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Comprehensive Genomic Analysis of Two *Avibacterium paragallinarum* Strains with Significantly Different Virulence

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ABSTRACT

Background: Infectious coryza, caused by *Avibacterium paragallinarum*, presents economic challenges to the global poultry industry. Previous research highlighted a highly virulent *Av. paragallinarum* strain (ZJ-C), which demonstrated a thousandfold increased virulence compared to a standard strain (Modesto) and evaded existing vaccine protection. This study aimed to conduct a comprehensive genomic analysis of two distinct *Av. paragallinarum* serogroup C strains, focusing on the pathogenic potential and resistance profiles. **Methods:** High-throughput sequencing technology was employed to assemble and analyze the genomic sequences of the ZJ-C (highly virulent) and Modesto (reference) strains. Comparative genomic analysis was facilitated by sophisticated tools like Mauve and BLAST Ring Image Generator and comprehensive databases such as the Virulence Factor Database and Comprehensive Antibiotic Resistance Database. Additionally, the virulence differences predicted by genomic analysis were experimentally validated using a chicken infection model. **Result:** The study identified variations in gene composition, including virulence genes, antibiotic resistance genes and metabolic pathways. Comparative analysis showed that the ZJ-C strain demonstrates increased resistance to antibiotics and higher infectivity compared to the Modesto strain. Unique virulence genes and resistance pathways in ZJ-C suggest evolutionary adaptability. Significant disparities in protein ortholog gene clusters and metabolic pathways were uncovered, indicating strain-specific adaptations. Animal infection experiments confirmed that the ZJ-C strain induced significantly more severe and prolonged clinical symptoms compared to Modesto, validating genomic predictions regarding virulence differences. Our findings offers valuable insights of the genomic landscape and pathogenicity of *Av. paragallinarum*, important for informing future vaccine research and disease control strategies. The study highlights the importance of genomic analysis in understanding pathogenicity, resistance profiles and host interactions of *Av. paragallinarum*, crucial for improving disease management in the poultry industry.

Key words: Antibiotic resistance, *Avibacterium paragallinarum*, Comparative genomics, Vaccine development, Virulence genes.

INTRODUCTION

Avibacterium paragallinarum (Av. Paragallinarum), previously classified as *Haemophilus paragallinarum* (Blackall *et al*., 2005), is the causative agent of Infectious Coryza (IC) in chickens, leading to nasal discharge, facial swelling and significantly decreased growth rates and egg production, causing considerable economic losses to the poultry industry worldwide. Phenotypically, *Av. paragallinarum* isolates are classified into three distinct serogroups (A, B and C) following Page's scheme and further divided into nine serovars (A-1 to A-4, B-1 and C-1 to C-4) based on the Kume classification (Soriano *et al*., 2004). Current vaccines, primarily consisting of inactivated bacteria, provide limited cross-protection between different serogroups and serovars, significantly complicating IC control strategies (Xu *et al*., 2019). Emerging variants of *Av. paragallinarum*, including multidrug-resistant and nicotinamide adenine dinucleotide (NAD)-independent strains, have further intensified the complexity of disease management (Mouahid *et al*., 1992; Blackall *et al*., 2011).

Recent research has highlighted the importance of specific virulence factors in *Av. paragallinarum* pathogenicity. For example, Tu *et al*. (2015) successfully generated a gene knockout mutant targeting the fimbrial protein gene (*flfA*), significantly reducing the bacterium's virulence, there by illustrating the role of fimbrial structures in host colonization and disease progression (Liu *et al*., 2016). Similarly, the discovery of AvxA toxin exhibiting cytotoxic effects on avian macrophage-like cell lines (Küng and Frey, 2013), as well as the identification of a cytolethal distending toxin (Chen *et al*., 2014), underscores the complexity and diversity of virulence determinants in this pathogen. Additional recognized virulence attributes include hemagglutinins, polysaccharide capsules, crude

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polysaccharides and repeats-in-toxin (RTX) proteins (Ramón Rocha *et al*., 2006).

Advances in high-throughput sequencing and genomic analysis technologies have significantly improved our understanding of bacterial pathogenesis, evolutionary dynamics and antibiotic resistance mechanisms (Wichmann *et al*., 2013). Whole-genome sequencing has been performed on numerous pathogenic bacteria, including various species within the genus *Avibacterium* available in the NCBI database. Comprehensive genome sequencing and comparative genomic analyses have already provided crucial insights into the pathogenic mechanisms of various bacterial species, including those belonging to the genus *Avibacterium*. However, genomic information for *Av. paragallinarum* remains relatively sparse, particularly concerning the genetic basis underpinning the pronounced differences in virulence among strains.

In this study, we addressed this knowledge gap by sequencing and comparatively analyzing the complete genomes of two Page serovar C *Av. paragallinarum* isolates: the highly virulent ZJ-C strain and the less virulent reference strain, Modesto. Our previous research has demonstrated clear differences between these strains regarding minimum pathogenic doses and their ability to elicit cross-protection (Mei *et al*., 2023). The findings from this study offer further insights into these differences, which may contribute to the development of more effective vaccines and therapeutics (Morales-Erasto *et al*., 2015). By conducting comprehensive genomic analyses, this study aims to elucidate the underlying genetic determinants contributing to these observed phenotypic differences. Understanding the genomic variations associated with strain-specific pathogenic traits will significantly enhance our ability to develop targeted and effective IC vaccines and therapeutic strategies (Morales-Erasto *et al*., 2015), ultimately improving poultry health management worldwide.

