Title

Convergence of resistance and evolutionary responses in *Escherichia coli* and *Salmonella enterica* co-inhabiting chicken farms in China

Authors

Michelle Baker[1‡], Xibin Zhang[2‡], Alexandre Maciel Guerra[1‡], Kubra Babaarslan[1], Yinping Dong[3], Wei Wang[3], Yujie Hu[3], David Renney[4], Longhai Liu[5], Hui Li[6], Zhiqin Tong[6], Nicole Pearcy[1], Meimei Zhang[7], Yingzhi Geng[7], Li Zhao[8], Zhihui Hao[9], Junshi Chen[3], Zixin Peng[3*], Fengqin Li[3*], and Tania Dottorini[1,10*]

Affiliations

[1] School of Veterinary Medicine and Science, University of Nottingham, College Road, Sutton Bonington, Leicestershire, UK, LE12 5RD

[2] Shandong New Hope Liuhe Group Co., Ltd., and Qingdao Key Laboratory of Animal Feed Safety, Qingdao, Shandong, P.R. China, 266000

[3] NHC Key Laboratory of Food Safety Risk Assessment, China National Center for Food Safety Risk Assessment, Beijing, P. R. China, 100021

[4] Nimrod Veterinary Products Limited, 2, Wychwood Court, Cotswold Business Village, Moreton-in-Marsh, GL56 0JQ, United Kingdom.

[5] Shandong Kaijia Food Co. LTD, Weifang, P. R. China

[6] Luoyang Center for Disease Control and Prevention, No. 9, Zhenghe Road, Luolong District, Luoyang City, Henan Province, P. R. China, 471000

[7] Liaoning Provincial Center for Disease Control and Prevention, No. 168, Jinfeng Street, Hunnan District, Shenyang City, Liaoning Province, P. R. China, 110072

[8] Agricultural Bio-pharmaceutical Laboratory, College of Chemistry and Pharmaceutical Sciences, Qingdao Agricultural University, No. 700 Changcheng Road, Chengyang District, Qingdao City, Shandong Province, P. R. China, 266109

[9] Chinese Veterinary Medicine Innovation Center, College of Veterinary Medicine, China Agricultural University, Haidian District, Beijing City, P. R. China, 100193

[10] Centre for Smart Food Research, Nottingham Ningbo China Beacons of Excellence Research and Innovation Institute, University of Nottingham Ningbo China, Ningbo, P. R. China, 315100

[*] Corresponding authors (tania.dottorini@nottingham.ac.uk)

[‡] These authors contributed equally to this work.

Abstract

Sharing among different pathogens and commensals inhabiting same hosts and environments has significant implications for antimicrobial resistance (AMR), especially in settings with high antimicrobial exposure. We analysed 661 *E. coli* and *S. enterica* isolates collected within and across hosts and environments, in 10 Chinese chicken farms over 2.5 years using novel data-mining methods. Most isolates within same hosts possessed same clinically relevant AMR-carrying mobile genetic elements (plasmids: 70.6%, transposons: 78%), which also showed recent common evolution. Machine learning revealed known and novel AMR-associated mutations and genes underlying resistance to 26 antimicrobials and primarily associated with resistance in *Escherichia coli* and susceptibility in *Salmonella enterica*. Many of these genes were essential and affected same metabolic processes in both species, albeit with varying degrees of phylogenetic penetration. Multi-modal strategies are crucial to investigate the interplay of mobilome, resistance and metabolism in cohabiting bacteria, especially in ecological settings where community-driven resistance selection occurs.

MAIN TEXT

Introduction

AMR is a major global health problem and livestock farms and their surrounding environment have been highlighted as a potential source of AMR infections (*1*). Plans to tackle AMR fall into three major categories: surveillance, prevention and treatment (*2*). The development of AMR in individual bacterial species is dependent not only on antibiotic exposure but also on the presence of other bacteria within their environment and with which they interact (*3*). Hence, acknowledging the extent to which bacteria within the same environment are able to co-evolve and share their genome could help the development of more efficient treatments to fight AMR (*4, 5*). Tracking and understanding the spread of mobile genetic elements and their AMR-associated gene content in human and animal pathogens has been suggested as an important factor to help mitigation efforts (*4*). In this study, we have focused on two important opportunistic pathogens found in livestock *Escherichia coli* and *Salmonella enterica*, which display high levels of drug resistance, and both have zoonotic potential (*6, 7*). These species can share genetic material both within and potentially between species, a mechanism by which AMR is spread (*8*).

A recent study found that in model experimental systems *E. coli* and *S. enterica* co-cultures evolved different antimicrobial resistance mechanisms than monocultures of either species subject to the same experimental conditions (*9*). It has also been shown that within the same environment *E. coli* and *Salmonella spp*. are able to directly communicate using bacterial signalling, increasing antibiotic tolerance (*10*). A previous study showed that plasmids carrying ARGs, can be transferred from *S. enterica* to *E. coli* (*11*) and proposed that similarity of the plasmids in S. enterica isolated from a chicken gut, to plasmids found in pathogenic *E. coli*, another gut resident, could indicate transmission between the species (*11*). Additionally, bacteria can benefit from antibiotic inactivation mechanisms of other species (such as beta lactamase production) (*12*) altering the cost-benefit drivers of resistance evolution. Hence, both bacterial signalling and transfer of genomic material could impact AMR for species within the same environments. However, AMR phenotypes in *E. coli* are not a good indicator of AMR phenotypes in *S. enterica* even when taken from the same sample, with the authors suggesting that complex AMR dynamics need to be further studied on a genomic level to understand this disparity (*13*).

In this study, by taking isolates from the same biological samples, from animals and surrounding environments, we have been able to dissect patterns of similarities and the diversities, at a larger scale involving antimicrobial susceptibility profiles against 28 antimicrobials and phylogeny as well as, at a finer scale searching for resistomes associated to AST phenotypes, metabolic adaptations and mobile genetic elements and their co-evolution between these two species in real-world settings which pose a high risk of AMR transfer to humans and the environment (*14*). We hypothesise that: (i) Resistance and evolutionary responses to antibiotic treatments of bacteria is significantly influenced by other bacteria inhabiting the same microbiota and the ecological context. In this respect, responses to antibiotic treatments should not be considered as traits of individual bacteria species but the common evolutionary trajectory of resistance of coexisting and interacting bacteria; (ii) This collective evolutionary resistance trajectory exist between focal pathogens and resident commensal, subject to the same antibiotic pressure, and can propagate in interconnected but different communities, such as those in the gastrointestinal tract of animals and the natural microbial communities present in environment where the animals live; (iii) We further hypothesise that the selection for antimicrobial resistance is influenced by the ecological context and it is associated with different levels of changes occurring at the mobilome, resistome and metabolism found similarly correlated to AMR in different bacterial species within the same environment; and (iv) an ML-powered approach that integrates culture-based AST, culture-independent methods (whole genomic data, WGS), Bayesian divergent analysis and genome-scale metabolic models can be used to identify the traits and evolutionary responses to antimicrobials in coexisting bacteria such as *E. coli* and *S. enterica* across interconnected communities exposed to high antibiotic usage.

To test these hypotheses, we collected isolates of two important opportunistic pathogens *E. coli* and *S. enterica*, common in agricultural settings and representing a significant cause of diarrheal disease-associated mortality in humans, particularly low-to-middle income countries(*15*). We first, characterised the population structure, evolution and AMR phenotypes of both *E. coli* and *S. enterica* strains circulating on ten commercial poultry farms and four connected slaughterhouses in three provinces of China over a two-and-a-half-year period, also identifying any mobile genetic elements potentially able to transfer AMR determinants between the two species. We found that most isolates of *E. coli* and *S. enterica* within the same host possessed the same AMR-carrying mobile genetic elements, which also appear to have co-evolved by Bayesian divergence analysis. Secondly, using a machine-learning pipeline integrating a wide repertoire of genetic features covering the whole genome (SNPs within core genes and intergenic regions and accessory gene content) we identified genes, mutations and regulatory elements that were strongly associated with the experimentally determined resistance to a panel of 26 different antimicrobials in each isolate of each species. Thirdly and finally, when analysing these AMR-associated features using genome-scale modelling and protein-protein interaction networks, we found that they were linked to the same functional pathways, which were essential for growth, and which were affecting biochemical fluxes within both bacteria indicating potential metabolic adaptions.

