Veterinary Microbiology 290 (2024) 109995

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

GWAS and comparative genomics reveal candidate antibiotic resistance genes in the avian pathogen *Gallibacterium anatis* for six widespread antibiotics

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ARTICLE INFO

Keywords:
Gallibacterium anatis
Avian infections
Multidrug resistance
GWAS
Phylogenomics

ABSTRACT

Gallibacterium anatis is a Gram-negative bacterium found in the respiratory and genital tracts of various animals, primarily poultry. Its association with septicemia and high mortality in poultry, along with the rise in multidrug-resistant strains, has amplified concerns. Recent research uncovered significant variability in antibiotic resistance profiles among *G. anatis* isolates from different Austrian flocks, and even between different organs within the same bird. In response, in the present study 60 of these isolates were sequenced and a combination of comparative genomics and genome-wide association study (GWAS) analysis was applied to understand the genetic variability of *G. anatis* across flocks and organs and to identify genes related to antibiotic resistance. The results showed that each flock harbored one or two strains of *G. anatis* with only a few strains shared between flocks, demonstrating a great variability among flocks. We identified genes associated with resistance to nalidixic acid, trimethoprim, cefoxitin, tetracycline, ampicillin and sulfamethoxazole. Our findings revealed that *G. anatis* may develop antibiotic resistance through two mechanisms: single-nucleotide mutations and the presence of specific genes that confer resistance. Unexpectedly, some tetracycline-resistant isolates lacked all known tetracycline-associated genes, suggesting the involvement of novel mechanisms of tetracycline resistance that require additional exploration. Furthermore, our functional annotation of resistance genes highlighted the citric acid cycle pathway as a potential key modulator of antibiotic resistance in *G. anatis*. In summary, this study describes the first application of GWAS analysis to *G. anatis* and provides new insights into the acquisition of multidrug resistance in this important avian pathogen.

1. Introduction

Gallibacterium anatis, a member of the *Pasteurellaceae* family, is a versatile Gram-negative bacterium known to infect a wide range of avian species, where it is considered a normal inhabitant of the upper respiratory and lower genital tracts (Persson and Bojesen, 2015). However, it has become increasingly linked to septicemia and elevated mortality in chicken flocks (Varan Singh and R Singh, 2015; Narasinakuppe Krishnegowda et al., 2020). Additionally, it has been known to occasionally infect other mammals such as cattle (Driessche et al., 2020), and even humans (Ghislain Aubin et al., 2013; de Moreuil et al., 2017). The treatment of *G. anatis* infections with antibiotics has become more challenging in recent years, due to the emergence of multidrug-resistant strains (Nhung et al., 2017). Resistance to a variety of antibiotics has been reported, with the most common being ampicillin, clindamycin, oxytetracycline, penicillin, sulfamethoxazole, tetracycline, and tylosin (Johnson et al., 2013; Jones et al., 2013; El-Adawy et al., 2018; Elbestawy et al., 2018; Nassik et al., 2019; Hess et al., 2020; Allahghadry et al., 2021; Algammal et al., 2022). Tetracycline resistance genes, such as *tet*B and *tet*C, have been reported in strains from Mexico (Bojesen et al., 2011a), Denmark (Bojesen et al., 2011b), USA (Jones et al., 2013), and Iran (Allahghadry et al., 2021). However, these genes represent only a small portion of the antibiotic resistance genes catalogue of *G. anatis*, as their discovery was based on PCR of primers specific for tetracycline resistance genes or BLAST searches on sequenced genomes. Given this limited exploration of the genetic components of antibiotic resistance in *G. anatis*, there is a need for more comprehensive research. Therefore, a systematic analysis of the genetic basis of antibiotic resistance is crucial for understanding the evolution of antibiotic resistance in this species.

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https://doi.org/10.1016/j.vetmic.2024.109995
Received 3 April 2023; Received in revised form 20 November 2023; Accepted 11 January 2024
Available online 26 January 2024

In a recent study we determined the antibiotic resistance (AR) profiles of 213 isolates from 29 chicken flocks with and without antibiotic treatments (Hess et al., 2020). The study found a high level of variation in antibiotic resistance profiles among isolates from the same bird, as well as between different organs of the same bird, raising questions about the origins of such diversification. To this purpose, we sequenced a subset of 60 isolates from that study and employed a combination of comparative genomics and genome-wide association study (GWAS) analysis to describe the genetic variability of *G. anatis* among flocks and organs and to identify genes linked to antibiotic resistance for six widespread antibiotics. This previously unexplored approach in the context of *G. anatis* provides a deeper understanding of the evolution of antibiotic resistance in this important avian pathogen.

2. Methods

2.1. Genome assembly and phylogenetic analysis of G. anatis isolates

All isolates were sequenced using Illumina MiSeq technology (150 bp paired ends). The sequencing reads were then uploaded to the PATRIC web application (https://www.patricbrc.org/) for assembly and annotation using the Comprehensive Genome Analysis service with default parameters. Genome sequences were submitted to NCBI under the BioProject PRJNA951522 and BioSamples ranging from SAMN34045049 to SAMN34045108. Phylogenetic analysis was performed using the software parsnp (Treangen et al., 2014) with the strain UMN179 (accession ASM20967v1) as reference. The choice of UMN179 was primarily based on its well-annotated status and the availability of various functional resources like protein-protein interaction networks and KEGG pathways. In this analysis, we opted to filter out SNPs situated in recombination regions by setting the -x parameter to 'YES'. To ensure the robustness of our methodology, we performed a secondary run of parsnp, this time with the -x parameter set to 'NO', thereby not excluding potential recombination regions. Interestingly, the alignments generated from both runs—with and without SNP filtering in recombination regions—were indistinguishable. This outcome suggests the absence of significant recombination events within our dataset, reinforcing the validity of our initial approach. To define strains, we computed Average Nucleotide Identity (ANI) with a custom R script between all pairwise sequences obtained from the parsnp alignment. Isolates with ANI values greater than or equal to 99.9% were considered to belong to the same strain.