MATERIALS AND METHODS

Media and bacterial strains

The *Av. paragallinarum* strains used in this study were Modesto and ZJ-C. The Modesto strain was originally isolated in the early 1970s in California (Matsumoto and Yamamoto, 1975) and it has since been subjected to extensive laboratory handling. The strain was passed through several laboratories, including Dr. Rick Rimler's group in the early 1980s, before being provided to us by Dr. P.J. Blackall (Queensland University) in the 1990s, GenBank accession number GCA_004931865 (Thornton and Blackall, 1984; Rimler and Davis, 1977). Due to concerns about potential genomic drift from long-term laboratory subculture (>50 passages) and the fragmented nature of the previously published Modesto genome (536 contigs, CheckM completeness=86.55%), we performed re-sequencing to obtain a complete circular chromosome for reliable comparative analysis. The ZJ-C strain, a local isolate from 2021, was obtained from a clinical case of IC in China. Both strains are preserved at the Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Science, China.

Bacterial isolation, propagation and maintenance were performed on tryptic soy agar (TSA) and tryptic soy broth (TSB) containing 10% chicken serum and 25 µg/mL reduced NAD. The agar cultures were incubated in 5% CO_2 and the broth cultures were grown aerobically in a shaking incubator at 170 rpm. All incubations were carried out at 37°C.

Whole-genome sequencing, assembly, finishing and annotation

Genomic DNA from both the ZJ-C and Modesto strains was extracted using the TIANamp Bacterial DNA Kit (Tiangen Biochemical Technology, Beijing, China). Whole-genome sequencing was then performed using the Nanopore sequencing platform (Rand *et al*., 2017). (Oxford Nanopore PromethION R9.4.1 flow cells, 50× coverage) with simultaneous Illumina NovaSeq 6000 sequencing (2×150 bp, 100×coverage) for hybrid assembly. The sequencing data for the ZJ-C strain has been deposited in the NCBI Sequence Read Archive (SRA) under accession number SRR33154522 (BioSample accession: SAMN22999571) and will become publicly available following standard processing by NCBI. Assembly of Nanopore long reads was performed using Flye v2.9 (-nano-hq mode), followed by three rounds of polishing using Medaka v1.7.0 with default parameters. Subsequently, Illumina short reads were integrated for hybrid correction using Unicycler v.0.4.3 in hybrid assembly mode with default parameters (Zhang *et al*., 2025). combined with the hierarchical genome-assembly process (HGAP) (Chin *et al*., 2013). Final genome assembly polishing was performed with Pilon v.1.22 (Broad Institute, Cambridge, MA, USA) using Illumina reads to correct any residual errors.

Gene prediction was performed with multiple tools, including Prodigal (PROkaryotic DYnamic programming Gene-finding ALgorithm; University of Georgia, Athens, GA, USA), Glimmer (The Center for Computational Biology, Johns Hopkins University, Baltimore, MD, USA) and GeneMark HMM (Georgia Institute of Technology, Atlanta, GA, USA) (Delcher *et al*., 2007; Shmatkov *et al*., 1999). Genome sequence annotation was carried out on the RAST online platform (http://rast.theseed.org/FIG/rast.cgi). To further refine the annotation, we used an in - house Python script to run NCBI - BLAST + v2.13.0 with an E - value cutoff of 1e - 5 and 70% identity threshold against the RefSeq non - redundant database (2023 - 04 release) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This automated process efficiently identified and classified genes, improving the accuracy of annotation.

Genomic characterization

Genomic characterization reveals the genetic composition and functional components of an organism. In this study,

we used various tools to obtain a detailed genomic profile. Genome comparison circle diagrams were generated using BLAST Ring Image Generator software (Alikhan *et al*., 2011) and further enhanced with Circos software (Marx and Coon, 2019), highlighting genomic regions, variations and functional domains. Gene functions and metabolic pathways, crucial for understanding the biological roles of the genes, were predicted using Blast2GO software (Conesa *et al*., 2005). Transmembrane domains (TMDs), essential for cellular processes, signaling and interactions, were identified using the TMHMM method (http://www.cbs.dtu.dk/services/TMHMM/).

Genome comparison

Comparative genomics provides essential insights into evolutionary relationships, functional capacities and the pathogenic potential of bacterial strains. To accurately determine the directionality of gene gain or loss, we included *Av. paragallinarum* strain ESV-135 (Genome assembly ASM1176560v1) as an outgroup in ortholog cluster analyses using the OrthoVenn2 platform (https://orthovenn2.bioinfotoolkits.net/home). Genome collinearity, reflecting conservation of gene order, was analyzed using Mauve software (Darling *et al*., 2004) to compare genomes of ZJ-C and Modesto strains. Core and unique protein families were identified by Cluster of Orthologous Groups of proteins (COGs) analysis using OrthoVenn2.