Results

Differences in antimicrobial-resistant phenotypes, phylogeny, SNPs, and evolution are observed between the *E. coli* and *S. enterica* isolates across farms, source, and time.

Altogether, we collected 518 *E. coli* isolates and 143 *S. enterica* isolates from a total of 692 animal and 285 environmental samples (see Methods and Materials) taken from both farms and abattoirs; with an overlap of 113 samples where both species were cultured from the same sample. Samples were taken from ten farms and four connected abattoirs in three

provinces of China: Henan (three farms and two abattoirs), Liaoning (three farms and one abattoir) and Shandong (four farms and one abattoir) over a 25-month period, between March 2019 and April 2021. **Table S1**. In each location samples were taken at three points over the six-week broiler production cycle: t_1 - mid-life approximately three weeks old, t_2 - full grown approximately six weeks old and t_3 - end-of-life approximately six weeks and 1-5 days, post slaughter sampling. The most represented province by isolate count for *E. coli* was Henan (*n*=219) followed by Shandong (*n*=209) then Liaoning (*n*=90). Analogously, for *S. enterica* the most represented province was Henan (*n*=111), followed by Shandong (*n*=25) then Liaoning (*n*=7). The 518 *E. coli* and 143 *S. enterica* isolates were laboratory tested for resistance/susceptibility to up to 28 antimicrobials, **Fig. S1**. In general, most isolates in both species were resistance to penicillins, monobactams, cephalosporins and aminoglycosides whilst resistance to carbapenems and polymyxins was much less frequent, **Fig S1**. To understand the genetic relatedness of the isolates and its relationship with AMR, maximum likelihood phylogenetic trees were constructed for each species, using the core genome (based on genes present in >99% of isolates), **Fig 1**. In *E. coli* resistance phenotypes were not related to phylogroup, however in *S. enterica* the serotype Enteritidis was more susceptible than other serotypes to all antibiotics except for polymyxins for which it was more resistant, **Fig. 1**. When considering individual antibiotics, the three tetracycline antibiotics (tetracycline, doxycycline and minocycline) were significantly different between species (adjusted *p*-value <0.001, chi-squared test with Bonferroni correction), with DOX and MIN showing greater resistance in *S. enterica* and TET showing greater resistance in *E. coli*. Similarly, both polymyxins (polymyxin B and colistin) had significantly more resistance in *E. coli* (corrected *p*-value <0.001, chi-squared test with Bonferroni correction). Two 3rd generation cephalosporins (cefepime and ceftazidime) were proportionally more resistant in *S. enterica* (corrected *p*-value <0.001, chi-squared test with Bonferroni correction). Finally, the aminoglycoside streptomycin was found to have a similar proportion of resistant cases, but more intermediate and less susceptible isolates in *S. enterica* compared to *E. coli* (corrected *p*-value <0.001, chi-squared test with Bonferroni correction).

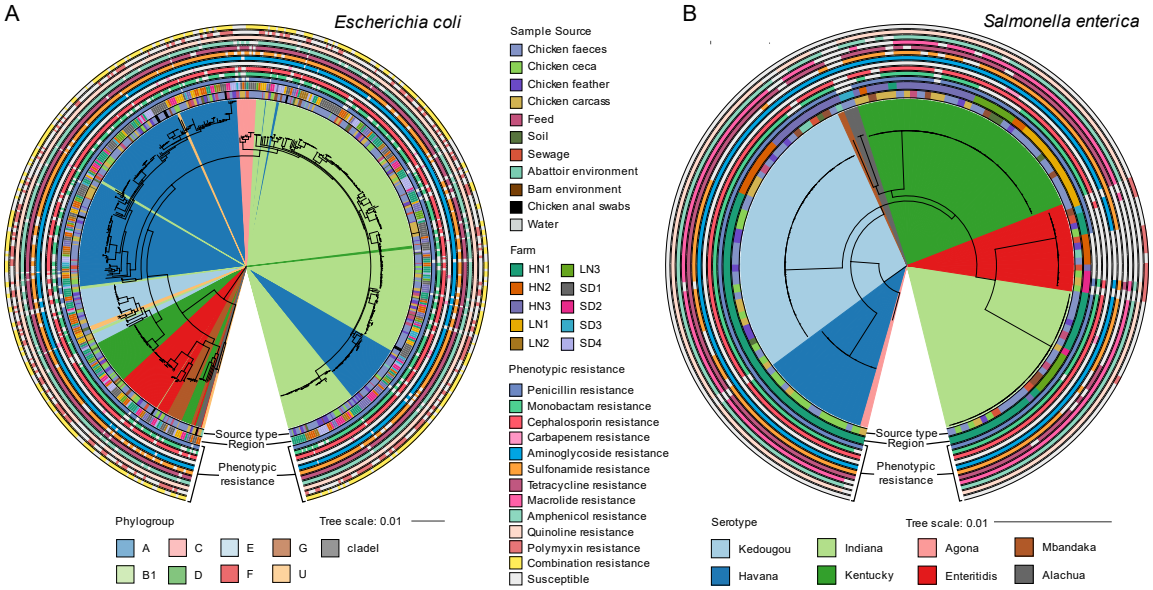

Figure 1. Phylogeny of *E. coli* and *S. enterica* isolates collected from 10 commercial broiler farms in China (A) Maximum likelihood phylogenetic tree of the whole cohort of *E. coli* isolates based on core genome of the 518 isolates, cultured from the animal and environmental samples collected from the 10 farms and 4 abattoirs. Phylogroups are shown as coloured sections. Sample source, region and phenotypic resistances are shown as rings around the tree. (B) Maximum likelihood phylogenetic tree of the whole cohort of *S. enterica* isolates based on core genome of the 143 isolates, cultured from the animal and environmental samples collected from the 10 farms and 4 abattoirs. Serotypes are shown as coloured sections. Sample source, region and phenotypic resistances are shown as rings around the tree. In both panels A and B the phenotypic resistance rings are grouped by antibiotic class, with presence (coloured) defined as resistance to at least one antibiotic in the class and absence (grey) as susceptibility to all tested antibiotics in the class.

The phylogeny results showed that *E. coli* isolates (**Fig. 1A**) were highly diverse with no clustering observed across isolates sourced from the sample farm or source type. Isolates were primarily from phylogroups A or B1, both associated with commensal *E. coli*, however all other phylogroups were also seen in smaller numbers. Sample source type, farms, provinces, and collection dates were not associated with phylogroups (p values > 0.05, Fishers exact test for count data with simulated p value). Using the standard association index (I^S_A) to measure for clonality in the population (*16, 17*), we found the I^S_A to be 0.2126 (p value < 0.0001) at whole cohort level and 0.1313 (p value < 0.0001) at ST type level, indicating there was little clonality observed amongst our *E. coli* cohort. Conversely, for *S. enterica* (**Fig. 1B**) statistically significant differences were observed among isolates in relation to serotypes per farm, source type and collection date (p values < 0.0001, Fishers exact test for count data with simulated p value), with Havana enriched in ceca samples and Enteritidis enriched in waste water (adjusted p values < 0.05, chi-square), Kentucky enriched in Liaoning 1 and Henan 3 (adjusted p values < 0.05, chi-square) and Enteritidis enriched in Henan 2 and Shandong 2 (adjusted p values < 0.05, chi-square). June 2020 was associated with an enrichment of Havana serotype samples; this collection month was associated with t_1 collections from Henan 1, however Henan 1 collections across all timepoints were not enriched for Havana serotype. The I^S_A of 0.9077 (p value < 0.0001) at whole cohort level and 0.3883 (p value < 0.0001) at ST type level indicated stronger clonality.

To further assess the genomic relatedness of both the *E. coli* and *S. enterica* isolates in our cohort we measured the number of different core genome SNPs, in a pairwise manner across all isolates. Creating a network based on clusters of related isolates with less than 15 SNPs, as done previously (*17, 18*), we observed again differences between the two species, with *S. enterica* forming larger clusters compared to *E. coli*, highlighting the greater clonality of the *S. enterica* isolates. For *S. enterica* (**Fig. 2A**), ten large clusters (mean SNPs difference within cluster = 6, range 0-10) were mainly defined by serotype, with poor intermixing of timepoints, whilst the source types were largely intermixed (91%), however an enrichment for ceca samples in Havana isolates is observed, as in the phylogeny. For the *E. coli* (**Fig. 2B**) ninety smaller clusters of highly related isolates (mean SNPs difference within cluster = 4, range 0-15) were present and 71% of these small clusters were composed of more than one sample type.

