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RESEARCH ARTICLE

Transcriptomic Profiling of Avian Macrophages HD11 Reveals the Immunomodulatory Role of HutZ in *Avibacterium paragallinarum* Infection

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ABSTRACT

Avibacterium paragallinarum (*Av. Paragallinarum*) infection causes infectious coryza, which is a widespread and serious respiratory disease in chickens. Although the HutZ protein has been linked to heme utilization and virulence in various pathogens, a specific role in host-pathogen interactions during *Av. paragallinarum* infection remains unclear. In this study, to investigate host immune responses and the regulatory role of HutZ following *Av. paragallinarum* infection, we performed the transcriptomic profiles of avian macrophage HD11 following wild and HutZ mutant of *Av. paragallinarum* infection using RNA-seq, respectively. The differentially expressed genes (DEGs) in the HD11 macrophages between the two groups were identified. Compared with the uninfected group, more DEGs involved in signaling pathways and cellular functions were identified in the infected groups. Our results revealed extensive transcriptional changes following infection, with both bacterial strains activating core immune pathways including PI3K-Akt and MAPK signaling. Notably, the HutZ mutant elicited stronger activation of pattern recognition receptor pathways such as Toll-like, NOD-like, and RIG-I-like signaling, suggesting that HutZ may help the pathogen evade host immune detection. This work provides novel insights into the immunomodulatory role of HutZ and offers a foundation for developing targeted interventions against *Av. paragallinarum*.

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INTRODUCTION

Avibacterium paragallinarum is the aetiological agent of infectious coryza, which is a severe respiratory disease which causes significant economic losses in the poultry industry (Blackall, 1999). The main clinical symptoms include blindness, facial swelling, nasal discharge, conjunctivitis, anorexia and diarrhea (Xu *et al*., 2019; Blackall *et al.*, 2005). *Av. paragallinarum* belongs to the bacterial *Pasteurellaceae* family and has three serogroups including A, B and C based on the Page schemes (Page,1962). *Av. paragallinarum* infection is prevalent worldwide such as USA (Kuchipudi *et al.*, 2021; Byukusenge *et al.*, 2020), South Africa (El-Gazzar, *et al.*, 2024), Japan (Shibanuma, *et al.*, 2023), Korea (Han *et al.*, 2016), Peru (Falconi-Agapito *et al.*, 2015), Mexico (Luna-Castrejón *et al.*, 2021), and China (Sun *et al.*, 2018; Guo *et al.*, 2022) in recent years. Due to the severe economic losses caused by *Av. paragallinarum* in poultry industry, it is of urgent necessity to investigate the molecular mechanisms of microbial pathogenesis and develop new strategies to control infectious disease.

Iron is one of the most important micronutrients for most organisms. It plays critical roles in growth and colonization of pathogenic bacteria. Pathogenic bacteria have utilized multiple iron uptake systems to acquire adequate iron through siderophores, hemophores or iron-binding proteins (Parrow *et al*., 2013; Uchida *et al*., 2012). The heme utilization system is one of the most important ways for Gram-negative pathogens such as *Vibrio cholerae* to acquire iron (Sekine *et al*., 2016). In the heme utilization system, heme utilization Z (HutZ) encodes a heme-degrading enzyme. It can cleave heme to biliverdin, which has been demonstrated to be critical for heme utilization in

V. cholerae (Uchida *et al.*, 2012). In addition, HutZ is also involved in pathogenesis and biofilm formation of *Edwardsiella piscicida* (Shi *et al*., 2019). Our previous study demonstrated the presence of HutZ in *Av. paragallinarum* under iron-limited conditions using RNA-seq analysis (Huo *et al*., 2021). Moreover, HutZ has also proved to be essential for heme utilization and pathogenesis of *Av. paragallinarum* (Huo *et al*., 2023). Therefore, HutZ can be considered as one of the virulence factors in *Av. paragallinarum*.

Iron is an essential nutrient for growth of pathogenic bacteria as well as play a crucial role in the physiological function of the host (Nakamura *et al*., 2019). In the case of a pathogen infection, there is a competitive relationship between the host and the pathogen in the utilization of iron. It has been reported that the immune cell macrophage plays an important role in maintaining iron homeostasis by a precise regulatory system during infection of pathogenic bacteria. Studies have shown that most of the iron of the host exists in the hemoglobin of red blood cells, while aged red blood cells are degraded and recycled by macrophages. Macrophages can produce 20-25 mg of iron daily, resulting in a turnover of plasma iron for approximately every 2 hours (Ganz and Nemeth, 2012). Therefore, macrophages modulate the systemic iron homeostasis by recovering and returning iron to the circulation system. In terms of the relationship between bacterial HutZ protein and macrophages, previous studies have confirmed that, in *Edwardsiella piscicida,* the HutZ protein can affect the immune response of host macrophages by reducing the level of reactive oxygen species in macrophages, thus blocking the activation of macrophages (Shi *et al.*, 2019). In *Av. paragallinarum*, our previous pathogenicity analysis showed that the bacteria can invade into avian macrophages, with the process being positively regulated by the HutZ protein (Huo *et al*., 2023). However, the detailed mechanisms during the interactions between host macrophages and *Av. paragallinarum* infection are still unknown.

Therefore, to better understand the relationship between macrophages and *Av. paragallinarum*, as well as to explore the specific roles of HutZ in bacteria-infected macrophages, a comprehensive transcriptomic analysis of macrophages (HD11) following *Av. paragallinarum* infection was performed using RNA-seq. In addition, the transcriptomic and bioinformatic characterizations in HD11 macrophage in response to *Av. paragallinarum* infection were elucidated. Our findings provide mechanistic insights into HutZ function and suggest its potential as a target for future therapeutic interventions.