2.2. Comparative genomics screening of genes linked to tetracycline resistance

To identify genes associated with tetracycline resistance, we collected known tetracycline resistance genes from literature (Bojesen et al., 2011b, 2011a; Allahghadry et al., 2021). The compiled list included the following genes: *tet*B, *tet*C, *tet*D, *tet*H, *tet*L, *tet*M and *tet*(31). Subsequently, we retrieved the corresponding protein sequences of these genes from UniProt and performed sequence alignment against our isolate dataset using BLASTP, employing a significance threshold of $E < 10^{-5}$. Furthermore, to ensure thorough screening for resistance genes, we utilized two widely accepted tools: ResFinder (Zankari et al., 2012) and the CLC Find Resistance tool with the Nucleotide DB module, to complement our sequence alignment approach.

2.3. GWAS-based identification of antibiotic resistance genes

For the GWAS analysis, a SNP matrix was generated by reformatting the parsnp vcf output. In addition, a gene presence/absence matrix was generated using Panaroo (Tonkin-Hill et al., 2020) (parameters –clean-mode strict –remove-invalid-genes). Both SNPs and genes matrices were correlated with antibiotic resistance profiles using the software TreeWAS (parameters p-value = 0.05) applying Bonferroni correction. In this analysis, resistant isolates were encoded as 1, while intermediate and susceptible isolates were encoded as 0, following the commonly used binary encoding approach. We also explored an alternative encoding scheme where resistant and intermediate isolates were encoded as 1, and susceptible isolates were encoded as 0. However, the former encoding approach resulted in a slightly higher number of candidates associated with antibiotic resistance. Data on antibiotic resistance profiles was taken from our previous study, by selecting antibiotic resistance profiles with at least five data points in each resistance class with respect to the sequenced isolates. For each significant SNP from the SNP-based GWAS, the corresponding gene name was extracted from the parsnp output and used as input for the STRING database (string-db.org) and KEGG pathways database (https://www.genome.jp/kegg/pathway.html) for functional annotation.

3. Results

3.1. Genetic variability of G. anatis among flocks and organs

A total of 60 *G. anatis* isolates were sequenced from a previous study monitoring the antibiotic resistance variability in 213 *G. anatis* isolates from Austrian chicken flocks within the period 2013–2016 (Hess et al., 2020). For our analysis, we randomly selected 15 flocks with available data for at least two organs. From each flock, four isolates were chosen, representing two replicates from two distinct organs of two different individuals (15 flocks x 2 organs x 2 replicates = 60 isolates) The sole exception was flock 9, where we sequenced four replicates from the same organ due to the observed variability in antibiotic resistance patterns within that organ (Hess et al., 2020). The isolates were labelled using a combination of the flock number, organ abbreviation, and a progressive number to maintain a clear and organized naming system. The phylogenetic tree of the 60 isolates showed that many isolates form distinct clusters with long internal branches (Fig. 1). To define strains, we performed pairwise ANI calculations and considered isolates with ANI values equal to or greater than 99.9% as members of the same strain. Based on this definition we found that, of the 15 flocks, 9 were associated with a single strain (flocks 4, 5, 22, 24, 31, 32, 33, 51, 61) and 6 with two different strains (flocks 6, 8, 9, 20, 52, 63). Additionally, some strains were shared between flocks: one strain was shared by flocks 4 and 5 and one was shared by flocks 9 and 33. When considering the relationship between organ and strain, we found that samples from the same organ were associated with a single strain, except for flock 9 where two strains were found in the same organ. Notably, flock 9 is also peculiar in that two of the four isolates from this flock represent a single strain that is associated with flock 33.

3.2. Comparative genomics screening of genes linked to tetracycline resistance

Tetracycline resistance genes are a unique class of resistance genes that can be accurately annotated through homology searches, as demonstrated in previous genomic reports in *G. anatis* (Bojesen et al., 2011b, 2011a; Allahghadry et al., 2021). This is because the resistance is conferred by the presence of the gene itself, rather than genetic mutations in conserved genes. Tetracycline resistance genes in *G. anatis* encode efflux pumps or ribosomal protection proteins that confer resistance. Efflux pumps actively remove tetracycline from the bacterial cell, while ribosomal protection proteins interact with the ribosome to prevent tetracycline from binding and inhibiting protein synthesis (Roberts, 2005). These mechanisms enable *G. anatis* to withstand the antibiotic pressure posed by tetracycline. At first, we screened for known tetracycline resistance genes among our 60 isolates: *tet*B, *tet*C, *tet*D, *tet*H, *tet*L, *tet*M and *tet*(31). The results revealed four tetracycline-susceptible isolates devoid of any tetracycline resistance genes. On the other hand, among the 55 tetracycline-resistant isolates, 51 contained at least one tetracycline resistance gene. Intriguingly, four of the resistant isolates,