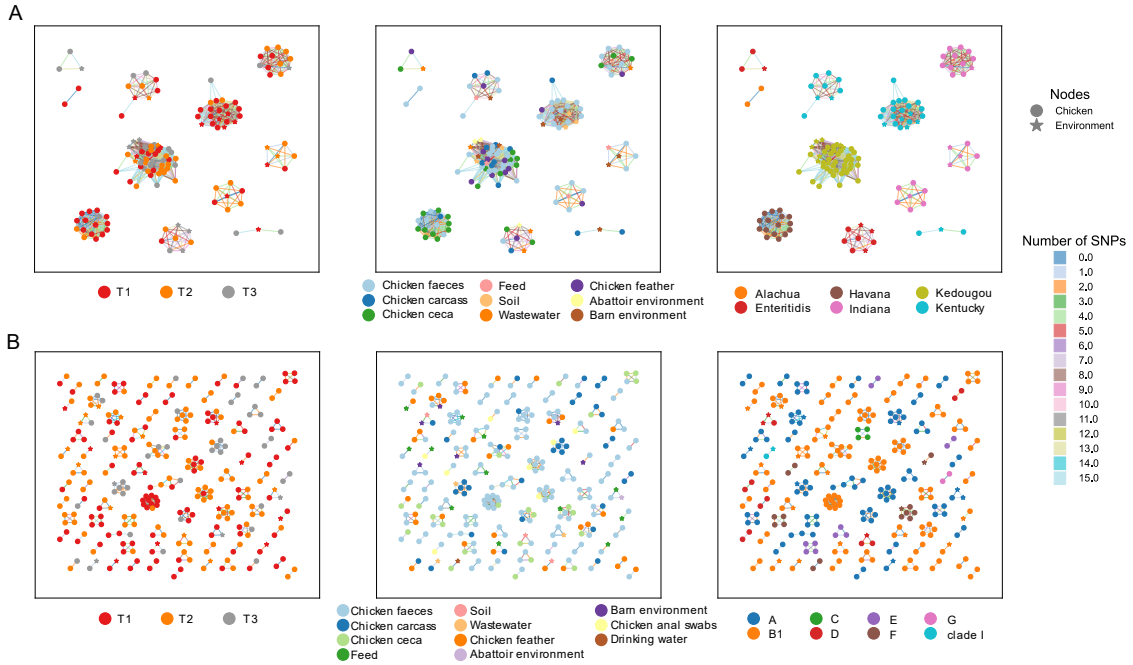

Figure 2. SNP network analysis of highly connected isolates in *S. enterica* and *E. coli*. Network diagram showing pairwise connections between chicken (circle) and environmental (star) isolates with less than 15 pairwise SNP differences for (A) *S. enterica* and (B) *E. coli*. The panels in each row show the same network with the nodes colour-coded according to time point (left), source type (centre) or serotype/phylogroup (right). The lines between pairs of isolates are colour-coded by SNP number.

As phylogroups can encompass large genomic variations that might be related to geographic distance or time, we analysed the clustering pattern of *E. coli* and *S. enterica* strains using a core genome distance of <100 (SNPs) as done previously (*19*). Overall, we found that isolates, of both species, from the same farm were generally more closely related than those of different farms, similarly, isolates from the same province were more closely related than those further apart, **Fig S2**. Analogously, when isolate pairs were compared from different collection dates, we found that strain-level diversity can persist over time and that there was a general trend of increasing SNP distance the further apart the sampling, **Fig. S3**. These results indicate a geographic diversity and evolution in both species. Finally, to better understand the phylogenetic and SNP distance differences observed between *E. coli* and *S. enterica* and to explain the evolution of these two species, we performed a Bayesian inference of phylogeny for the major phylogroups and serotypes in our cohort. The vast phylogenetic and genomic (SNPs) diversity of *E. coli* and *S. enterica* was supported by the Bayesian divergence analysis results showing that *S. enterica* strains were more recently evolved, with MCRAs of *S. enterica* of around 5 years (**Fig. S4**), compared to MRCAs for *E. coli* (**Fig. S5**) in the range of hundreds of years.
Together, these results show a diverse, non-clonal and evolutionarily distantly related cohort of *E. coli* isolates potential indicative of large circulation populations of commensal *E. coli* in these farms, with many samples carrying multidrug resistance. In contrast *S. enterica* shows highly clonal, region specific, and evolutionarily closely related isolates, more indicative of outbreaks of bacterial growth, and as with *E. coli* many isolates are multidrug resistant.

Within-host favours sharing of plasmids and mobile genetic elements carrying AMR genes between *E. coli* and *S. enterica*

Genomic mobility through plasmids and mobile genetic elements is correlated to antimicrobial resistance, giving the opportunity for genomic content to be shared between bacteria (*20*). In our cohort 99.4% of *E. coli* isolates and 88.1% of *S. enterica* isolates carried plasmids **Table S2**. Using MOBsuite (*21*) plasmids were reconstructed and typed from both species. In *E. coli*, 3169 plasmids were reconstructed (mean of 6.1 per isolate) whilst for *S. enterica* 572 plasmids were reconstructed (mean of 4 per isolate). Plasmids found in *S. enterica* were also significantly smaller (p value <0.0001) with a size range of 977bp-261kbp in *S. enterica* compared to 983bp-448kbp in *E. coli*. Gene content was found to significantly differ between plasmids from each species (p value < 0.0001) with *E. coli* plasmids carrying on average 0.82% (±0.33%) of the genome and *S. enterica* plasmids carrying 0.45% (±0.22%). Similar proportions of plasmids with no identifiable replicon and multiple replicons were found in both species. In *S. enterica* plasmids with no replicon were enriched in Liaoning province (LN1 and LN3), and Havana and Kentucky serotypes. In *E. coli* no-replicon plasmids were enriched in farms from each of the provinces (HN1, LN3, and SD3) and phylogroups B1 and G. However, *E. coli* carried significantly more novel plasmids (n=59) compared to *S. enterica* (n=4), *p* value = 0.047. In *E. coli* the most prevalent plasmid replicon was IncFIB found in 402 isolates, followed by IncHI2 found in 239 isolates. In *S. enterica*, Col(pHAD28) was most prevalent found in 79 isolates followed by IncHI2 found in 58. All these plasmids have previously been associated with AMR gene carriage (*17, 22-24*). When comparing plasmids type and content in the 113 *E. coli* and *S. enterica* strains isolated from the same samples we found that many 70.6% of isolate pairs cultures from the same sample carried the same plasmid types. In the plasmid types that were shared in isolate pairs, we observed more sharing than would be expected by chance compared to typical plasmid prevalence in isolates collected from chicken in China (p < 0.0001**).** Among these the most common shared replicon types were Col(pHAD28) and IncHI2, **Fig. S6**. In particular, Col(pHAD28) and IncHI2 were found in 29 and 27 isolate pairs respectively, **Fig. S6**. For IncHI2, a major AMR carrier (*25*), a Bayesian phylogenetic analysis of reconstructed IncHI2 plasmid sequences within our samples, showed that generally *E. coli*-sourced plasmids and *S. enterica*-sourced plasmids fell in different clades with a MRCA of 16 years, **Fig. 3**. However, there were two regions of more recent evolution, with the MRCA dating back to only 2019, and within these we have pairs of *E. coli* and *S. enterica* strains isolated from the same sample, potentially indicative of recent transmission of this plasmid between species.