MATERIALS AND METHODS

Bacterial strain and growth conditions: *Av. paragallinarum* serogroup C strain 3005, originally isolated in China in 2018, was used in this study. The strain was cultured in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA), with both media being enriched by the addition of 0.0025% nicotinamide adenine dinucleotide (NAD) and 10% chicken serum to ensure optimal growth.

Construction of the HutZ mutant: The HutZ mutant strain of *Av. paragallinarum* was generated via homologous recombination as described previously (Huo *et al.*, 2023). The targeted deletion of the HutZ gene was confirmed by sequencing.

HD11 macrophage infection assay: Chicken HD11 macrophage cells were plated in 12-well plates and infected separately with the wild or HutZ mutant strain of *Av. paragallinarum* at a multiplicity of infection (MOI) of 10:1. Following a 6-hour incubation at 37°C, cells were rinsed with phosphate-buffered saline (PBS) and then incubated with 50 μg/mL cephalexin for 2 hours to eliminate extracellular bacteria. Subsequently, cells were rinsed with PBS, collected, and stored at −80°C. Each infection group included three biological replicates.

RNA isolation and quality assessment: Total RNA was extracted from the harvested cells post-infection using TRIzol reagent, strictly adhering to the manufacturer's specified instructions. The integrity of the extracted RNA was assessed using the Agilent 2100 Bioanalyzer, while its concentration was accurately measured using a NanoDrop ND-2000 spectrophotometer.

Transcriptome library construction and sequencing: The poly(A)+ mRNA was isolated through the utilization of oligo(dT)-conjugated magnetic beads. Subsequently, the isolated mRNA was fragmented and reverse-transcribed into cDNA. Following adapter ligation, libraries underwent sequencing on the Illumina NovaSeq 6000 platform (Majorbio Co., Shanghai). Raw sequencing data obtained were filtered to eliminate low-quality sequences, and the remaining high-quality reads were then aligned to the *Gallus gallus* reference genome (GRCg7b).

Differential expression analysis: Transcript abundance was normalized using transcripts per million (TPM) with RSEM. DESeq2 was used to identify differentially expressed genes (DEGs), applying thresholds of $|\log2 \text{ fold change}| \geq 1$ and adjusted p-value < 0.05 (Benjamini-Hochberg correction). Genes with a fold change ≥ 2 were subjected to downstream enrichment analyses.

Functional enrichment and bioinformatics analysis: DEGs were functionally annotated by referencing the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases via the DAVID platform (http://david.abcc.ncifcrf.gov/). Enriched GO terms (biological processes, cellular components, and molecular functions) and KEGG pathways with adjusted $P \leq 0.05$ were considered statistically significant. Heatmaps were generated using the "pheatmap" package in R to visualize DEG expression patterns.

RESULTS

Sequencing quality and alignment efficiency: Following 6-hour infections with either wild or HutZ mutant of *Av. paragallinarum* strains, total RNA was extracted from HD11 macrophage cells for transcriptome analysis. Each sample generated 41.7–45.7 million raw reads, of which 41.4–45.3 million clean reads remained after quality filtering using the FastQC tool and data pre-processing (Table S1). Base-call quality was high, with Q20 and Q30 values exceeding 95.5%. The average length was approximately 150 bp（Fig. S1). Then, the filtered high-quality reads (clean reads) were aligned to

the *Gallus gallus* reference genome (GRCg7b) with alignment rates above 90% (Table S2). The proportion of uniquely mapped reads ranged from 91.87 to 94.37%, indicating reliable mapping suitable for downstream expression analysis. The distribution and numbers of gene expression are shown in Fig. 1A. Density plots and distribution profiles showed consistent expression across replicates, supporting the validity of subsequent DEG analysis.

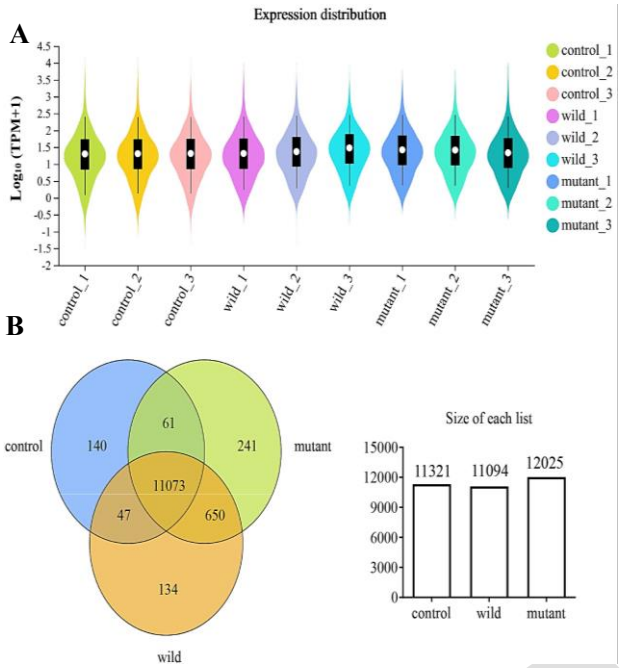

Fig. 1: Quantitative statistics of gene expression. (A and B) The violin diagram and Venn diagram showing the distribution of gene expression and the overlap of genes in HD11 cells following wild and HutZ mutant *Av. paragallinarum* infection, respectively. TPM: Transcripts Per Million reads.