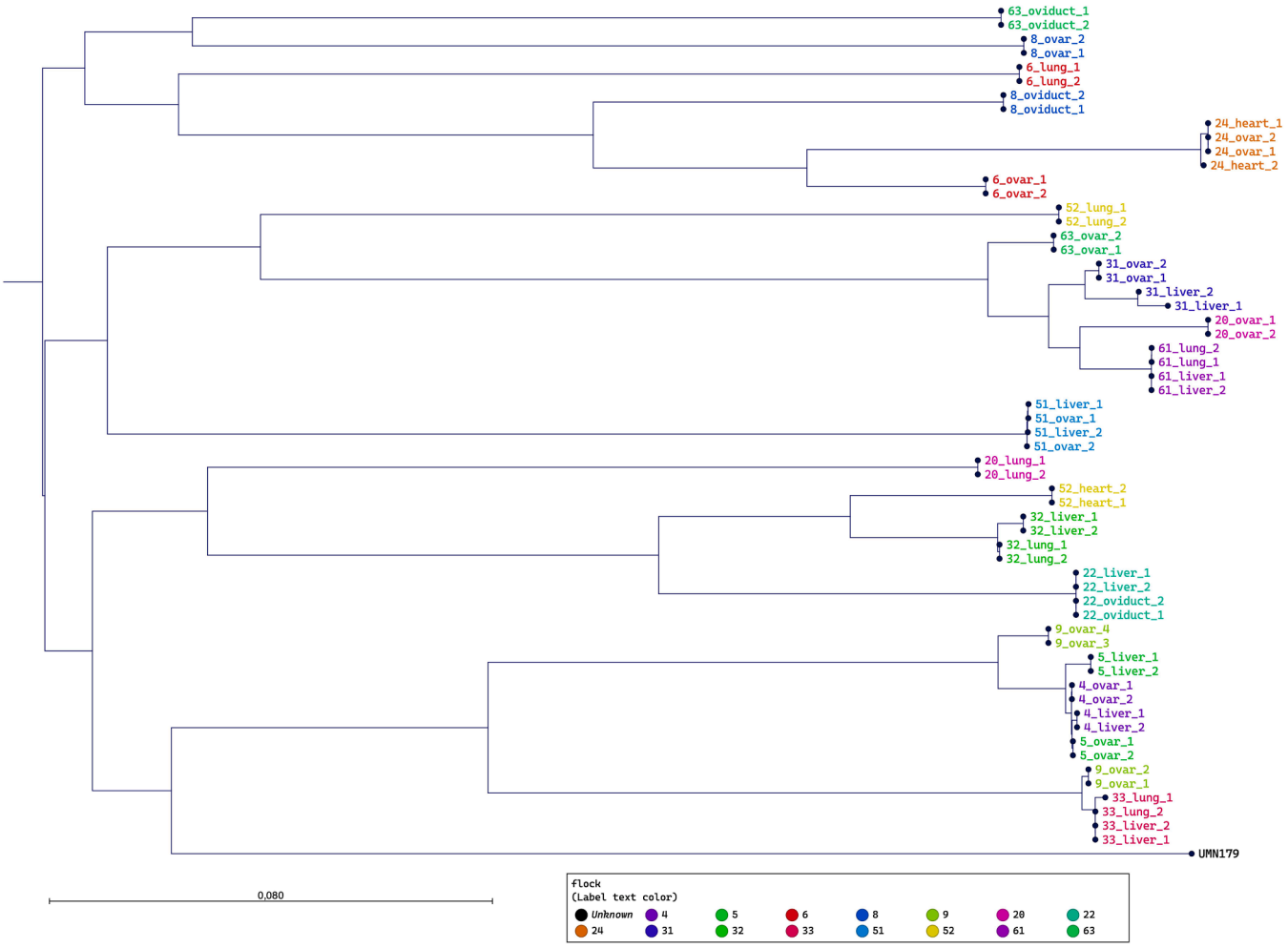


Fig. 1. Phylogenetic tree of 60 *G. anatis* isolates from 15 different Austrian flocks. Phylogenetic tree of 60 *G. anatis* isolates constructed with the software parsnp with the strain UMN179 as reference and filtering out SNPs located in recombination regions. Isolates were labelled using flock number, organ abbreviation and a progressive number and color-coded by flock number.

specifically: 8_ovar_1, 8_ovar_2, 9_ovar_3, 9_ovar_4, were found to be devoid of any known tetracycline resistance genes (Supplementary Table 1 – columns starting with tet*). Using ResFinder for analysis, we confirmed the presence of the *tet*B gene in 38 resistant isolates and the *tet*M gene in 10 resistant isolates (Supplementary Table 1 – ResFinder column). Interestingly, ResFinder also detected the beta-lactamase gene $bla_{\text{TEM-1B}}$ in 19 isolates. It should be emphasized that this gene is known to confer resistance to beta-lactam antibiotics (Shaheen et al., 2011) and not to tetracycline. Its presence in our dataset serves to underscore the broad scope of our antibiotic resistance screening. To corroborate our findings, we employed the CLC Find Resistance tool in conjunction with the Nucleotide DB module. Intriguingly, the results mirrored those obtained from ResFinder precisely (Supplementary Table 1 – Nucleotide DB column). Upon further investigation of the four tetracycline-resistant isolates that lacked any known tetracycline resistance genes, neither ResFinder nor Nucleotide DB could identify any such genes. This points towards the possibility of alternative, or even hitherto undiscovered, mechanisms of tetracycline resistance at play in these isolates.

3.3. GWAS-based identification of genes linked to resistance to widespread antibiotics

In contrast to tetracycline resistance genes, there are no online databases that currently catalogue other antibiotic resistance genes specifically associated with *G. anatis*. To address this issue, we applied a GWAS to correlate a SNPs matrix and a gene presence/absence matrix with 11 antibiotic resistance profiles determined earlier (Hess et al., 2020) (Fig. 2). As a result of this dual GWAS approach, we identified genetic elements associated with resistance to five different antibiotics, the number of isolates for each resistance class of which is detailed in Table 1A. The SNP-based GWAS revealed 19 SNPs linked to resistance to four different antibiotics across eight different genes (Table 1B). The prevalence of resistant alleles varied, but was notably high for nalidixic acid and sulfamethoxazole, with all resistant isolates having the associated SNP in citrate synthase and multidrug efflux system subunit *mdtA*, respectively. Cefoxitin resistance was linked to multiple genes, including phosphotransferase, histidine kinase, hydrogenase 2 protein HybA, biotin synthase, and glutamate synthase subunit alpha, with the prevalence of the resistant allele in the isolates ranging from 63.64% to 90.91%. Trimethoprim resistance was associated with an SNP in the gene encoding oxoglutarate dehydrogenase (succinyl-transferring) with a prevalence of 86.96% in the resistant isolates. In the gene-based GWAS, the presence of specific genes was linked to antibiotic resistance or susceptibility (Table 1C). The gene *sttH*, encoding a cysteine hydrolase family protein, was detected in all (100%) ampicillin-resistant isolates, thereby suggesting a strong association with ampicillin resistance. Conversely, the gene group_4, encoding a DUF4268 domain-containing protein, was observed in 31.91% of sulfamethoxazole-resistant isolates but was more prevalent in the susceptible ones (61.54%). This observation suggests that the absence or