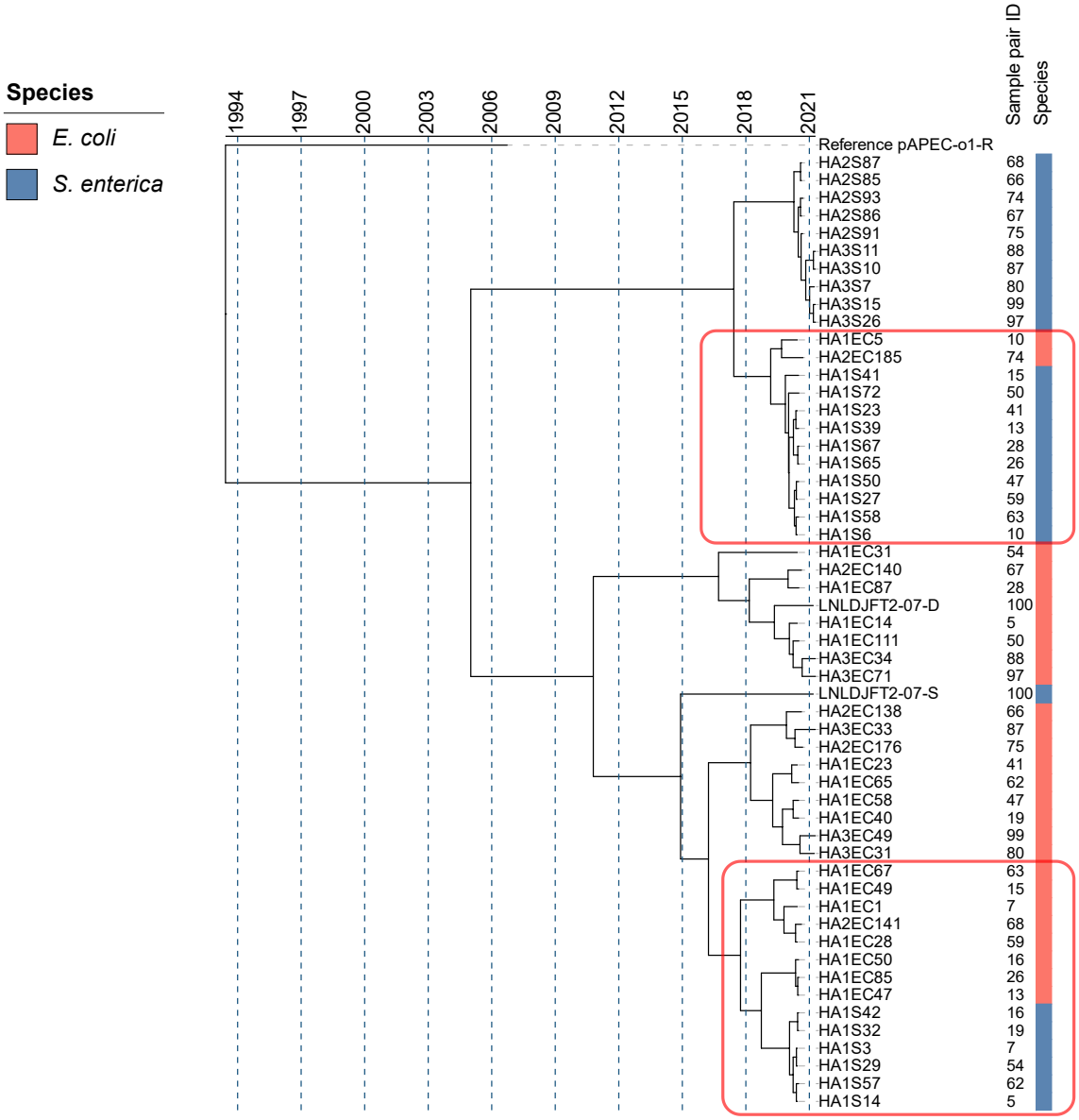

Figure 3. Bayesian evolutionary analysis shows recent common evolution of IncHI2 plasmids from *E. coli* and *S. enterica* isolates collected from the same samples and environments. Bayesian estimation of IncHI2 plasmid divergence times using sequencing data from 27 *E. coli* and 27 *S. enterica* strain pairs collected from the same samples, with the reference pAPEC-o1-R plasmid used as an outgroup (GenBank DQ517526.1). Sample pair numbers are given with each number corresponding to an *E. coli* and *S. enterica* isolate recovered from the same sample. Red rectangles indicate two areas of recent evolution containing IncHI2 plasmids from both species.

Next, we assessed whether the same mobile ARGs (AMR genes in the 5kb vicinity of an MGE) (*26-30*) were found in pairs of *E. coli* and *S. enterica* isolates collected from the same sample. Considering known ARGs according to the CARD database (*31*), we found 88 *E. coli-S. enterica* isolate pairs (78%) carrying the same mobile ARG (**Table S3)**. These mobile ARGs included clinically relevant *CTX-M*, *APH(3)*, *floR*, *mphA*, and *qnrS1* genes, all known important to AMR in human health (*32*) (**Fig. S7**). Overall, 14 different mobile ARG patterns were found (**Table S3**). The spread of mobile ARGs across farms was broadly reflective of the distribution of isolate pairs (*p* value = 0.064, chi-squared test), **Fig. S7A**. The spread of mobile ARGs across source types significantly differed from the

expected distribution (p value < 0.001, chi squared test) driven by a higher-than-expected number of mobile ARGs in feed samples (standardized Pearson residual = 4.468), **Fig S7B**.

Gene structures of the ARG containing contigs for mobile ARGs showed highly conserved structures in both *E. coli* and *S. enterica* isolates, from the same sample and those from different samples, as seen for example in the gene structures of the *QnrS1* gene (**Fig. 4)**.

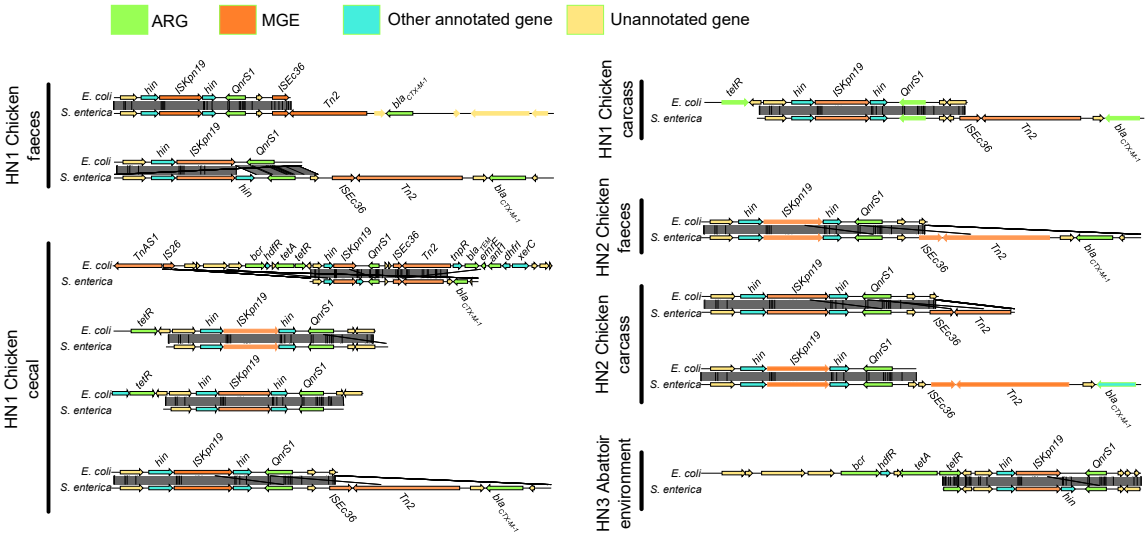

Figure 4. Genomic structure patterns of *QnrS1* mobile ARG patterns in *E. coli* and *S. enterica* isolates collected from the same samples. Each pair of samples was aligned by *QnrS1*. Coding DNA sequences are coloured according to the function of their encoded protein, as follows: ARGs were coloured in green; MGEs were coloured in orange, other annotated genes were coloured in cyan and other open reading frames (ORF) were coloured in yellow.

Overall, the patterns of plasmid and mobile ARG presence suggest there is high potential for transfer between *E. coli* and *S. enterica* in the farm environments that we have studied which could lead to the spread of clinically important ARGs, and suggestions that historically this is likely to have occurred. However, we have not seen any evidence of this happening on a large scale in the timeframes we have studied.