To assess bacterial resistance genes, the ResFinder tool on the CGE platform (https://cge.cbs.dtu.dk/services/) was used with default parameters (≥90% identity, ≥60% coverage). Amino acid sequences of the isolated strains were compared to gain further insight into protein functions. Annotations were performed through databases such as the Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al*., 2020), Non-Redundant Protein Sequence Database (Nr) (Pruitt *et al*., 2012), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*., 2008) and Swissprot (Watanabe and Harayama, 2001), which helped elucidate gene functions, resistance profiles and metabolic pathways. Additionally, COGs (Powell *et al*., 2014), KEGG and Virulence Factors of Pathogenic Bacteria databases (VFDB) were used for protein function annotation with NCBI-BLAST+. Potential genomic islands, often associated with genes of adaptive significance, were predicted using the GIHunter tool (http://www5.esu.edu/cpsc/bioinfo/software/GIHunter/).

To ensure the observed genomic differences between ZJ-C and Modesto were not assembly artifacts, we aligned our newly completed Modesto genome with the previously published fragmented assembly (GCA-004931865, 536 contigs, completeness=86.55%) using MUMmer v4.0 (-maxmatch -mincluster 500) and confirmed completeness by BUSCO v5.4 (Bacteria-odb10 dataset) to normalize gene content assessments.

Animal virulence testing

The virulence differences between the *Av. paragallinarum* strains ZJ-C and Modesto were systematically evaluated using animal infection experiments. Specific-pathogen-free (SPF) chickens (n = 105, 42-day-old) were obtained from Beijing Boehringer Ingelheim Viton Biotechnology Co., Ltd. Chickens were divided into seven groups (n=5 chickens/group) (Four ZJ-C groups with doses ranging from 5×10^2 to 5×10^5 CFU; two Modesto groups with doses ranging from 5×10^4 to 5×10^5 CFU and one non-infected control group) and acclimated for one week before experimentation. All chickens were housed in individual SPF units and provided free access to feed and water throughout the experimental period.

Infection doses were determined based on preliminary pathogenicity data (Mei *et al*., 2023). Chickens were inoculated with bacteria by spinning the infraorbital sinus. Clinical monitoring, including severity scoring of nasal discharge and facial swelling, was performed daily for 7 days. The severity of clinical symptoms was scored according to a previously validated scale (Zhi *et al*., 2025): 0 (no symptoms), 1 (mild: slight nasal discharge and mild swelling), 2 (moderate symptoms) and 3 (severe symptoms, obvious facial swelling with abundant nasal discharge and ocular secretion). The detailed experimental design of the animal virulence testing is presented in Fig 5A. To confirm reproducibility and experimental rigor, animal trials were independently repeated three times at different intervals. This study received ethical approval from the Institute of Animal Husbandry and Veterinary Medicine's Animal Care and Use Committee (Permit number: IHVM11-2402-05).

RESULTS AND DISCUSSION

Genomic comparisons and variations

Understanding the genomic distinctions between the *Av. paragallinarum* isolate ZJ-C and the Modesto reference strain was a key goal of our study, aiming to determine their evolutionary trajectories and functional differences. High-throughput sequencing technology was used to sequence and assemble the genome of ZJ-C, which was found to be 2.67 Mb in size with a GC content of 40.93%. The genomic structure of the serogroup C Modesto strain served as the reference for comparative analyses (Table 1, Fig 1). Using Mauve software, we identified notable collinearity between the genomes of ZJ-C and Modesto, with largely similar structural arrangements. Circular genome diagrams generated using BLAST Ring Image Generator (Fig 2A) showed genome coverage of 82% and 96.41% sequence similarity in the covered regions. Genomic collinearity between the strains is shown in Fig 2B.

Further analysis of ortholog gene clusters revealed that ZJ-C had 92 fewer genes than Modesto. Notably, a more pronounced difference was observed in protein-coding genes, with ZJ-C harboring 503 fewer genes than Modesto (Table 1). This disparity highlights the conservative nature of Modesto's protein-coding genes, while suggesting greater evolutionary dynamism in ZJ-C, potentially involving mutations (Rodriguez-Larrea *et al*., 2010). The genomic

variation observed here highlights the evolutionary dynamics of *Av. paragallinarum*, providing insights into its pathogenicity and potential control measures (Baltrus *et al*., 2011). Orthologous gene cluster analysis indicated that ZJ-C contained significantly fewer protein-coding genes compared to Modesto, with a difference of 503 genes (Table 1). The considerable genomic divergence between these two strains of *Av. paragallinarum* may reflect distinct evolutionary pressures and adaptation strategies. Such genomic differences underscore the evolutionary plasticity within strain ZJ-C and emphasize the need for future functional studies to experimentally verify the precise impact of these gene variations on virulence and pathogenicity. Clarifying the role of these differential genes will enhance our understanding of bacterial adaptation mechanisms and inform strategies for disease control and prevention (Gupta, 2016).