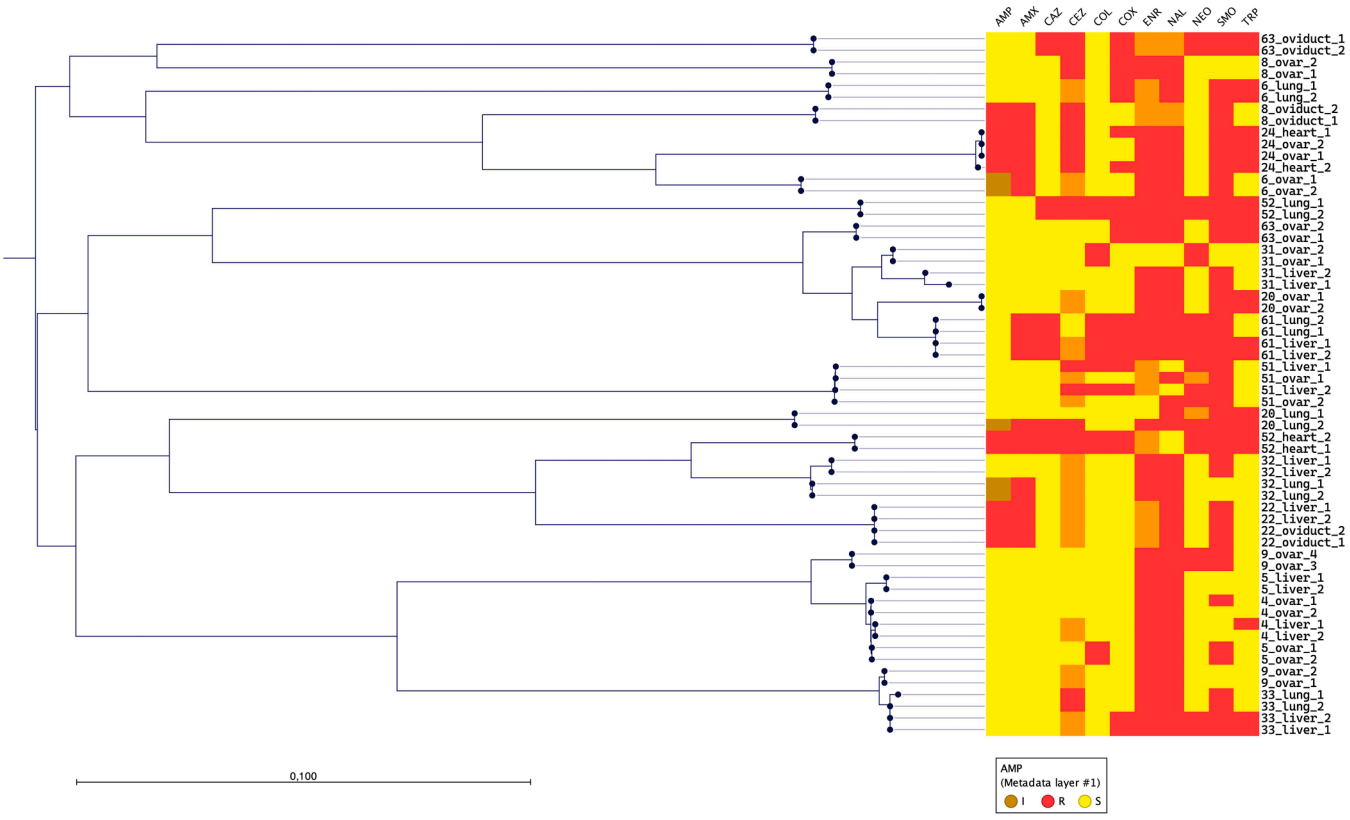


Fig. 2. Antibiotic resistance profiles for the 60 *G. anatis* isolates. The figure displays the evolutionary relationships among the 60 *G. anatis* isolates analyzed in this study using a phylogenetic tree on the left side. On the right side, a heatmap is presented, showing the antibiotic resistance profiles for 11 antibiotics. Each cell is color-coded according to the level of resistance: I = intermediate (orange), R = resistant (red), S = susceptible (yellow).

loss of this gene may be associated with resistance to sulfamethoxazole. In conclusion, our combination of SNP-based GWAS and gene-based GWAS identified genes linked to resistance to five other antibiotics.

3.4. Genetic profile of cefoxitin resistance

For the five genes associated with cefoxitin resistance, we investigated the protein-protein interactions using the STRING database. Our analysis found that four of these five genes belong to the same genetic network (Fig. 3A). The original and alternative gene names for these four genes in the UMN179 strain are: histidine kinase UMN179_01263 (AEC17282.1), hydrogenase 2 protein HybA UMN179_01264 (AEC17283.1), biotin synthase UMN179_02116 (*bioB*), and glutamate synthase subunit alpha UMN179_00528 (AEC16564.1). We report the alternative gene names as they are displayed in the genetic network constructed by the STRING database. The resulting genetic network is composed of four distinct clusters (Fig. 3B), each of which is made up of different subnetworks associated with different functional terms (Fig. 3C). Cluster 1 includes the subnetwork that connects UMN179_01263 (AEC17282.1) and UMN179_01264 (AEC17283.1), which is associated with the two-component system and sulfur metabolism. Cluster 2 includes the gene AEC16564.1 associated with glutamate biosynthesis. Cluster 3 includes the gene *bioB* associated with biotin biosynthesis and fatty acid transport. In addition to the genetic network, we extracted information about metabolic pathways from the KEGG database for the five genes associated with cefoxitin resistance. Both histidine kinase (UMN179_01263) and hydrogenase 2 protein HybA (UMN179_01264) belong to the two-component system pathway (Fig. 4A) and are associated with tetrathionate respiration; biotin synthase (UMN179_02116) belongs to the biotin biosynthesis pathway (Fig. 4B); glutamate synthase subunit alpha (UMN179_00528) connects the citrate cycle to the amino acids biosynthesis (Fig. 4B). In contrast, the fifth gene associated with cefoxitin resistance, a phosphotransferase (UMN179_01176), did not belong to any of the identified genetic networks or metabolic pathways in our STRING and KEGG analyses. This gene contains several PFAM motifs: Hydrolase_3, associated with hydrolytic reactions; S6PP, related to sugar phosphatase activity; HAD, which stands for haloacid dehalogenase-like hydrolase; and GARS_N, indicative of glycyl-tRNA synthetase N-terminus.

4. Discussion

Examining the genetic diversity of *G. anatis* in 60 Austrian chicken flocks highlighted the presence of one or two *G. anatis* strains associated with a single flock with two cases where strains were shared between unrelated flocks. These results extend early diversity estimations obtained through amplified fragment length polymorphisms (AFLP) analysis (Bojesen et al., 2003) and MALDI-TOF spectrometry (Alispahic et al., 2012), which showed that a single strain was usually associated with each flock. These findings are also concordant with a recent genomic survey of 21 isolates from Iran, which reported up to three strains associated with a single flock and provided evidence for strains shared between epidemiologically unrelated flocks (Allahghadry et al., 2021).