Machine learning unravels known and novel AMR-associated core genome SNPs and accessory genes correlated with resistance/susceptibility profiles to 26 antimicrobials in both species

Given the similar proportions of resistance, against 26 antimicrobials, across the 518 *E. coli* and 143 *S. enterica* isolates, despite phylogenetic differences, we further investigated which genetic determinants (features) were underlying the experimentally determined resistance/susceptibility profiles and if they were in common between the two species. To do this we employed supervised machine learning pipelines (**Fig. S8A** and **S9A**). The method is aimed at data mining sequencing data to identify the genetic elements that more strongly correlate with observed phenotypic differences, which in this case are resistance/susceptibility to antibiotics. For this analysis we improved our original ML-based core methods (*17*, *33*, *34*) in a notable way (see **Fig. S8A**): Information about different genetic features (SNPs (both from coding and non-coding regions) and presence/absence of accessory genes), have been fed all at the same time as input into the same model. This should capture the co-occurrence of multiple mechanisms (mutations, HGT) as well as their

additive effect on resistance. To ensure the best performance and avoid any bias both species underwent population structure correction and were tested against a panel of seven ML methods, five classifiers and two meta-methods (Linear SVM, RBF SVM, Random Forest, Extra tree classifier, Logistic regression, Adaboost and XGBoost), with the best classifier performance compared using the Friedman and Nemenyi tests (see **Methods** and **Fig S10** for details). To correct for unbalanced classes SMOTE (*35*) or SMOTEENN (*36*) was used to synthetically oversample the minority class under sample the majority class, and nested cross validation was employed. This procedure necessitated a minimum of 12 samples in the minority class for each predictive model, hence some antimicrobial datasets were insufficient for ML. For *E. coli*, ML was able to be carried out on 21 antimicrobials. Of these 21 models, 17 achieved high performance with an AUC greater than 0.9, and 13 of those achieved an AUC greater than 0.95, with the doxycycline performing best (AUC = 0.985), **Fig. 8B**. Similarly, all other performance metrics were good for these 17 models, **Fig. 8C-G**. All four poorly performing predictive models (amoxicillin-clavulanic acid, ceftazidime, cefotaxime-clavulanic acid and cefepime) were beta-lactam class antimicrobials, and these models also had a small number of features selected as predictive by the data pre-processing, **Fig. S11A**.

For *S. enterica* a different ML pipeline was used as shown in **Fig. 9A** with pre-processing of all three feature types separately using an extra tree classifier and resampling of classes using SMOTEENN (*36*), see Methods for details of ML pipeline choice. Due to the relatively low sample number and unbalanced nature of the resistance-susceptibility profile of the isolates predictive models were built for only the thirteen antimicrobials with enough samples in the minority class. Of these thirteen models, all had good AUC performance, greater than 0.9, with ten achieving an AUC greater than 0.95, **Fig. 9B**. Performance across all metrics was high, **Fig. 9B-G**, and gentamycin (GEN) performed best across all metrics. Unlike in *E. coli* the poorer performing antibiotics came from three different antibiotic classes beta lactams (AMC), diaminopyrimidines (SXT) and tetracycline (TET) and poorer performing models did not have a low number of features, **Fig. S11B**.

The AMR-related features selected by the machine learning showed differences according to the type and genomic localization (i.e. SNPs/accessory genes) correlated to the resistance against a specific antibiotic. In both species, accessory gene presence-absence tended to be selected in multiple AMR models, whilst SNP features originating from the core genome tended to be more specific to a single antibiotic, **Fig. S12**. For each species, and each antibiotic model, SHAP (SHapley Additive exPlanations) values were calculated. SHAP values will disclose the individual contribution of each gene mutation/accessory gene on the output of the model, for each isolate. **Figs 5** and **6** use a bee swarm plot to show the correlation of each of the top ten most important genes for each model for *E. coli* and *S. enterica* respectively, to predict the resistant phenotype. For *E. coli*, in most cases the presence of the SNP in genes/accessory gene was positively correlated to the resistance phenotype, though there were some genes for which the absence of the SNPs/accessory gene was positively correlated to the resistance phenotype, most notably in the prediction of CAZ-C and AMI resistance, **Fig 5**. For *S. enterica*, a more mixed pattern emerged, with many gene SNPs/accessory genes negatively correlated to the resistance phenotype i.e. correlated to susceptibility, **Fig 6**. Most notably, for gentamycin, the presence of 9 of the top 10 genes were positively correlated to the susceptible phenotype.

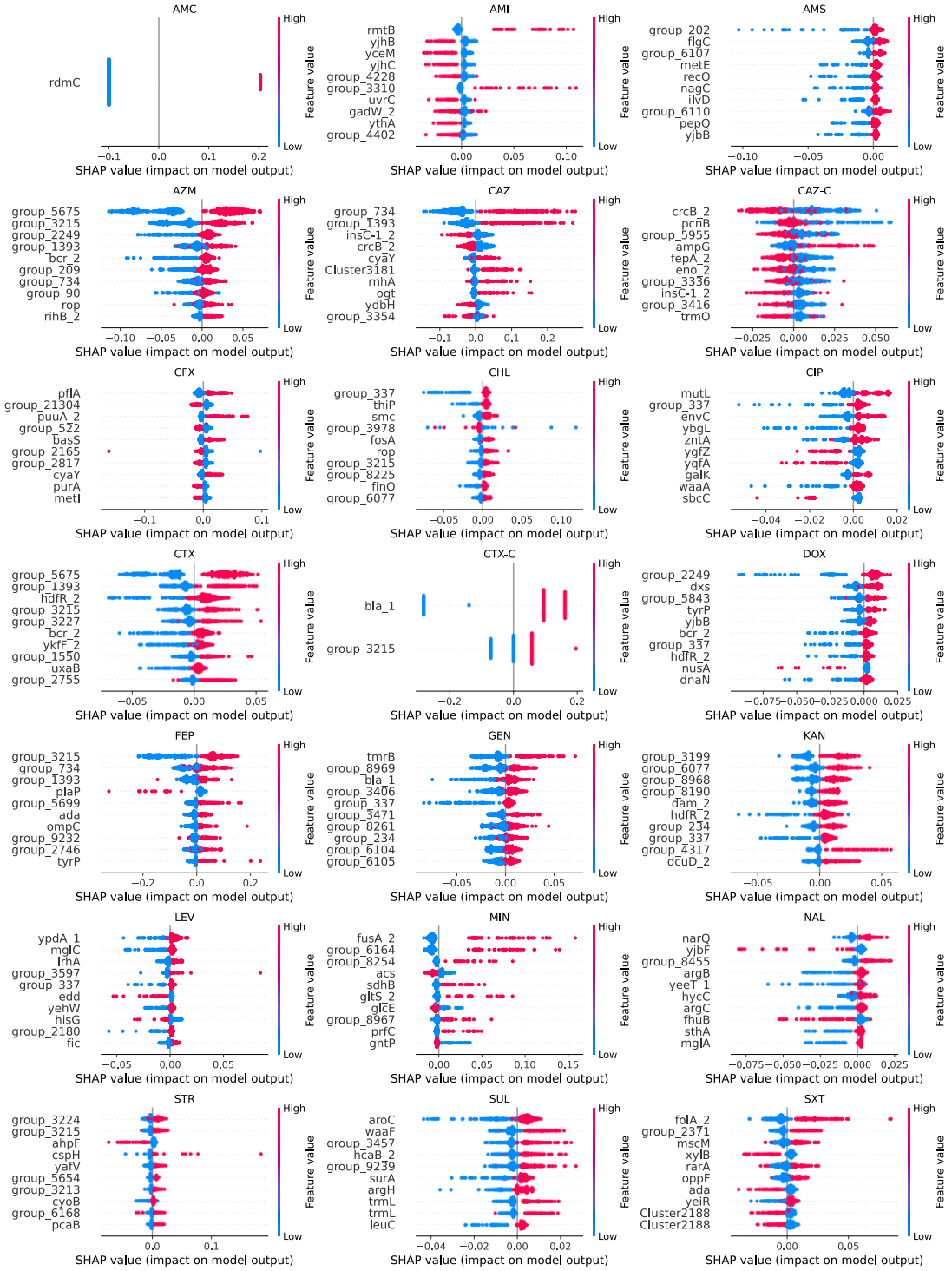

Figure 5. Bee swarm plot of SHAP-calculation for the ten highest ranking genes for each of the *E. coli* antibiotic ML models. Genes are sorted by their mean absolute SHAP value in descending order with genes carrying most important features (SNP or accessory gene) at the top. Genes labels as 'group_' are unannotated genes. Each dot corresponds to one isolate in the study. The colour red indicates the presence of the feature while the colour blue indicates its absence. The bee swarm plot shows how the different feature in each isolate affects the prediction of the ML model towards resistance to the respective antibiotic. Positive SHAP values indicate a change in the expected model prediction towards resistance, while negative SHAP values indicate a change towards susceptibility. The plot is based on the ML model with all selected features included.