Virulence gene analysis

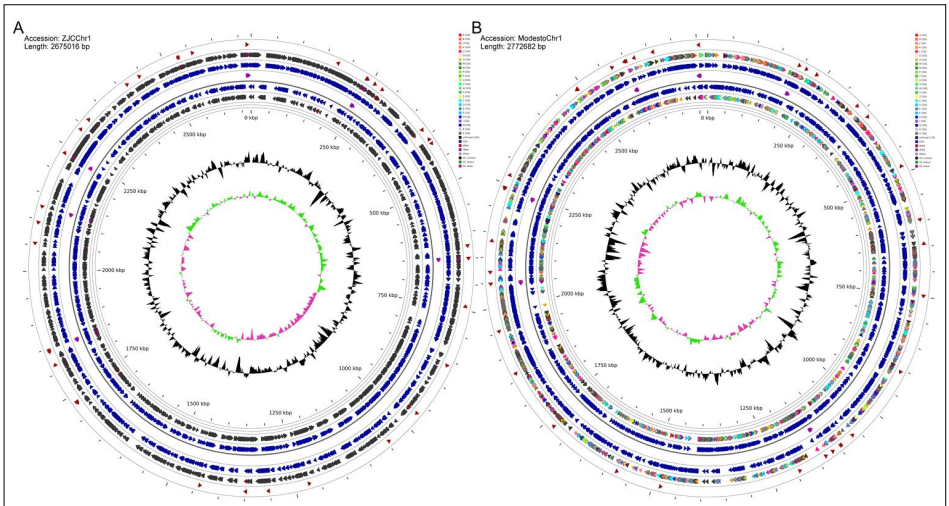
Comparative analysis of virulence genes between *Av. paragallinarum* strains ZJ-C and Modesto helps elucidate their pathogenic potential and resistance profiles. According to the VFDB, ZJ-C harbored 210 virulence genes, while Modesto had 212. Some virulence genes had multiple copies in both strains. Specifically, we found that a pilus guide protein gene, which is often implicated in urinary tract infections caused by *A. baumannii* (Mahapatra *et al*., 2022), was replicated four times in ZJ-C. This gene replication may suggest enhanced host colonization or infection by *Av. paragallinarum*, warranting further investigation.

Further analysis revealed that the two strains shared 168 identical virulence genes, but ZJ-C and Modesto had 42 and 44 unique virulence genes, respectively (Fig 3). Refined KEGG pathway analyses, focused exclusively on bacterial metabolism, pathogenicity and antibiotic resistance pathways, revealed distinct differences. Unique virulence genes in ZJ-C predominantly belonged to membrane transport systems and carbohydrate metabolism (Fig 4A), potentially indicating adaptive advantages in nutrient acquisition and host interaction. Conversely, Modesto-specific genes primarily mapped to metabolic pathways including glycan biosynthesis, lipid metabolism and nucleotide metabolism (Fig 4B), suggesting different adaptive strategies in this strain.

ResFinder analysis, sourced from the drug resistance database (Zankari *et al*., 2012), revealed that ZJ-C contained the tetracycline resistance gene *tet(B)*, which was absent in Modesto. This suggests that ZJ-C may have an increased ability to invade and establish infections in chickens at a potentially more aggressive rate than Modesto (Guillard *et al*., 2016) Additionally, ZJ-C's increased drug resistance profile further strengthens its adaptability in

Table 1: Sequence annotations of the ZJ-C and Modesto strains.

Bacterial strain	ORF	Contig	Bases	CDS RNA	Misc	rRNA	tRNA	tmRNA	GC%	Gene GC%	Gene length
ZJ-C	2740	1	2675016	2630	35	15	59	1	40.93	41.99	869
Modesto	3262	1	2772682	3133	50	19	59	1	41.03	42.05	751



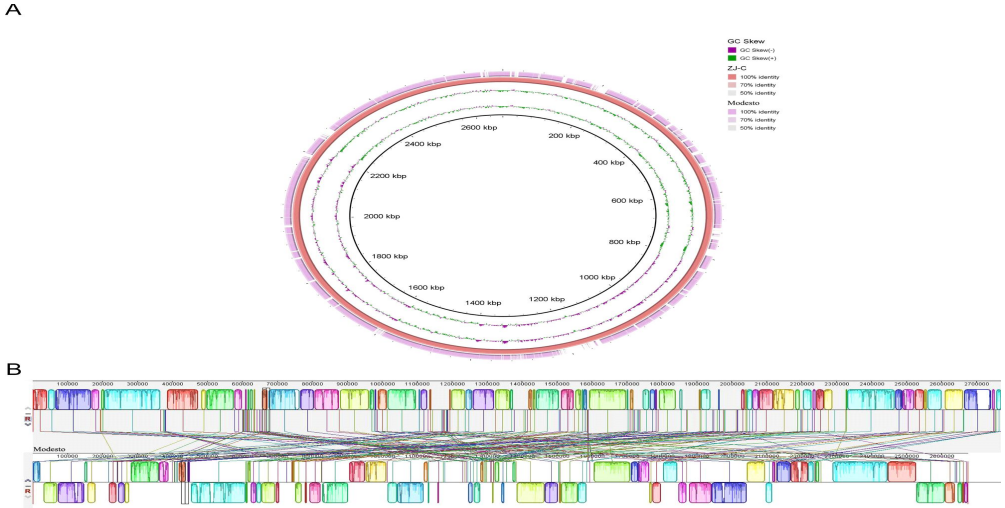
Note: Circular representation indicating (from outside to inside): COG functional annotations on positive-sense strands (circle 1), genes on positive strands (circle 2), rRNA genes (circle 3), genes on reverse strands (circle 4), COG annotations of reverse strands (circle 5), GC skew and GC content distribution (innermost circles).