Differential gene expression profiles: In total, 25,680 genes were detected, including 25,060 annotated genes and 620 novel transcripts. The Venn diagram analysis showed 11,073 genes were commonly expressed in all groups (Fig. 1B). In addition, by analyzing the DEGs among different groups, we obtained 140, 134, and 241 unique DEGs in non-infected, wild-infected, and HutZ mutant-infected HD11 cells, respectively.

As shown in Fig. 2A and Fig. 2B, compared to uninfected controls, infection with the wild strain resulted in 2,013 upregulated and 764 downregulated genes, while the HutZ mutant strain induced 2,591 upregulated and 702 downregulated genes. Furthermore, a total of 125 significantly DEGs were identified between HutZ mutant and wild infections, comprising 73 upregulated and 52 downregulated genes. Hierarchical clustering and volcano plots illustrated clear transcriptomic separation between infected and uninfected samples (Fig. S2 and Fig. 3). Together, a larger number of DEGs were identified in HD11 cells following wild or HutZ mutant of *Av. paragallinarum,* which demonstrated that HD11 infection with *Av. paragallinarum* resulted in significant changes in the global transcriptome. Notably, the HutZ mutant provoked a broader transcriptomic response, suggesting that deletion of HutZ elicits additional host responses beyond those caused by the wild strain.

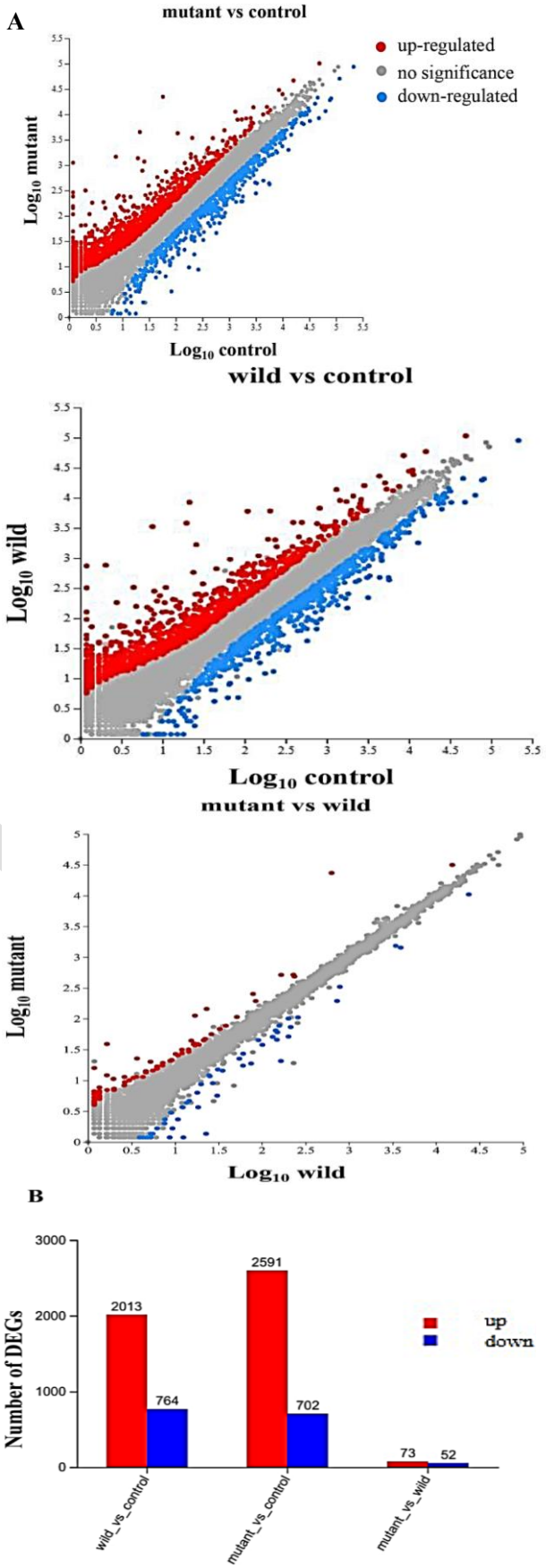

Fig. 2: Identification and quantification of differentially abundant genes. (A) Scatter plots of gene abundance ratio in wild vs control, mutant vs control and mutant vs control, respectively. (B) Bar chart showing the up-regulated DEGs (Red) and down-regulated DEGs (Blue) in HD11 cells infected with *Av. paragallinarum* and uninfected cells (wild vs control, mutant vs control and mutant vs control), respectively.

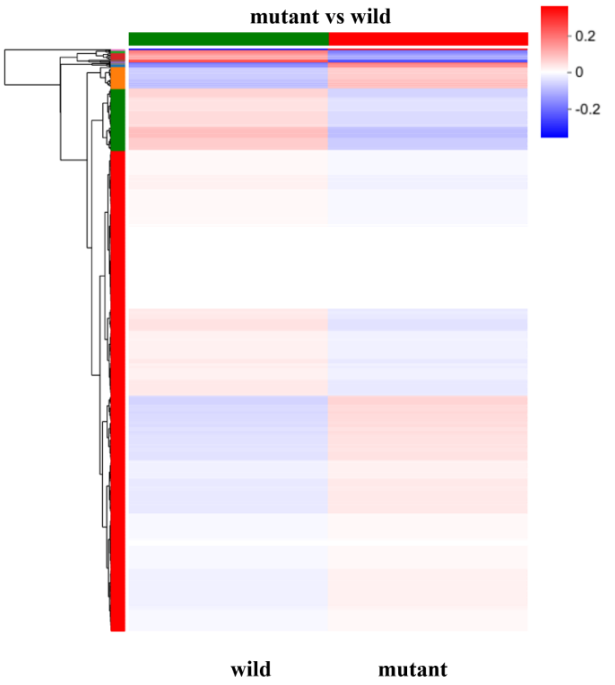

Fig. 3: Heat map of gene cluster enrichment analysis for DEGs screened in HD11 cells following infection with wild and HutZ mutant *Av. Paragallinarum*. Each column represents a sample, and each row represents a gene. Colors indicate normalized expression levels of the gene in each sample, with red denoting higher expression and blue denoting lower expression. The left panel displays a dendrogram of gene clustering and a module diagram of sub-clusters, where each color represents a sub-cluster. Closer gene branches indicate more similar expression profiles.

