with them for iron. It also has antimicrobial activity by detaching iron ions (Fe<sup>3+</sup>), which Gram-negative bacteria like *Salmonella* need (Martins et al. 2016). Another is the complement C3, which is involved in immune defense and immune regulation in humans (Yan et al. 2019). In response to LPS stimulation, C3 was the obvious DEP. The host body rapidly identifies the invading material through the C1 complex (classical pathway) and mannose-binding lectins and ficolins (lectin pathway) in the complement pathway to produce C3 lyase, and when the complement protein C3 is activated by C3 convertase, C3a

and C3b are formed (Cestari and Ramirez 2010, Zhao et al. 2021). The C3b binds to the bacterial surface and continues to create new C3 lytic enzymes while forming C5 convertases, which attract phagocytes or form membrane attack complexes (Tan et al. 2016). As predicted, they play a big role in LPS response.

By infecting chicken macrophages with LPS, we screened 31 differentially expressed proteins. Among them, 24 were up-regulated and 7 were down-regulated. Ten DEPs were strongly associated with inflammatory response and foreign pathogen clearance. In this experiment, we found that macrophages from LPS-infected chickens were involved in several immune pathways, including the *S. aureus* infection pathway and the B-cell receptor signaling pathway. These signaling pathways were up-regulated by complement C3, IgG, IgA and BCR, with complement C3 playing a significant role in macrophage clearance in LPS-infected chickens. We speculated that C3 was crucial to *Salmonella* pathogenesis. A high complement level may make the complement activation pathway a key therapeutic factor. These results provide a basis for understanding LPS infection with HD11 and contribute to a better understanding of *Salmonella*.

Furthermore, chickens' tolerance and susceptibility to LPS infection was studied.

### Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD034436. The authors confirm that the data supporting the findings of this study and available within the article.

### Acknowledgements

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