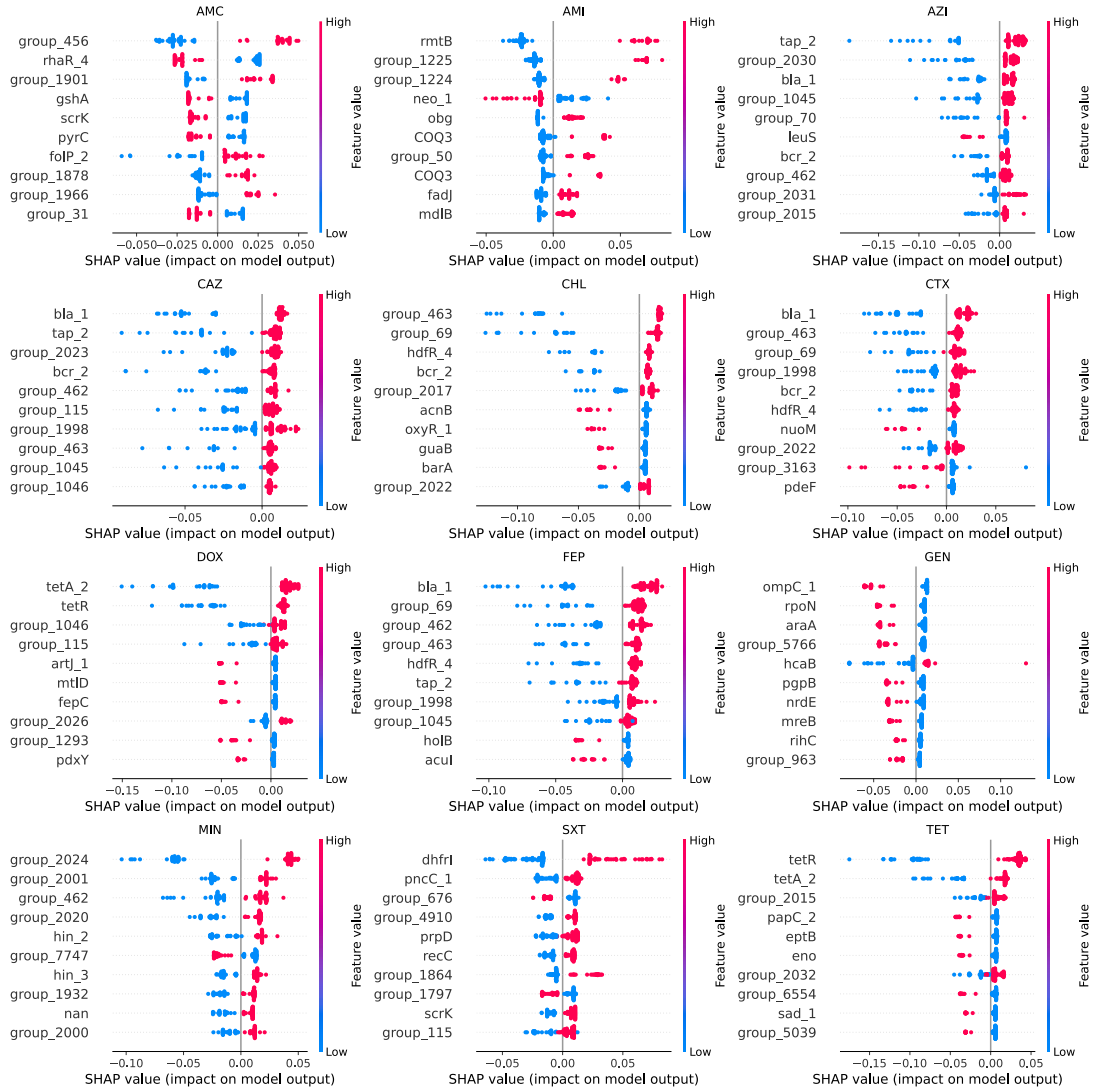

Figure 6. Bee swarm plot of SHAP-calculation for the ten highest ranking genes for each of the *S. enterica* antibiotic ML models. Genes are sorted by their mean absolute SHAP value in descending order with genes carrying most important features (SNP or accessory gene) at the top. Genes labels as 'group_' are unannotated genes. Each dot corresponds to one isolate in the study. The colour red indicates the presence of the feature while the colour blue indicates its absence. The bee swarm plot shows how the feature in each isolate affects the prediction of the ML model towards resistance to the respective antibiotic. Positive SHAP values indicate a change in the expected model prediction towards resistance, while negative SHAP values indicate a change towards susceptibility. The plot is based on the ML model with all selected features included.

To understand the relationship between AMR phenotype and genotype, we cross-referenced the SNPs that acted as predictors for AMR phenotype for each antibiotic to the pan-genome for each model data set and identified the corresponding genes. In total the *E. coli* features (SNPs and accessory genes) that correlated to AMR profiles across all antibiotic ML models, mapped back to 4419 genes, 20.3% of the pan genome. Genes correlated to *E. coli* resistance were enriched for plasmid located genes with 256 (5.6% of the 4419 genes) found in plasmids, compared to 2.4% of the pan genome. In contrast, genes correlated to *S. enterica* were less enriched for plasmid localisation, with 89 genes plasmid-located (2.7%) compared to 1.9% in the pan genome. Of these just 1% (44 genes) were

known AMR genes (defined as being found in public AMR databases, see Methods). For *S. enterica* ML selected features (SNPs and accessory genes from all ML models) mapped back to 3501 genes, 44.2% of the pan genome and 1.14% of these (40 genes) were known AMR genes. Considering these known AMR genes that were selected from the ML pipeline as being predicative of resistance/susceptibility (SNPs or accessory genes), many of these were found in multiple ML models (82% for *E. coli* and 78% for *S. enterica*), **Figure 7**. For *E. coli*, of the 44 known AMR genes selected, those relating to multidrug resistance (MDR) were most frequent (9 of 44 genes), followed by aminoglycoside genes (8 of 44). For *S. enterica* of the 40 known AMR genes, aminoglycoside genes (11 of 40) were most frequent, followed by beta lactam genes (8 of 40).