GO and KEGG analysis of the wild strain–induced DEGs: As shown in Fig. 4A, DEGs in wild-infected HD11 cells by GO enrichment analysis could be clustered into 3 categories, including biological processes (11), cellular components (2) and molecular functions (7). Then, the top 20 terms of GO enrichment analysis of these DEGs were summarized in Fig. 5A and Data Set S1. Among the top 20 terms, all these terms belonged to the biological process, which were mainly involved in signaling pathways such as regulation of actin filament-based movements, cytokine production, immune response, inflammatory response, cell adhesion, cell surface receptor signaling pathway.

Additionally, KEGG pathway analysis was conducted to further detect the related interactions of these DEGs in HD11 cells following *Av. paragallinarum* infection. As shown in Fig. 6A, DEGs in wild-infected HD11 cells were classified into 4 KEGG categories, including environmental information processing (2), cellular process (4), organismal systems (6) and human diseases (8). The top 20 KEGG-pathway items were mainly associated with ECM-receptor interaction, cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, and MAPK signaling pathway (Fig. 7A and Data Set S2). Taken together, these findings indicated that wild strain infection triggers immune activation and cytoskeletal remodeling in HD11 macrophages.

GO and KEGG analysis of HutZ mutant–induced DEGs: As shown in Fig. 4B, the 3 categories of GO enrichment analysis in HutZ mutant-infected HD11 cells were like those in wild-infected HD11 cells. Then, the top 20 terms of GO enrichment analysis were summarized in Fig. 5B and Data Set S3. Among the top 20 terms, all these terms belonged to the biological process, which were obviously classified into the cell-cell adhesion, enzyme-linked receptor protein signaling pathway, cell surface receptor signaling pathway. No cellular component or molecular function categories exhibited significant enrichment for these unique DEGs.

KEGG pathway analysis found that the DEGs in the HutZ mutant-infected HD11 cells could be classified into 5 KEGG categories, including genetic information processing (1), environmental information processing (2), cellular processes (4), organismal systems (5) and human diseases (2) (Fig. 6B). According to the KEGG enrichment analysis, the top 20 significantly enriched KEGG-pathway items were listed in Fig. 7B and Data Set S4. These KEGG-pathway terms were classified into the PI3K-Akt signaling pathway, ECM-receptor interaction, cytokine-cytokine receptor interaction. Importantly, PRR-related pathways such as RIG-I-like, Toll-like, and NOD-like receptor signaling were more prominently enriched in the HutZ mutant group. This suggests that loss of HutZ enhances host pattern recognition signaling, potentially amplifying immune surveillance.

Comparative analysis of HutZ mutant versus wild infections: As shown in Fig. 4C, GO enrichment analysis showed that DEGs could be classified into 3 categories, consisting of biological processes (9), cellular components (2) and molecular functions (4). The top 20 terms of GO enrichment analysis were summarized in Fig. 5C and Data Set S5. With the top 20 terms, 12 biological process terms, 2 cellular component terms and 6 cellular components terms were significantly enriched, which were mainly associated with CXCR chemokine receptor binding, cytokine activity, cytokine receptor binding, chemokine activity, chemokine-mediated signaling pathway, chemokine receptor binding, cellular response to lipopolysaccharide.

Based on the KEGG pathway, DEGs in the mutant-infected HD11 cells could be clustered into 5 KEGG categories, including metabolism (1), environmental information processing (2), cellular process (1), organismal system (1) and human diseases (9) (Fig. 6C). Our analysis revealed that the DEGs were enriched in a total of 43 KEGG pathways. Among these, 38 KEGG pathway categories exhibited significant enrichment (Data Set S6). Here, top 20 of significantly enriched KEGG-pathway items were shown in Fig. 7C, which mainly participated in the IL-17 signaling pathway, cytokine-cytokine receptor interaction, RIG-I-like receptor signaling pathway, Toll-like receptor signaling pathway and NOD-like receptor signaling pathway. Together, these data suggest that both bacterial strains activate core immune pathways, and HutZ deletion enhances PRR pathway activity and inflammatory signaling, indicating a modulatory role for HutZ in macrophage–bacteria interactions.