Fig 1: Circular representation of the *Av. paragallinarum* ZJ-C genome (A). Circular representation of the *Av. paragallinarum* Modesto genome (B).

challenging environments. The differences in virulence genes and resistance profiles hint at a complex interplay between the pathogen's ability to evade treatment and its capacity to cause disease. This potential association between resistance and virulence may reflect evolutionary pressures on ZJ-C (Andersson and Hughes, 2014). Currently, specific mechanisms employed by *Av. paragallinarum* to evade host defenses remain poorly characterized. However, considering the strategies utilized by closely related pathogens, such as *Haemophilus influenzae* and *Shigella*, it is plausible that similar mechanisms, including biofilm formation and Type III secretion systems (T3SS), might play roles in *Av. paragallinarum* pathogenesis. Biofilms could potentially facilitate persistent colonization and resistance to antimicrobial agents, whereas T3SS could assist in translocating virulence factors into host cells to modulate cellular processes essential for colonization and survival (Chatziparasidis *et al*., 2023; Deng *et al*., 2017; Flacht *et al*., 2023; Sowmiya *et al*., 2025). These potential pathogenic mechanisms in *Av. paragallinarum* warrant further experimental verification, which could contribute significantly to understanding its virulence and developing targeted therapeutic strategies.

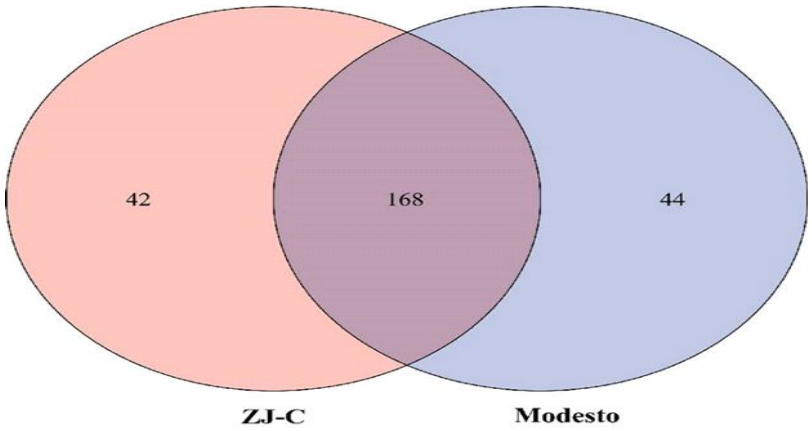
Experimental validation of ZJ-C and Modesto virulence differences

In Modesto-infected groups (5×10^4-5×10^5CFU), clinical symptoms were initially mild, with incomplete infection on day 1. Even at the highest inoculation dose (5×10^5 CFU),



(A) Circular genomic comparison showing genome structure alignment, GC skew, GC content and genomic coordinates. (B) Genome collinearity analysis illustrating gene order conservation and variations between the two strains.

Fig 2: Comparative genomic analysis between *Av. paragallinarum* strains ZJ-C and Modesto.

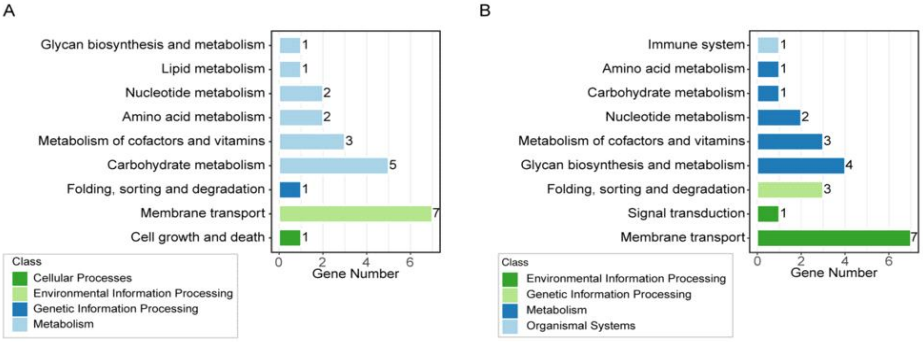


A Venn diagram showing unique *Av. paragallinarum* ZJ-C annotated virulence genes (pink) and unique *Av. paragallinarum* Modesto annotated virulence genes (purple). Virulence genes common to both *Av. paragallinarum* ZJ-C and Modesto are shown in the overlapping area.

Fig 3: Virulence gene analysis of ZJ-C and Modesto.