Our study employed comparative genomics and GWAS analysis to identify candidate genes linked to resistance to six widespread antibiotics, namely nalidixic acid, trimethoprim, cefoxitin, tetracycline, ampicillin and sulfamethoxazole. Our findings indicate that resistance to nalidixic acid and trimethoprim were both linked to mutations in a single gene, while resistance to cefoxitin was associated with mutations in five different genes. In contrast, we found that resistance to tetracycline and ampicillin was specifically linked to the presence of a single gene. Notably, resistance to sulfamethoxazole was linked to both mutations in a conserved gene and the existence of a specific gene. While

Table 1
Genes linked to antibiotic resistance in *G. anatis* for five widespread antibiotics (A) Number of resistant, intermediate, and susceptible isolates for each antibiotic where significant genes or SNPs have been identified in the GWAS. (B) Results from the SNP-based GWAS linking specific SNPs to antibiotic resistance in *G. anatis*. The table includes the antibiotic name, SNP identifier, p-value, resistant allele, prevalence of the resistant allele in resistant, intermediate, and susceptible isolates, gene ID, location of the SNP, and gene function. (C) Results from the gene-based GWAS identifying specific genes associated with antibiotic resistance in *G. anatis*. The table includes the antibiotic name, gene ID, p-value, prevalence of the gene in resistant, intermediate, and susceptible isolates, and gene function.

Table 1A

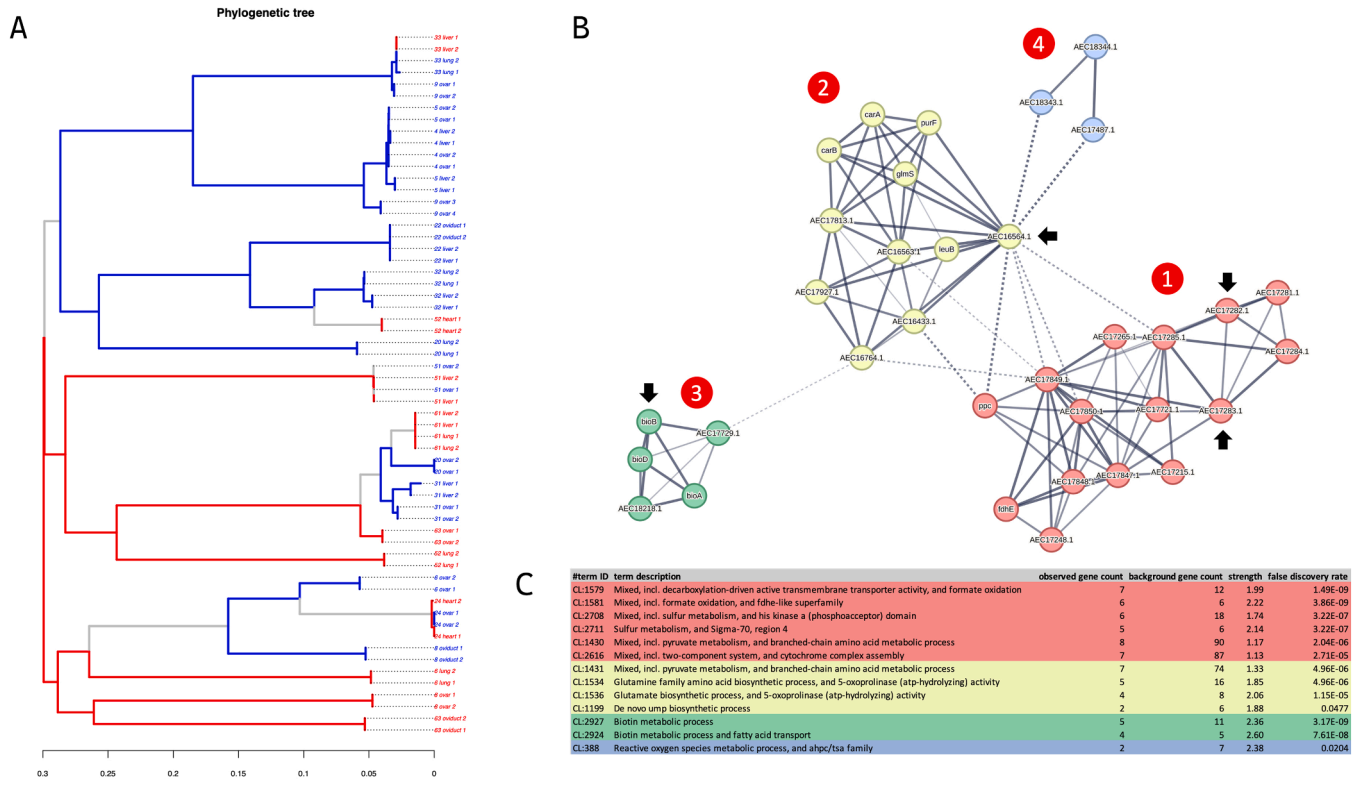
antibiotic	resistant_isolates	intermediate_isolates	sensitive_isolates
Cefoxitin	22	0	38
Nalidixic acid	50	4	6
Sulfamethoxazole	47	0	13
Trimethoprim	23	0	37
Ampicillin	12	5	43