DISCUSSION

Bacterial heme utilization system plays critical roles in iron acquisition, adaptation, and pathogenicity (Shi *et*

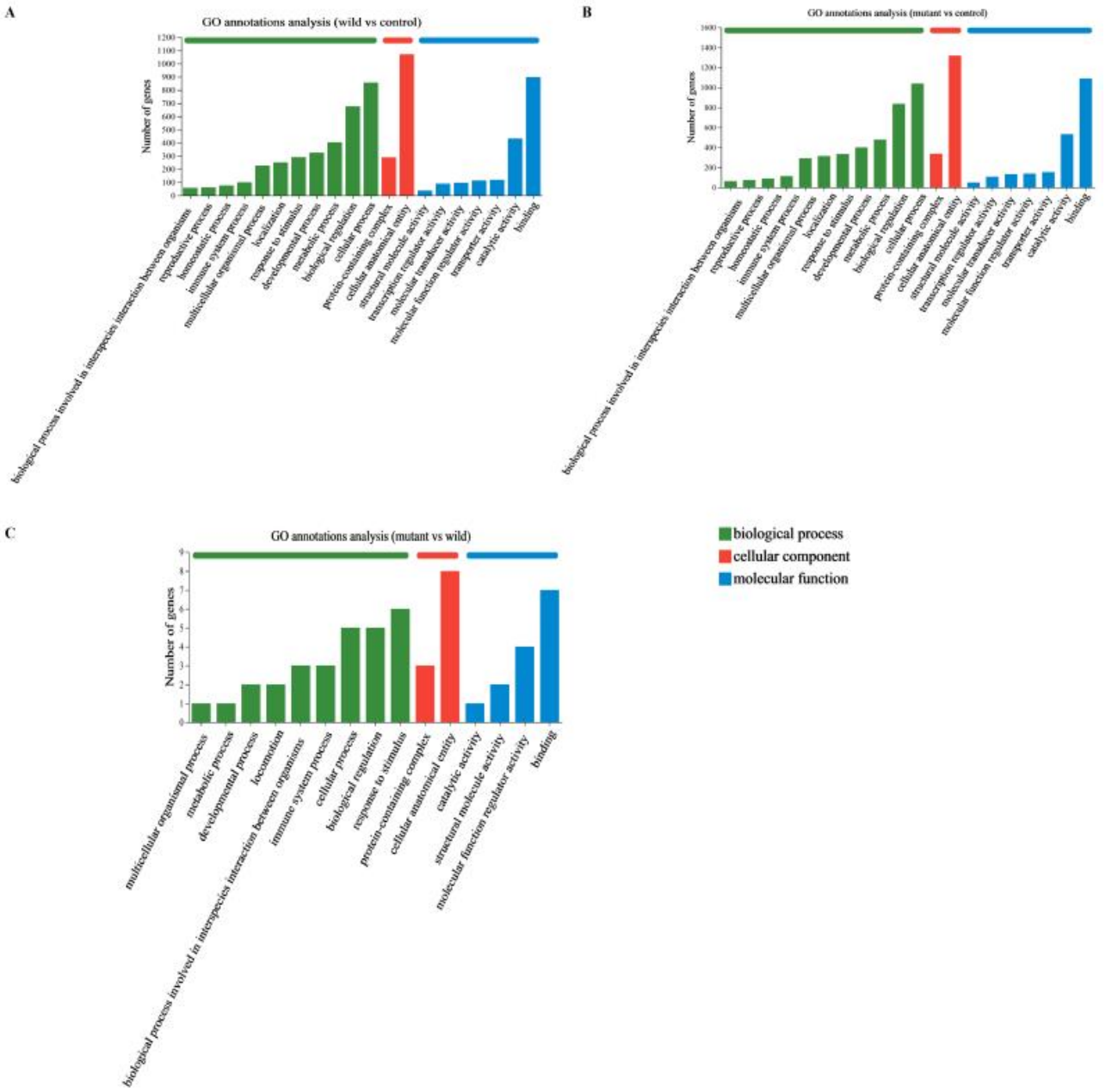

Fig. 4: GO annotations analysis of DEGs in HD11 cells following wild and HutZ mutant *Av. paragallinarum* infection. (A) wild vs control. (B) mutant vs control. (C) mutant vs wild.

al., 2019). HutZ was regarded as an essential protein for heme utilization as iron source in *V. cholerae* (Wyckoff *et al*., 2004). In terms of *Av. paragallinarum*, we have identified the HutZ protein in bacteria as well as the effects of this protein on the growth and pathogenicity (Huo *et al*., 2021; Huo *et al*., 2023). Interestingly, we found that the bacteria can invade into avian macrophages and cause the inflammatory response, which the process is positively regulated by the HutZ protein (Huo *et al*., 2023). However, the specific molecular mechanisms of this protein remain unclear.

Nowadays, transcriptome-based bioinformatic analyses have been extensively used to explore the transcriptome response of host to microbial pathogens (Pu *et al*., 2021). In this study, with the aim of gaining a more comprehensive understanding of the gene expression patterns in macrophages following *Av. paragallinarum* infection, we performed the RNA-Seq analysis. Firstly, we focused on the genes of avian macrophages regulated by *Av. paragallinarum* infection and carried out a meticulous examination of the relative functional activities associated with these genes. At the same time, the influence of HutZ on transcriptomic levels in macrophages was also assessed. Thus, the cellular dynamic processes of DEGs modulated by wild and HutZ mutant strains in HD11 cells were investigated. This data will be helpful to further understand the molecular mechanisms of *Av. paragallinarum* infection in immune cells and host defense.