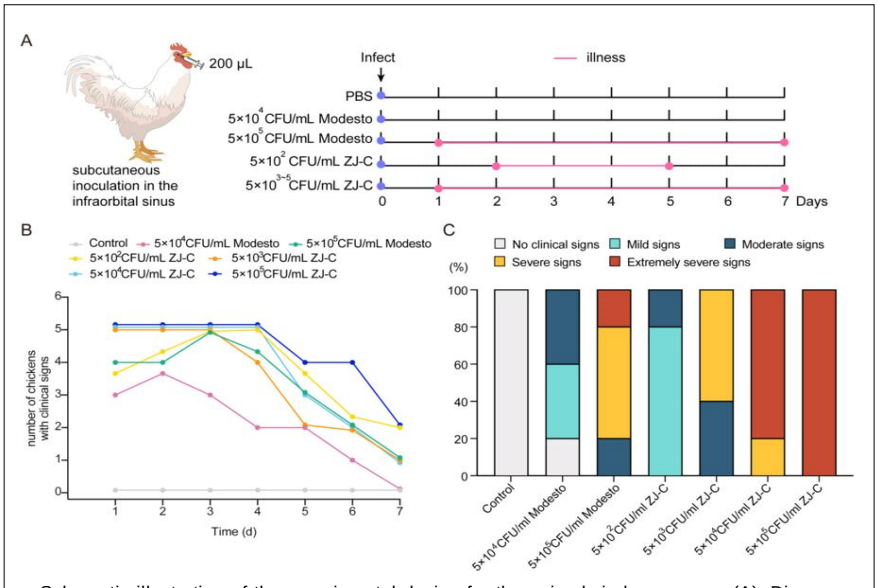
all chickens only displayed symptoms by day 3, followed by a rapid recovery. In contrast, ZJ-C infection at doses ranging from 5×10^3 to 5×10^5 CFU resulted in the onset of clinical symptoms in all chickens on the first day, with persistent severe symptoms lasting until day 5 before showing mild recovery. Notably, even a low-dose ZJ-C infection (5×10^2 CFU) led to 100% of chickens exhibiting symptoms by day 3. These findings indicate that ZJ-C exhibits significantly higher virulence than Modesto (Fig 5B). Using an established infection scoring model, we further assessed the clinical severity of symptoms. At equivalent doses, the Modesto-infected groups consistently displayed milder symptoms. Even at 5×10^2 CFU, ZJ-C infection resulted in more severe clinical signs compared to Modesto at 5×10^4 CFU (Fig 5C). Collectively, both the duration and severity of disease symptoms indicate that ZJ-C possesses substantially higher virulence than Modesto.

The testing of virulence *in vivo* supports our genomic findings, reinforcing the idea that ZJ-C possesses genetic characteristics that enhance its pathogenic abilities. The swift manifestation of disease symptoms and the extended duration of infection seen in chickens infected with ZJ-C indicate that the virulence factors of this strain facilitate effective colonization of the host and evasion of the immune response. Importantly, ZJ-C was capable of establishing infection and inducing severe illness at significantly lower doses compared to Modesto, emphasizing its increased pathogenic potential. Moreover, earlier research has shown



Bar plots illustrating enriched functional categories for unique virulence genes identified in ZJ-C (A) and Modesto (B).

Fig 4: KEGG pathway enrichment analysis of unique virulence genes.



Schematic illustration of the experimental design for the animal virulence assay (A). Disease incidence in chickens infected with different doses of ZJ-C and Modesto strains (B). Clinical severity scores of chickens infected with different doses of ZJ-C and Modesto strains, based on a standardized scoring model (C).

Fig 5: Animal virulence testing comparing ZJ-C and Modesto strains in chickens.

that hemagglutinins, fimbrial proteins and cytolethal distending toxins are essential to the virulence of *Av. paragallinarum* (Wang *et al*., 2014; Liu *et al*., 2016; Chen *et al*., 2014). The genome of ZJ-C includes numerous copies of genes related to these factors, which may enhance adhesion, invasion and modulation of the immune response. The extended persistence of disease in chickens infected with ZJ-C is also consistent with its anticipated metabolic adaptability, which aids in bacterial survival within the host environment. In summary, these findings offer solid experimental evidence that backs our genomic assessment and underline the necessity for focused strategies to address infections caused by hypervirulent strains of *Av. paragallinarum*. Future studies should aim to clarify the specific molecular mechanisms driving the heightened virulence of ZJ-C utilizing transcriptomic and proteomic methodologies.

Antibiotic resistance gene identification

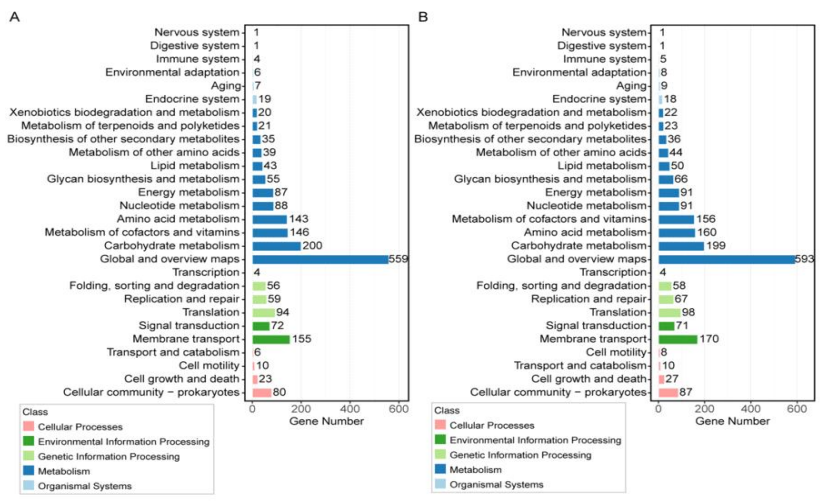
Identifying antibiotic resistance genes (ARGs) in *Av. paragallinarum* isolate ZJ-C is essential for understanding potential treatment challenges in IC management. Using the Resistance Gene Identifier (RGI) mode of CARD, six resistance genes were predicted in ZJ-C, including *qacJ* and *tet(B)*, as well as genes associated with resistance in *Klebsiella pneumoniae* (*e.g*., *KpnH*), *Haemophilus influenzae* (*e.g*., *PBP3*, conferring beta-lactam resistance) and *Escherichia coli* (*e.g*., *EF-Tu*, conferring resistance to pulvomycin and *tetR*). These genes mediate resistance through mechanisms such as antibiotic efflux (*qacJ, tet(B), KpnH, PBP3*) (Tristram *et al*., 2007) and target site alterations (*EF-Tu* and *tetR*) (Hummel *et al*., 2007).