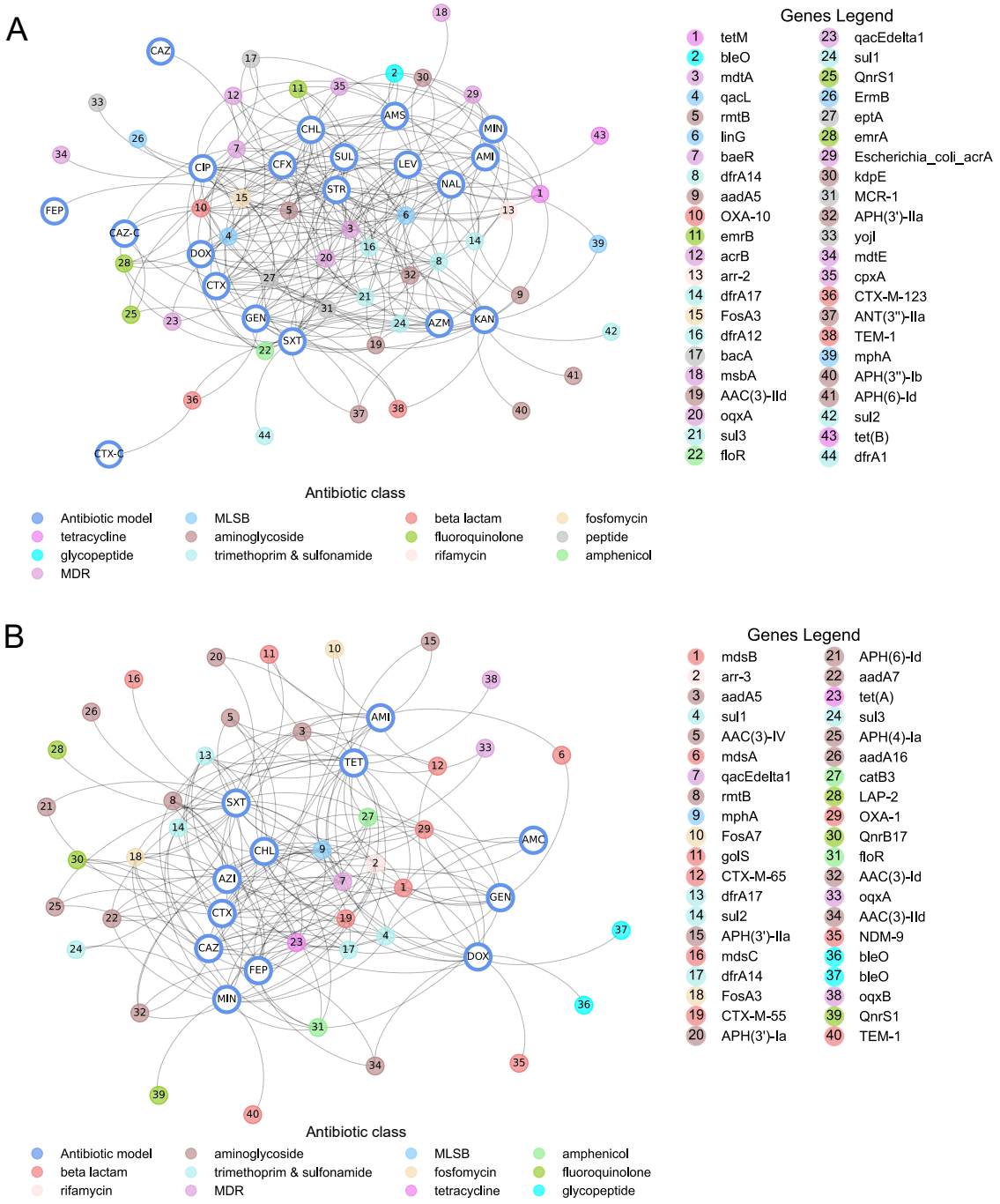

Figure 7. Known AMR genes predictive of resistance in antibiotic models (A) Undirected graph of genes found by ML as predictive of resistance-susceptibility profiles to a panel of 21 antimicrobials in *E. coli* and present in public AMR gene databases connected

to the antibiotic model in which they were selected. Nodes of the graph represent either the antibiotic model (indicated by three letter antibiotic abbreviation) or the gene. Gene nodes are colour-coded by antibiotic class. Edges of graphs connect antibiotic model nodes to gene nodes and are unweighted. (B) Undirected graph of genes found by ML as predictive of resistance-susceptibility profiles to a panel of 12 antimicrobials in *S. enterica* and present in public AMR gene databases connected to the antibiotic model in which they were selected. Nodes of the graph represent either the antibiotic model (indicated by three letter antibiotic abbreviation) or the gene. Gene nodes are colour-coded by antibiotic class. Edges of graphs connect antibiotic model nodes to gene nodes and are unweighted.

As done previously (*33*), we selected only the top-ranked AMR-related genetic determinants that most strongly contribute to the performance of the ML classifier. This was done by limiting our analysis on genes corresponding to the top 10% of ranked features recognized as discriminant by the AMR classifiers. This led to a total of 1089 genes in *E. coli* and 688 in *S. enterica*, that were correlated to at least one antibiotic. Of these 88 were correlated to at least one antibiotic in both *E. coli* and *S. enterica.* Protein-protein interaction networks of these 88 genes, **Fig. S13**, showed that in both species these genes were interacting with each other significantly more frequently than would be expected by chance (*E. coli* adjusted p value 1.17 x 10^{-11}; *S. enterica* adjusted p value 1.93 x 10^{-8}; hypergeometric test). These genes fell into multiple functional groups according to KEGG ontology, **Table S6**, including various metabolic processes, DNA repair, cellular transport, cellular community and drug resistance all of which have been linked to AMR (*33*, *37*, *38*). Of particular interest, the genes *ompC* and *oppC* which were selected in both species are involved in beta-lactam resistance (*39*). The *Opp* gene operon is also known to be involved in aminoglycoside resistance (*40*) and quorum sensing (*39*), whilst *ompC* is known involved also in signal transduction (*39*), hence these gene appears as interesting drug targets, hitting multiple pathways. The *cys* gene pathway had many genes selected by the machine learning suggesting its importance in resistance mechanisms, these genes were highly connected with other genes in the PPI networks and in the KEGG ontology were involved in purine and pyrimidine metabolism, previously suggested to be important in resistance (*33*).

Integration with GSM reveals that many top-ranked AMR-related genes are essential genes for growth and affect common metabolic pathways correlated to AMR in both species

To further investigate the systemic relationships connecting the identified AMR genetic signatures on a mechanistic level and to elucidate their mechanistic effects beyond genes encoding proteins targeted by drugs (i.e., positive selection in basal biosynthetic, regulation, and repair pathways), we integrated the top 10% ranked genetic determinants identified by ML with the genome scale models (GSM) of *E. coli* (K-12 MG1655, iML1515 (*41*)) and *S. enterica* (STM v1.0 (*42*)). Many accessory genes, as missing from these reference genomes, were not included in the analysis. For *E. coli*, for each antibiotic model on average 10% (*n* = 30, mean across 19 antibiotic models) of metabolic genes accounted for in iML1515 from the top-ranked 10% features (n=135, mean across 19 antibiotic models), whilst for *S. enterica* proportionally more metabolic genes were found, 28% (*n* = 24, mean across 12 antibiotic models). To investigate the importance of these metabolic genes to the bacteria we simulated 'loss of function' mutations for each gene using the GSM models. For *E. coli* 22 knockouts of 22 genes were predicted to be lethal in rich environmental conditions (*luxS, accD, hemE, dxs, ubiD, coaE, ispB, aroC, ispG, lptG, ribC, ispA, lpxD, waaA, murA, kdsC, cysG, folC, psd, hemD, mraY, ftsI. yrbG, hemL, ubiA*). The gene *lptG* is an ABC transporter and was selected in 15 of 19 antibiotic ML models so could be an important drug target for

multidrug resistance. Similarly, the genes *dxs* and *ubiD* were selected in ten ML models. For *S. enterica* only ten genes were essential in rich media (*dfp, fepD, ribF, fepB, luxS, hemB, aroA, fepC, entF, mrsA*) with three of those from the *fep* gene operon, the primary iron import transporter in *S. enterica*, important for virulence (*43*).

To investigate the system level effect of each gene on metabolism, beyond essentiality, we performed a flux balance analysis. In particular, we knocked out individual genes, blocking the flux through reactions associated with that specific gene and evaluated the change in flux span as a result of the gene knockout. In doing so, we can infer potential metabolic adaptation mechanisms that can be linked to a change in gene function (i.e., downregulation, overexpression, or deletion) and find clusters of genes affecting the same reaction pathways, which may indicate metabolic adaptions to antibiotic stress. Pathway enrichment on those genes significantly affecting the reaction fluxes revealed a number of significant pathways in each species and a core set of pathways significantly effected in both species (**Fig. 8**). The latter involving pentose phosphate pathway, cofactor metabolism, cysteine metabolism, histidine metabolism, lysine and threonine metabolism, tyrosine, tryptophan and phenylalanine metabolism, cell envelope biosynthesis, phospholipid metabolism, purine and pyrimidine biosynthesis, purine metabolism, nitrogen metabolism, methionine metabolism, proline metabolism, peptidoglycan metabolism, transport metabolism, and tRNA charging (**Fig. 8**).

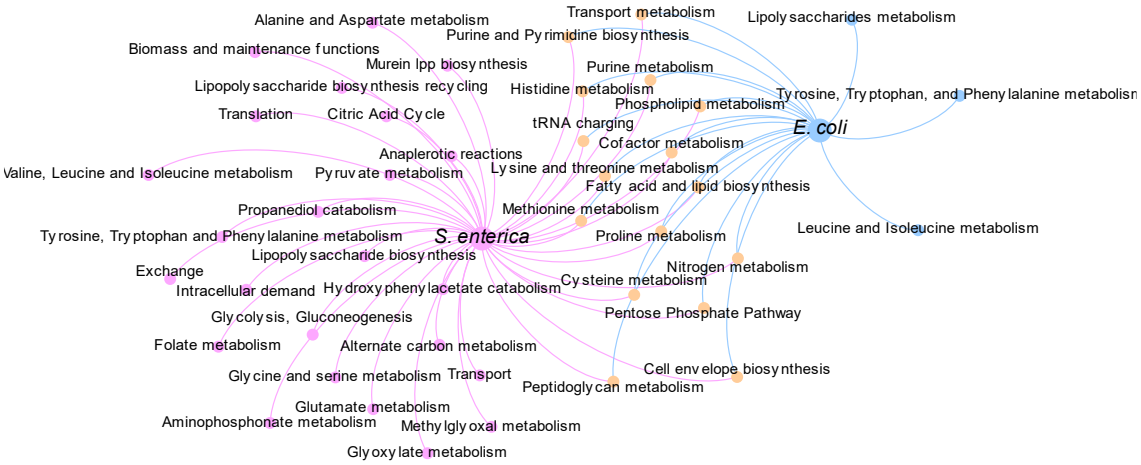

Figure 8. Flux balance analysis reveals the effects of AMR-conferring genes on metabolite yields and reaction fluxes and shows a great overlap of *E. coli* and *S. enterica* pathways enriched with genes correlated to AMR. Undirected graph network showing for the both *S. enterica* GSM model (STM v1.0) and the *E. coli* GSM model iML1515, pathways enriched with AMR-associated genes, that significantly affected the reaction fluxes though the pathway when knocked out. The pathway nodes (small circles) from each model are connected to the GSM model by unweighted edges. Pink nodes were significantly affected in the *S. enterica* model only, blue nodes in the *E. coli* model only and orange nodes in both models.