Table 1B

sul	SNP	p-value	resistant_allele	prevalence_resistant	prevalence_intermediate	prevalence_sensitive	gene_id	location	gene_function
Cefoxitin	snp_1279198	0	A	63.64	NA	0.00	UMN179_01176	CDS	Phosphotransferase
Cefoxitin	snp_1367510	0	C	90.91	NA	31.58	UMN179_01263	CDS	Histidine kinase
Cefoxitin	snp_1367537	0	A	90.91	NA	31.58	UMN179_01263	CDS	Histidine kinase
Cefoxitin	snp_1367579	0	A	90.91	NA	31.58	UMN179_01263	CDS	Histidine kinase
Cefoxitin	snp_1367585	0	T	90.91	NA	31.58	UMN179_01263	CDS	Histidine kinase
Cefoxitin	snp_1367588	0	G	90.91	NA	31.58	UMN179_01263	CDS	Histidine kinase
Cefoxitin	snp_1367714	0	G	90.91	NA	31.58	UMN179_01263	downstream	Histidine kinase
Cefoxitin	snp_1367835	0	C	90.91	NA	31.58	UMN179_01264	CDS	Hydrogenase 2 protein HybA
Cefoxitin	snp_2272142	0	A	81.82	NA	10.53	UMN179_02116	CDS	Biotin synthase
Cefoxitin	snp_2272145	0	C	81.82	NA	10.53	UMN179_02116	CDS	Biotin synthase
Cefoxitin	snp_2272226	0	G	90.91	NA	15.79	UMN179_02116	CDS	Biotin synthase
Cefoxitin	snp_2272229	0	G	90.91	NA	15.79	UMN179_02116	CDS	Biotin synthase
Cefoxitin	snp_2272232	0	C	90.91	NA	15.79	UMN179_02116	CDS	Biotin synthase
Cefoxitin	snp_551286	0	C	63.64	NA	15.79	UMN179_00528	CDS	Glutamate synthase subunit alpha
Cefoxitin	snp_551343	0	T	63.64	NA	15.79	UMN179_00528	CDS	Glutamate synthase subunit alpha
Nalidixic acid	snp_397817	0	C	100.00	100.00	33.33	UMN179_00397	CDS	Citrate synthase
Sulfamethoxazole	snp_400007_1	0	T	100.00	NA	0.00	UMN179_00399	CDS	Multidrug efflux system subunit MdtA
Sulfamethoxazole	snp_400007_2	0	T	100.00	NA	0.00	UMN179_00399	CDS	Multidrug efflux system subunit MdtA
Trimethoprim	snp_396139	0	C	86.96	NA	16.22	UMN179_00396	CDS	Oxoglutarate dehydrogenase (succinyl-transferring)

Table 1C

antibiotic	gene_id	p-value	prevalence_resistant	prevalence_intermediate	prevalence_sensitive	gene_function
Ampicillin	sttH	0	100.00	0.00	18.60	Cysteine hydrolase family protein
Sulfamethoxazole	group_4	0	31.91	NA	61.54	DUF4268 domain-containing protein



C

#term ID	term description	observed gene count	background gene count	strength	false discovery rate
CL:1579	Mixed, incl. decarboxylation-driven active transmembrane transporter activity, and formate oxidation	7	12	1.99	1.49E-09
CL:1581	Mixed, incl. formate oxidation, and fdhe-like superfamily	6	6	2.22	3.86E-09
CL:2708	Mixed, incl. sulfur metabolism, and his kinase a (phosphoacceptor) domain	6	18	1.74	3.22E-07
CL:2711	Sulfur metabolism, and Sigma-70, region 4	5	6	2.14	3.22E-07
CL:1430	Mixed, incl. pyruvate metabolism, and branched-chain amino acid metabolic process	8	90	1.17	2.04E-06
CL:2616	Mixed, incl. two-component system, and cytochrome complex assembly	7	87	1.13	2.71E-05
CL:1431	Mixed, incl. pyruvate metabolism, and branched-chain amino acid metabolic process	7	74	1.33	4.96E-06
CL:1534	Glutamine family amino acid biosynthetic process, and 5-oxoprolinase (atp-hydrolyzing) activity	5	16	1.85	4.96E-06
CL:1536	Glutamate biosynthetic process, and 5-oxoprolinase (atp-hydrolyzing) activity	4	8	2.06	1.15E-05
CL:1199	De novo ump biosynthetic process	2	6	1.88	0.0477
CL:2927	Biotin metabolic process	5	11	2.36	3.17E-09
CL:2924	Biotin metabolic process and fatty acid transport	4	5	2.60	7.61E-08
CL:388	Reactive oxygen species metabolic process, and ahpc/tsa family	2	7	2.38	0.0204

Fig. 3. Genetic profile of cefoxitin resistance. Panel A shows a phylogenetic tree of the analyzed isolates, with cefoxitin-resistant isolates highlighted in red and susceptible/intermediate isolates in blue. Panel B presents a genetic network obtained from the STRING database, with four clusters highlighted in different colors, which were identified using mcl clustering with an inflation parameter of 3. Panel C shows the enriched functional terms for each of the four clusters, with columns indicating the term ID, term description, observed gene count, background gene count, strength of enrichment, and FDR. This figure provides insights into the genetic basis of cefoxitin resistance in *G. anatis*, suggesting potential clusters of genes that may be involved in resistance mechanisms.

mutations in the conserved *mdtA* gene were commonly found in resistant isolates, the presence of a hypothetical protein gene, known as group_4, was notably higher in isolates that were susceptible to sulfamethoxazole. This highlights the importance to consider both conserved gene mutations and specific gene presence, as well as gene absence, in understanding the evolution of antibiotic resistance in *G. anatis*. In addition, our results also indicate that the genetic determinants identified in this study have a relatively large effect size since they were detected through screening of a relatively small number of isolates. Further analyses involving a larger number of isolates will be required to detect genetic variants associated with other widespread antibiotics and to identify genetic variants with smaller effect sizes. Overall, our study demonstrates that GWAS can be a powerful methodology to detect genes linked to antibiotic resistance in *G. anatis* and emphasizes the importance of sequencing new isolates to expand our understanding of the evolution of antibiotic resistance in this important avian pathogen. While the current study provides robust in silico evidence supporting the association of specific genes and mutations with antibiotic resistance in *G. anatis*, subsequent experimental validation is crucial for verifying their mechanistic implications. Experimental approaches, such as functional cloning or knock-out/knock-in studies, similar to those conducted by Kristensen et al. (2010), could help confirm the roles of these genetic elements in antibiotic resistance.

Our functional annotation of the resistance genes indicated a wide variety of mechanisms for antibiotic resistance in *G. anatis*. In this section, we aim to integrate our genetic network and metabolic pathway data with insights from other bacterial species to hypothesize plausible resistance mechanisms for the six antibiotics for which we have identified related genes.