Here, the HutZ mutant of *Av. paragallinarum* was initially developed by replacing the HutZ gene with a chloramphenicol resistant cassette. Through RNA-seq analysis, we examined the impact of HutZ on gene expression in HD11 cells during infection. Both wild and HutZ mutant strains elicited significant transcriptional reprogramming, but the mutant strain triggered a broader

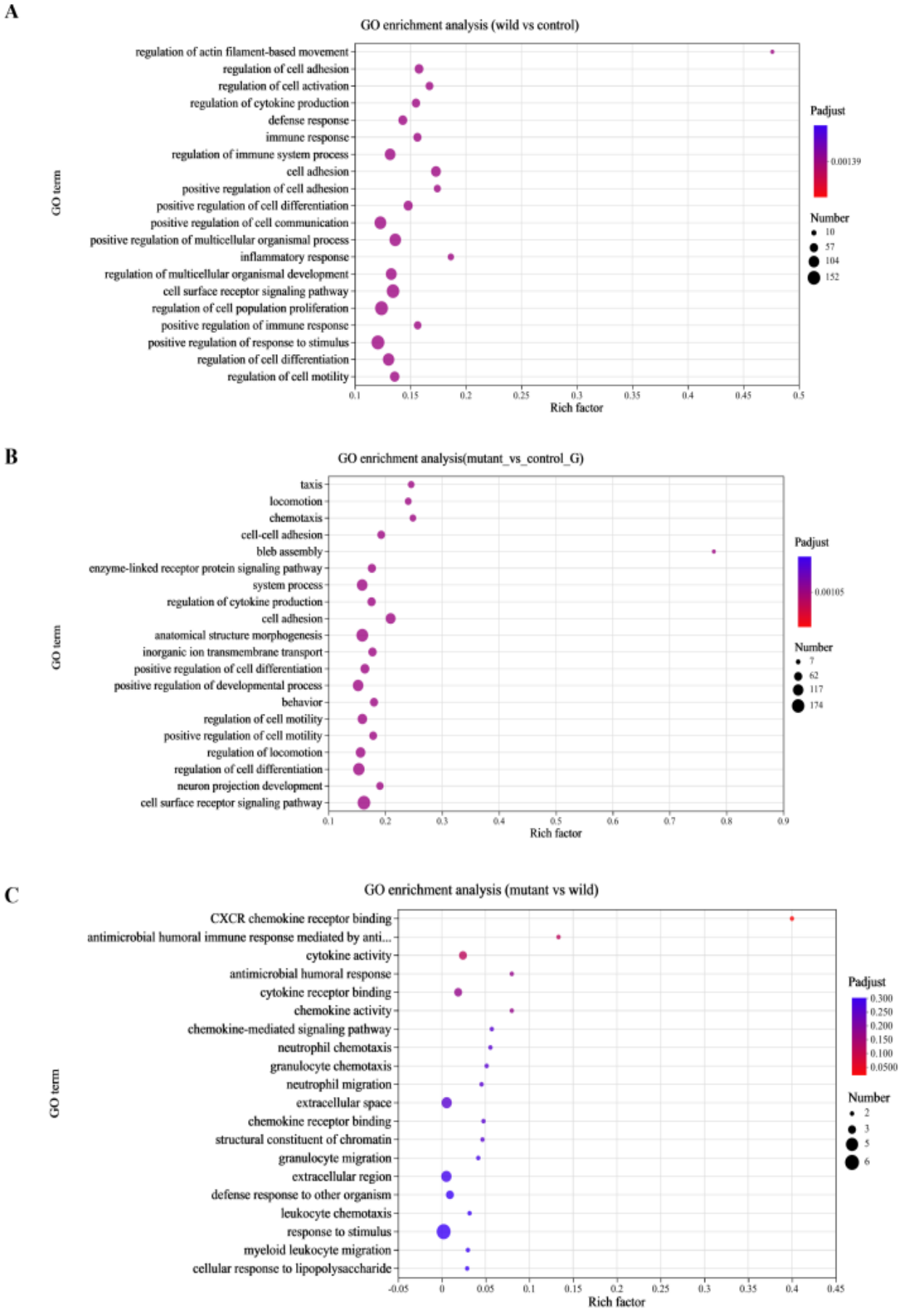

Fig. 5: GO enrichment analysis of DEGs in HD11 cells following wild and HutZ mutant *Av. paragallinarum* infection. (A) wild vs control. (B) mutant vs control. (C) mutant vs wild.