The presence of these resistance genes highlights ZJ-C's potential resilience to common therapeutic antibiotics, posing significant clinical challenges (Andersson and Hughes, 2014; Adwitiya *et al*., 2025). Although ZJ-C's additional ARGs such as tet(B) suggest higher resistance potential compared to Modesto, further studies are required to determine if these resistance mechanisms directly contribute to increased virulence. The potential relationship between resistance and virulence suggests an interplay between the pathogen's ability to evade treatment and cause disease (Martínez and Baquero, 2002; Romero *et al*., 2011). KEGG pathway analysis, refined to specifically highlight resistance-associated bacterial pathways, demonstrated significant enrichment differences: ZJ-C harbored 54 resistance-related proteins compared to Modesto's 45, underscoring ZJ-C's heightened potential for resisting therapeutic interventions (Fig 6) (Davies and Davies, 2010). Understanding the genetic basis of antibiotic resistance will inform targeted therapeutic strategies and facilitate the development of effective interventions against *Av. paragallinarum* infections (Alkatheri *et al*., 2023, Salyers, 2002).

Protein ortholog gene cluster analysis

Orthologous gene analysis is essential for understanding evolutionary relationships and functional similarities between genomes (Pan *et al*., 2009; Sivashankari and Shanmughavel, 2007). To accurately assess genomic differences, we included *Av. paragallinarum* strain ESV-135 (Ref_Strain) as an outgroup in the orthologous gene cluster analysis.

The refined analysis identified orthologous protein gene clusters across strains ZJ-C, Modesto and the outgroup (Fig 7). A total of 1839 conserved clusters were shared by all three strains, suggesting a substantial genomic core characteristic of the genus Avibacterium. Importantly, ZJ-C had 50 unique protein clusters and Modesto possessed 115 unique clusters, highlighting



The histogram highlights significant functional differences in key bacterial metabolic, pathogenicity-associated and antibiotic resistance pathways between the two strains.

Fig 6: KEGG pathway enrichment analysis of *Av. paragallinarum* strains ZJ-C (A) and Modesto (B).

distinct strain-specific evolutionary adaptations potentially linked to their differential phenotypes and pathogenic potential.

The identification of these unique gene clusters provides critical insights into the adaptive mechanisms and evolutionary pressures experienced by these strains. Although the limited number of publicly available *Av. paragallinarum* genomes restricts broader comparative analyses, these unique gene sets offer meaningful targets for future functional studies aimed at clarifying their roles in bacterial lifecycle management, virulence factors and host interaction dynamics. Thus, this comparative ortholog analysis not only enhances our understanding of genomic diversity within *Av. paragallinarum* but also establishes a solid foundation for targeted investigations into the genetic basis of pathogenicity and strain-specific adaptations.

Pathway enrichment analysis and functional implications

To explore the molecular mechanisms underlying differences in virulence and antibiotic resistance between *Av. paragallinarum* strains ZJ-C and Modesto, we conducted KEGG pathway enrichment analysis using the annotated genomic data (Kanehisa *et al*., 2017; Romero *et al*., 2011). Genes from both ZJ-C (2220 annotated genes) and Modesto (2255 annotated genes) were systematically classified into functional categories: Cellular Processes, Environmental Information Processing, Genetic Information Processing, Metabolism and Organismal Systems. Metabolic pathways represented the majority of annotated functions, accounting for 66.4% and 66.9% of genes in ZJ-C and Modesto, respectively. Notably, carbohydrate metabolism, amino acid metabolism and metabolism of cofactors and vitamins were among the most prominently enriched pathways, suggesting essential roles in bacterial adaptation and survival.

Detailed comparative analysis revealed significant functional divergence between the two strains, particularly in pathways associated with bacterial pathogenicity and antibiotic resistance. In ZJ-C, unique gene enrichment was notably prominent in membrane transport mechanisms and carbohydrate metabolism. These functional categories are crucial for bacterial nutrient acquisition, colonization efficiency and persistence in host environments. This enrichment supports experimental observations that ZJ-C exhibits enhanced colonization capability and persistent infection phenotypes. Furthermore, ZJ-C showed higher numbers of genes linked to antibiotic resistance pathways compared to Modesto, potentially reflecting an adaptive advantage that allows survival in antibiotic-exposed environments typical of poultry farms.

Among ZJ-C's unique virulence genes, approximately 62% (26 out of 42) were directly associated with metabolic