We observed that resistance to nalidixic acid was linked to a mutation in the gene encoding citrate synthase, a metabolic enzyme from the citric acid cycle, which catalyzes the formation of citrate and CoA from acetyl CoA and oxaloacetate. This reaction is the first committed step of the citric acid cycle and provides the cell with energy in the form of ATP. Additionally, citrate synthase is also involved in the regulation of the citric acid cycle. Limited research exists on the role of citrate synthase in antibiotic resistance in other bacterial species. One study has shown that mutations in citrate synthase in *Staphylococcus aureus* can confer tolerance to various antibiotics by decreasing its activity and inducing a global metabolic rewiring (Elgrail et al., 2022). Another study has found that down-regulation of citrate synthase in *Escherichia coli* is responsible for enrofloxacin resistance also through decreased metabolism (Piras et al., 2015). These findings suggest that a similar metabolic shift may occur in isolates of *G. anatis* with citrate synthase mutations, making them resistant to nalidixic acid. Interestingly, in a recent study on the *G. anatis* strain NCTC11413, citrate synthase was identified as a key virulence factor (Rajapaksha et al., 2022), suggesting that there may be a strong association between virulence and antibiotic resistance in *G. anatis*. The discovery provides new insights into the complex relationship between virulence and antibiotic resistance (Schroeder et al., 2017) and highlights the need for further research to better understand the underlying mechanisms and their impact on *G. anatis* biology.

Similarly, we found that resistance to trimethoprim was associated with a mutation in the oxoglutarate dehydrogenase, also a metabolic enzyme from the citric acid cycle, which catalyzes the conversion of oxoglutarate to succinyl-CoA. An in vitro evolution study in *Escherichia coli* showed that a mutation in the oxoglutarate dehydrogenase gene conferred resistance against streptomycin and ciprofloxacin (Lopatkin et al., 2021). This resistance was attributed to a reduction in basal respiration, which in turn limited the induction of the citric acid cycle by antibiotics, thereby decreasing metabolic toxicity and drug lethality. Taken together, these results show that resistance to nalidixic acid and

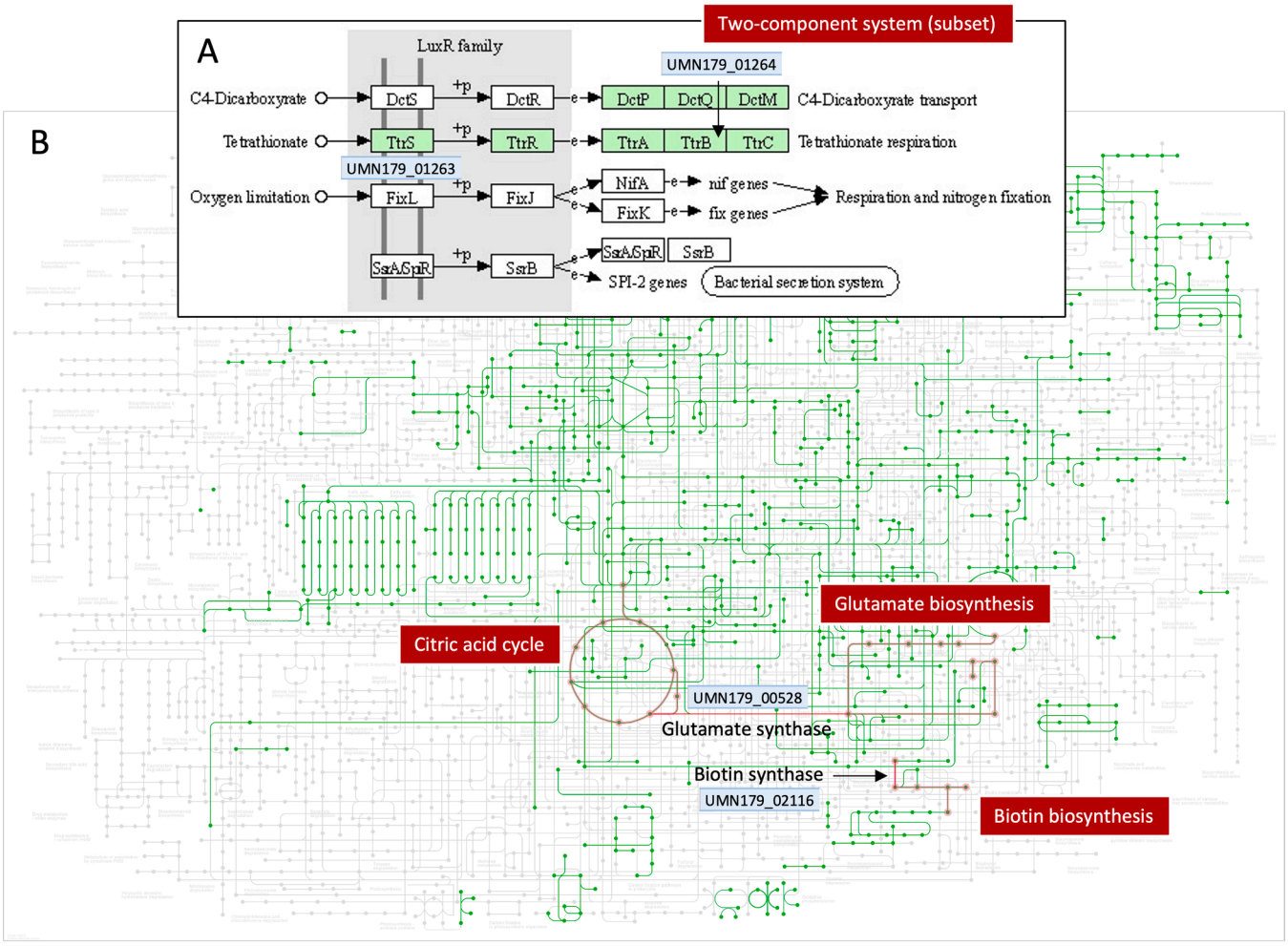


Fig. 4. KEGG pathways involved in cefoxitin resistance. (A) A close-up view of the two-component system pathway, highlighting genes *Ttrs* (UMN179_01263) and *TtrB* (UMN179_01264), which are involved in tetrathionate respiration. (B) An overview of primary metabolism in *G. anatis*, emphasizing the citric acid cycle, biotin biosynthesis, and glutamate biosynthesis pathways, along with the associated candidate genes that contribute to cefoxitin resistance.

trimethoprim in *G. anatis* might be linked with a metabolic shift involving the citric acid cycle. These findings underscore the importance of metabolic mechanisms in contributing to antibiotic resistance, alongside the more traditional resistance mechanisms (Jian et al., 2021). Furthermore, our results have significant implications for the development of novel treatment strategies for bacterial infections. Altering the metabolic state of bacteria has been shown to enhance the effectiveness of antibiotics (Stokes et al., 2019), suggesting that targeting the citric acid cycle could be a promising approach. In summary, our study suggests that the citric acid cycle could potentially play a key role in modulating antibiotic resistance in *G. anatis*.