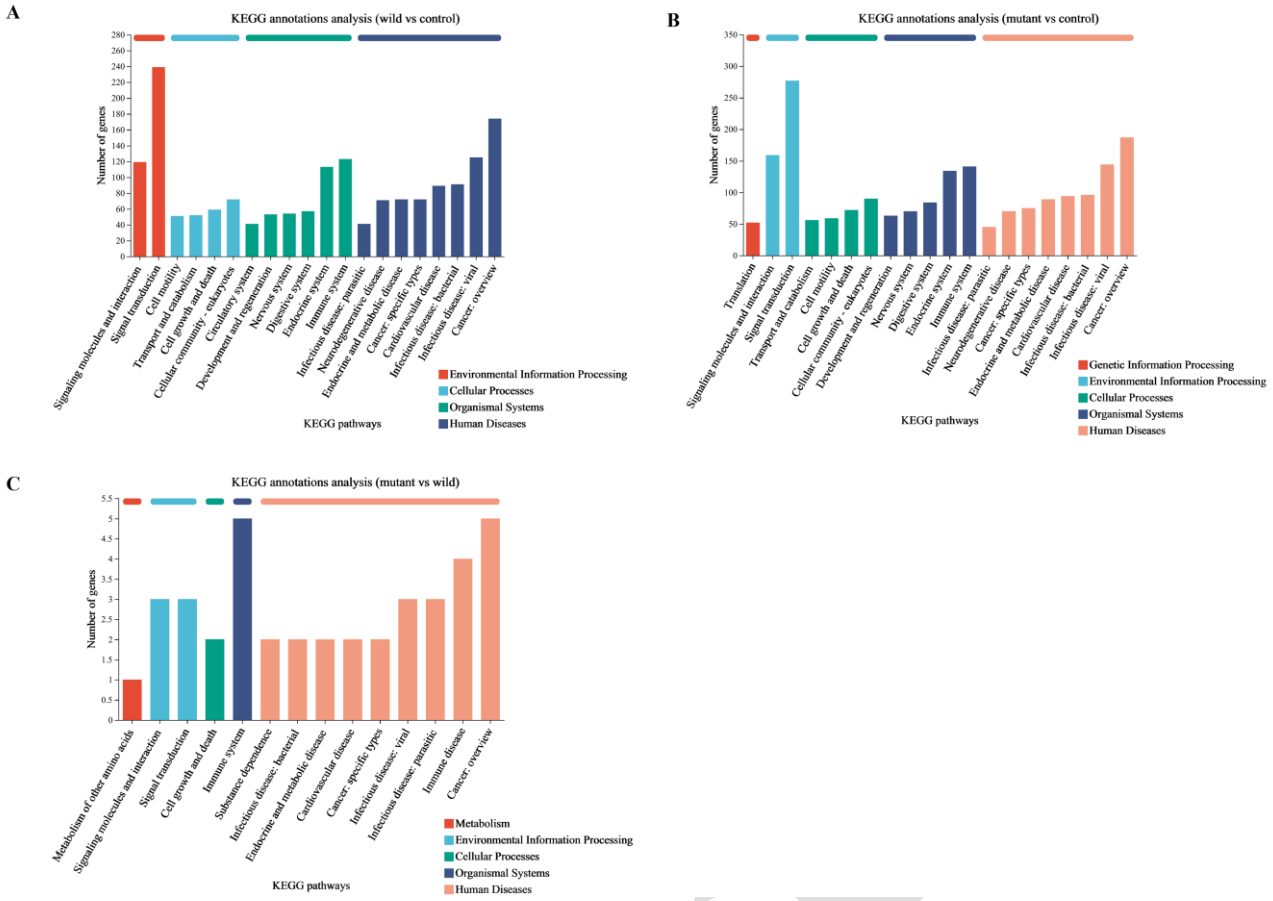

Fig. 6: KEGG annotations analysis of DEGs in HD11 cells following wild and HutZ mutant *Av. paragallinarum* infection. (A) wild vs control. (B) mutant vs control. (C) mutant vs wild.

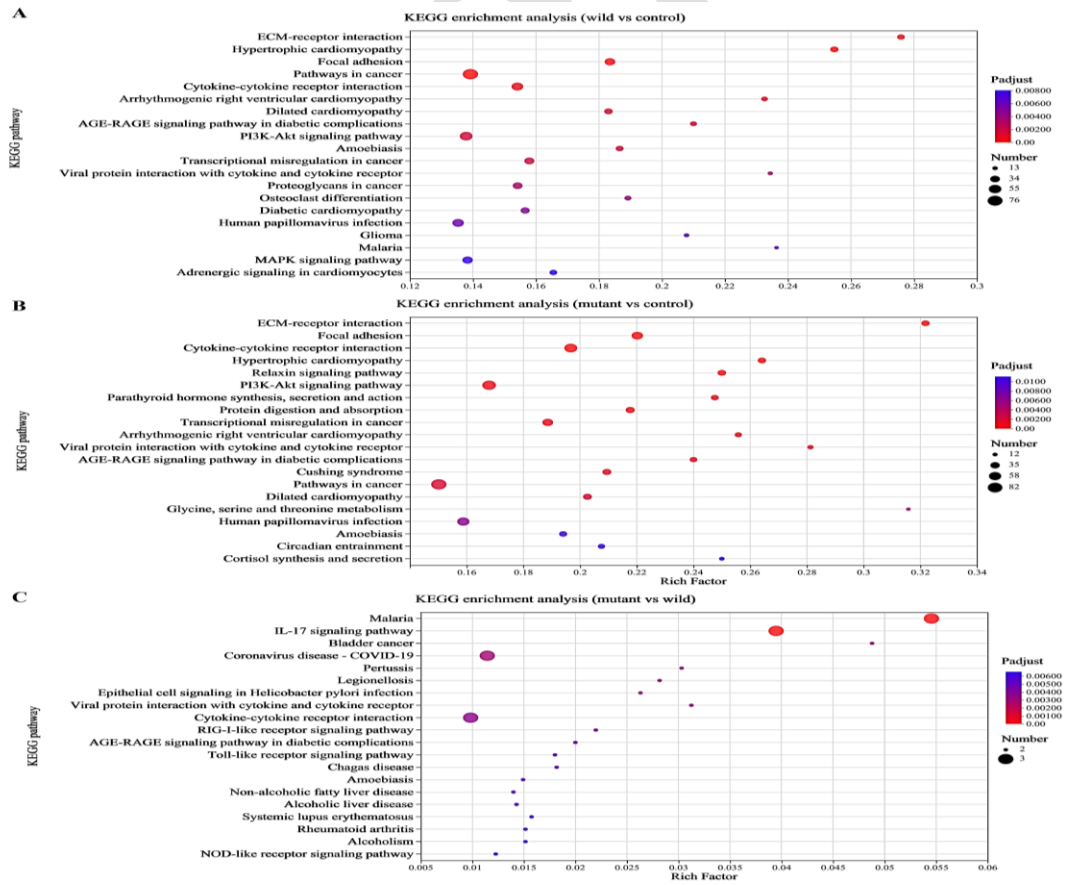

Fig. 7: KEGG enrichment analysis of DEGs in HD11 cells following wild and HutZ mutant *Av. paragallinarum* infection. (A) wild vs control. (B) mutant vs control. (C) mutant vs wild.

and more diverse host response. This suggests that HutZ influences not only bacterial physiology but also the magnitude and nature of host immune activation.