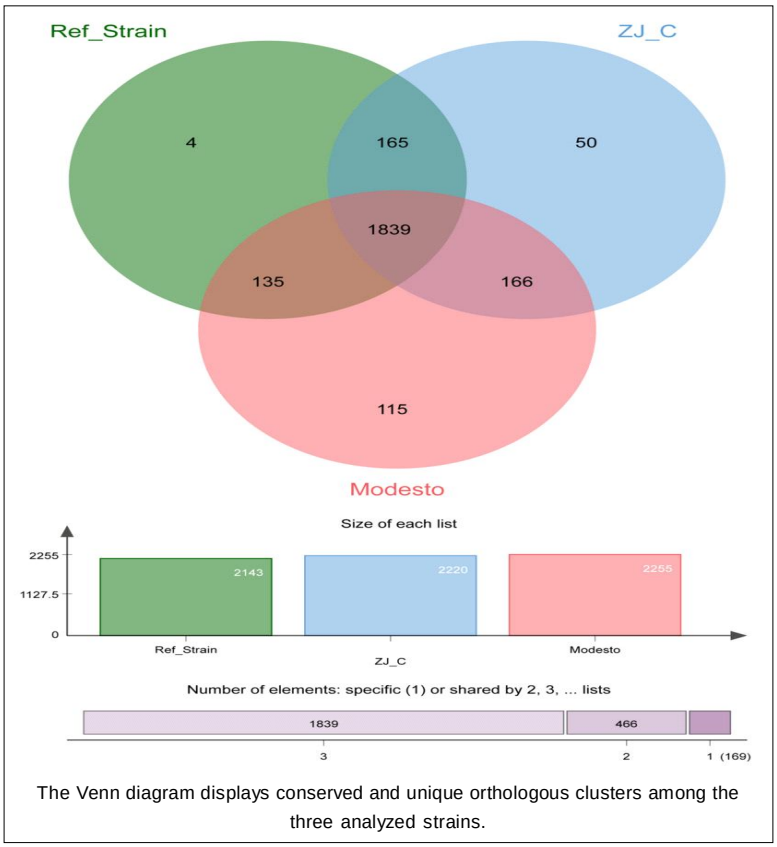


Fig 7: Orthologous protein gene cluster comparison among *Av. paragallinarum* strains ZJ-C and Modesto with *Av. paragallinarum* ESV-135 as an outgroup (Ref_Strain).

processes, especially membrane transport and carbohydrate utilization. In contrast, Modesto's unique virulence genes were primarily enriched in glycan biosynthesis, diverse metabolic processes and antibiotic resistance mechanisms. These genomic distinctions highlight differential evolutionary pressures acting on these strains and provide molecular insights into the distinct virulence phenotypes experimentally observed. Collectively, our pathway enrichment analysis underscores how genomic variations contribute to significant differences in virulence and antibiotic resistance traits between *Av. paragallinarum* strains (Fraser *et al*., 2010). The identification of specific metabolic and resistance-related gene clusters unique to ZJ-C offers critical insights and potential targets for developing more effective vaccines and therapeutic interventions against highly virulent *Av. paragallinarum* strains.

Implications for vaccine development and disease control

The comprehensive genomic comparison between *Av. paragallinarum* serovar C strains ZJ-C and Modesto provides critical insights with direct implications for vaccine development and the management of IC. Significant genomic differences identified between these two strains, particularly in virulence-associated genes, antibiotic resistance determinants and host-interaction pathways, underline the necessity for developing strain-specific or broadly protective intervention strategies (Plotkin, 2010). Distinct genomic profiles observed in ZJ-C and Modesto strains, especially unique gene clusters and differential enrichment in metabolic, pathogenicity and antibiotic resistance pathways, suggest differing pathogenic potentials and therapeutic challenges. Specifically, unique gene clusters in the highly virulent ZJ-C strain related to membrane transport, carbohydrate metabolism and antibiotic resistance mechanisms may confer enhanced capabilities for host colonization, persistent infection and resistance to conventional antibiotics used in poultry farming (Petersen *et al*., 2007), which can affect the efficacy of existing vaccines and treatments. Consequently, these genomic distinctions highlight a potential limitation in the protective efficacy of existing serovar C vaccines, which may not adequately address such highly pathogenic and drug-resistant strains like ZJ-C (Hendriksen *et al*., 2019).

The presence of strain-specific ARGs, such as those identified in ZJ-C, underscores the critical need for tailored antibiotic stewardship practices and novel therapeutic strategies specifically designed to target these resistance determinants. Conversely, the absence or reduced expression of certain genes in less virulent strains, like Modesto, presents opportunities to leverage these genetic vulnerabilities in new vaccine formulations or targeted treatments, potentially reducing pathogenicity and improving vaccine-induced protection. Our findings further advocate for incorporating comparative genomic analyses routinely into IC vaccine development protocols. Considering the substantial genomic variability even within the same serovar, incorporating locally prevalent or genetically representative strains into vaccine formulations might significantly enhance protection efficacy. Additionally, exploring genomic data across multiple *Av. paragallinarum* serovars would help identify conserved or universally critical antigens, facilitating the development of broadly protective vaccines with potential cross-serovar effectiveness.

Finally, the increasing availability of genomic sequences of *Av. paragallinarum* strains in public databases, such as those reported by (Byukusenge *et al*., 2020), provides valuable resources for expanding comparative genomic studies. Integrating these genomic resources into a broader analytical framework will enable more comprehensive understanding of virulence mechanisms, adaptive evolution and resistance emergence in *Av. paragallinarum*. Such integrated genomic approaches are pivotal for designing robust disease control measures and developing next-generation vaccines and therapeutics, ultimately improving poultry health and productivity.

CONCLUSION

This study identified significant genomic differences between the *Av. paragallinarum* serovar C strains ZJ-C and Modesto, particularly in virulence genes and antibiotic resistance genes. The comparative analysis provided insights into the genetic basis of pathogenicity and resistance in these strains, contributing to a better understanding of their evolutionary dynamics. These findings offer useful data for future studies on *Av. paragallinarum* and may inform more targeted approaches in disease control and prevention.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or

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