While in most cases antibiotic resistance was linked to mutations in single genes, we found that cefoxitin resistance was associated with mutations in five different genes, suggesting a more complex mechanism compared to other antibiotics. To better understand this mechanism, we analyzed the genetic network and metabolic pathways associated with these five genes. One possible explanation is that *G. anatis* may use tetrathionate respiration, which involves the histidine kinase and hydrogenase 2 protein HybA, to gain a growth advantage under stressful conditions such as antibiotic exposure. Mutations in these genes could enhance the bacteria's ability to utilize tetrathionate metabolism, potentially increasing their resistance to antibiotics. In support to this, previous research on the ortholog of *hybA* in *Escherichia coli*, tetrathionate reductase, has shown that it is involved in tetrathionate metabolism and contributes to bacterial growth in anaerobic conditions (Adsit et al., 2022). Additionally, mutations in biotin synthase may also contribute to cefoxitin resistance. Biotin is a cofactor for various essential enzymes in the cell, including those involved in cell wall synthesis. Alterations in the availability of biotin due to mutations in biotin synthase may indirectly affect the function of these enzymes, leading to changes in cell wall structure or integrity that could affect the efficacy of cefoxitin. Mutations in glutamate synthase subunit alpha, which is involved in the synthesis of glutamate, may also contribute to cefoxitin resistance. Disruptions to glutamate metabolism could affect the balance of amino acids required for proper cell function, and thus contribute to the development of resistance to cefoxitin by affecting cell growth or the function of proteins targeted by the antibiotic. Interestingly, the fifth gene implicated in cefoxitin resistance codes for a phosphotransferase that is not part of the same genetic network as the other four genes. This gene contains several PFAM motifs, including Hydrolase_3 (a common hydrolase domain), S6PP (S6 pyrophosphatase domain), Hydrolase HAD (haloacid dehalogenase-like hydrolase domain), and GARS_N (glycyl-tRNA synthetase N-terminal domain). Given its absence from known KEGG pathways and its unique domain composition, this phosphotransferase might be operating through a distinct, yet unidentified, mechanism to confer resistance. Moreover, it is important to note that these are hypothetical scenarios, and further research is needed to fully understand the mechanisms of antibiotic resistance against cefoxitin in *G. anatis*.

In some cases, the presence of specific genes rather than mutations

was linked to antibiotic resistance. We found that resistance to tetracycline was attributed to the presence of various tetracycline resistance genes, such as *tet*B, *tet*C, *tet*D, *tet*H, *tet*M and *tet*(31). Notably, the *tet*L gene was absent in all resistant isolates. Further investigation using UniProt (Bateman et al., 2023) revealed only one entry for this gene in *G. anatis*, indicating that *tet*L is rare in this species. In contrast, no tetracycline resistance genes were identified in any of the four susceptible isolates, which was consistent with their phenotype. Unexpectedly, four resistant isolates lacked the aforementioned resistance genes, suggesting the involvement of novel mechanisms of tetracycline resistance that requires additional exploration. Similarly, resistance to ampicillin was also associated with the presence of a specific gene, encoding for a cysteine hydrolase family protein. Studies have revealed that certain cysteine hydrolases possess the ability to deactivate beta-lactam antibiotics in bacteria such as *Pseudomonas aeruginosa* (Grøftehauge et al., 2015) and *Streptomyces/Acinetobacter* (Maruyama and Hamano, 2009). This suggests that *G. anatis* isolates that exhibit resistance to ampicillin might also have the ability to inactivate the antibiotic through a similar mechanism.

Finally, resistance to sulfamethoxazole was linked to mutations in the gene encoding the multidrug efflux subunit *mdtA*, identified in our SNP-based GWAS. We found that all resistant isolates had mutations in the *mdtA* gene, implying a crucial role for this gene in antibiotic resistance. The *mdtA* gene is often expressed together with other subunits, such as *mdtB* and *mdtC*, to form a functional heteromultimeric protein that pumps out drugs from the bacterial cell (Nagakubo et al., 2002). This suggests that mutations in *mdtA* from resistant *G. anatis* isolates could enhance the bacterium's ability to actively transport the antibiotic out of the cell, thereby conferring increased resistance to sulfamethoxazole. The *mdtA* gene was firstly characterized in *Lactobacillus lactis* and *Escherichia coli* in 2001 (Perreten et al., 2001), where it was found to confer resistance to antibiotics belonging to the macrolide, lincosamide, and tetracycline classes. Recent studies have also shown that the *mdtA* gene is present in the gut microbiome of broiler chickens from China (Xiong et al., 2018; Rui and Qiu, 2022), indicating that it can also be found in commensal bacteria from poultry. These findings suggest that monitoring the presence of *mdtA* in bacterial populations is important for understanding and controlling antibiotic resistance. Conversely, our gene-based GWAS revealed that the DUF4268 domain-containing protein (group_4) was more frequently present in sulfamethoxazole-susceptible isolates than in resistant ones. Although the role of this protein and its uncharacterized domain in antibiotic susceptibility remains unclear, its lower prevalence in resistant strains suggests that its absence may be linked to resistance mechanisms in *G. anatis*.

In conclusion, our GWAS and comparative genomics analysis suggest that antibiotic resistance in *G. anatis* may be associated with both mutations in conserved genes and the presence of specific candidate resistance-conferring genes, which might allow *G. anatis* to swiftly adapt to the detrimental effects of antibiotics. Our analysis also points to the potential significance of the citric acid cycle in modulating antibiotic resistance in this species. These findings contribute to a deeper understanding of the mechanisms of multidrug resistance in *G. anatis* and have implications for future strategies aimed at combating this issue.

CRediT authorship contribution statement

Palmieri Nicola: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Hess Claudia:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Hess Michael:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT 4.0 in order to improve the text. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

The authors of this study declare that they have no conflicts of interest, financial or otherwise, that could influence the results or interpretation of the research presented. All authors have contributed to the work and approved the final manuscript for submission.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.109995.

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