The PI3K-Akt signaling pathway can catalyze the phosphorylation of hydroxyl groups and produces inositol ester products, which governs diverse functions including survival, metabolism, and inflammation in immune cells (An *et al*., 2024; Hrincius *et al*., 2011). In addition, PI3K/AKT is an important upstream axis involved in the activation of NLRP3 inflammasomes (Ives *et al*., 2015) and inhibiting activation of this pathway can suppress the inflammation injury such as endometriosis in rats (Ma *et al*., 2021). Here, RNA-seq analysis also showed that the differentially expressed genes in *Av. paragallinarum*-infected macrophages were related to the PI3K/AKT signaling pathway when compared with the non-infected cells, which is one of the main enriched pathways. At the same time, we found that mutation of HutZ in bacteria could also lead to the enrichment of this pathway in macrophages. Thus, our results support previous findings that microbial pathogens can engage PI3K-Akt signaling to enhance cytokine secretion and cell survival. Activation of this pathway in both infection groups points to its central role in macrophage responses to *Av. paragallinarum*.

The MAPK signaling pathway, a signal transduction pathway widely present in various cells, participates in cell proliferation, differentiation, cell death and immune response (Chen *et al*., 2001; Pearson *et al*., 2001). The activation of MAPK signaling pathway regulates various functional activities of cells through the phosphorylation of cascade threonine and tyrosine residues (Dong *et al*., 2002). There are many kinases responsible for the regulation of MAPK protein, which are all uniformly named MAPKK. Among them, MKK3/6 and MKK4/7 are mainly responsible for the regulation of P38 and JNK pathways, respectively, and are involved in the downstream apoptosis and the expression of various inflammatory cytokines (Ludwig *et al.*, 2001). Another MAPKK protein (MEK) activates the downstream ERK1/2 pathway and is mainly involved in regulating cell proliferation and differentiation and viral RNP output (Xing *et al*., 2010). Accumulating evidence has demonstrated the tight interaction between MAPK signaling pathway and infectious diseases (Cheng *et al*., 2020). For example, *Glaesserella parasuis* stimulation induced the activation of the p38/MAPK pathway and led to the transcription factor Ets2 upregulation for initiating the expression of resistin in porcine alveolar macrophages, a process considered as one of the molecular mechanisms of pig resistin production induced by pathogenic bacteria (Hua *et al*., 2022). In the current study, RNA-seq analysis effectively validated the preferential expression of MAPK in both wild-infected and HutZ mutant-infected HD11 cells. Our findings offer novel insights, indicating that the MAPK signaling pathway has an essential role in macrophages following *Av. paragallinarum* infection, which might be associated with inflammation. The MAPK activation observed here further supports its role as a conserved immune mechanism in response to bacterial challenges.

Pattern recognition receptors (PRRs) present in immune cells can detect pathogen-associated molecular patterns (PAMPs). These PAMPs represent evolutionarily conserved structural features unique to pathogens (Mogensen, 2009; Boucher *et al.*, 2014). Once PRRs recognize PAMPs, they initiate proinflammatory and antimicrobial responses through the activation of a cascade of intracellular signaling pathways. There are four main types of PRRs: Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and DNA-dependent activator of interferon-regulatory factors (DAI) (Akira and Takeda, 2004; Brinkmann and Schulz, 2006; Rehwinkel *et al*., 2010; Le Goffic *et al*., 2006; Kanneganti *et al.*, 2007). In the current study, the HutZ mutant induced stronger enrichment of PRRs signaling pathways, including Toll-like, RIG-I-like, and NOD-like receptors. These pathways are vital for initiating innate immune responses by recognizing PAMPs. Upregulation of PRR-related genes in HutZ mutant infections suggests that HutZ may suppress innate sensing, helping the bacterium evade early immune detection. This is consistent with findings in *Edwardsiella*, where HutZ mitigates ROS generation and reduces macrophage activation (Shi *et al*., 2019). Thus, our data proposes a dual function for HutZ by facilitating bacterial iron metabolism as well as attenuating host immune recognition. Further study is needed to determine whether HutZ directly interferes with PRR signaling or alters the bacterial surface to reduce immunogenicity.

Conclusions: This study presents a transcriptomic overview of the immune response of avian macrophage HD11 cells to infection with *Av. paragallinarum*, revealing distinct responses triggered by the wild and HutZ mutant strains. Infection by either strain activated core immune pathways, notably PI3K-Akt and MAPK signaling. However, the HutZ mutant elicited stronger enrichment of PRR-related pathways, including Toll-like, RIG-I-like, and NOD-like signaling, suggesting an immunomodulatory function for HutZ. These findings support a dual role for HutZ in bacterial virulence with facilitating heme acquisition as well as modulating host immune recognition. The data provide mechanistic insight into how HutZ contributes to immune evasion, potentially enabling intracellular survival. This work offers a molecular framework for exploring HutZ as a therapeutic or vaccine target in strategies to control *Av. paragallinarum* infection.

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Author Contributions: Conceptualization, TX, FX and HS; methodology, FX and HS; software, DL, and QZ; validation, DL, GL and QZ; resources, HS; data curation, FX and HS; writing—original draft preparation, DL and QZ; writing—review and editing, FX and HS; supervision, HS; project administration, HS; funding acquisition, HS. All authors have read and agreed to the published version of the manuscript.

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