Next, for all significantly affected pathways in the flux balance analysis, **Fig. 8**, we considered which genes were responsible. In *E. coli* many genes were found to be reducing the flux span in each pathway, **Fig. 9A**, including a large number of genes in the purine and histidine pathways, highlighting the potential importance of these as potential gene targets. To understand whether these pathways are consistently important across the phylogenetic distribution of samples we looked at whether the mutation identified with the ML in the AMR-associated genes and correlated to each pathway were present in every *E. coli* isolate,

Fig. 9B. Interestingly, with the exception of a small group of isolates within phylogroup E, all *E. coli* isolates had accessory genes and/or mutations in core genes present in every significant pathway indicating that these AMR-associated genetic determinants are broadly present across the *E. coli* phylogeny. However, the lack of mutated genes/accessory genes in phylogroup E is relevant as this phylogroup is associated with human pathogenic *E. coli* including the highly virulent O157:H4 (*44*).

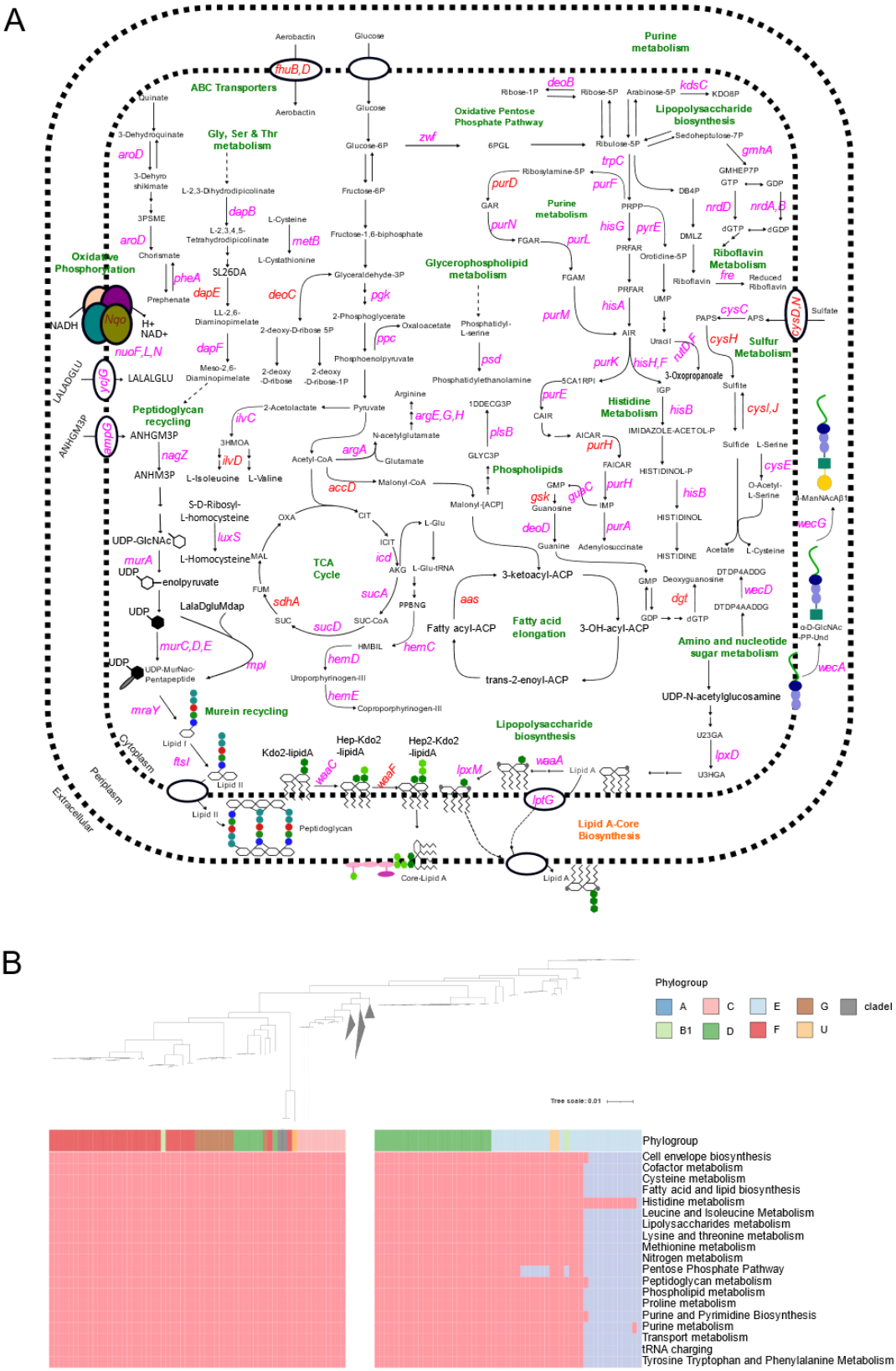

Figure 9. AMR-associated genes have been found to impact numerous metabolic pathways in *E. coli*, many of them are also considered essential metabolic genes, and are prevalent across all metabolic pathways throughout the phylogeny of *E. coli*. (A) An overview of the metabolic pathways affected by those AMR-associated genes

significantly influencing reaction fluxes in *E. coli*. All genes annotated in figure were found to have reduced the flux span through the metabolic system when knocked out, 11 of which were essential genes (i.e. gene knockouts were lethal in rich media). Genes highlighted in pink carried mutations that were selected in the *E. coli* ML models only, genes in red carried mutations that were selected in both the *E. coli* and *S. enterica* models. (B) Heatmap showing, for each *E. coli* isolate (columns of the heatmap), whether the isolate contained an AMR-associated gene that also significantly affected associated to one or more of the significantly affected pathways, found via flux variability analysis to reduce the flux span if knocked out. The top part of the heatmap shows the phylogenetic relationship between each isolate. The first row of the heatmap indicates the phylogroup to which each isolate belongs. The subsequent rows indicate the metabolic pathways into which the genes were grouped, with only pathways significantly enriched with ML selected genes shown. Pink represents the presence of a mutated gene/accessory gene, blue represent the absence. For brevity, A and B1 clades, which showed presence of genes in every pathway, were collapsed.

For *S. enterica*, many of the same pathways as for *E. coli* were significantly affected, with 16 genes on which AMR-associated mutations were selected from both *E. coli* and *S. enterica* ML predictive models **Fig, 10A.** However, the genes within these pathways carrying the AMR-associated mutations showed different phylogenetic patterns to those selected by ML in *E. coli*, **Fig. 10B**. When examining individual isolates, Figure **10B** reveals a distinct pattern of AMR feature presence and absence in *S. enterica* phylogeny. Among the various serotypes, only the Enteritidis serotype isolates exhibit the ML-selected AMR features across all pathways, whereas the remaining serotypes display features associated to a considerably smaller number of metabolic pathways. This indicates that the interplay between metabolism and AMR in *S. enterica* is influenced by serotype specificity. In summary, a high number of the same metabolic pathways are correlated to AMR in both *S. enterica* and *E. coli*, with genes correlated to AMR and significantly underlying metabolic function overlapping in both species. However, these AMR-associated determinants show a different phylogenetic pattern between the two species: prevalent across all metabolic pathways throughout the phylogeny of *E. coli*, whilst, in *S. enterica*, these determinants are prevalent to certain serotypes across all pathways.

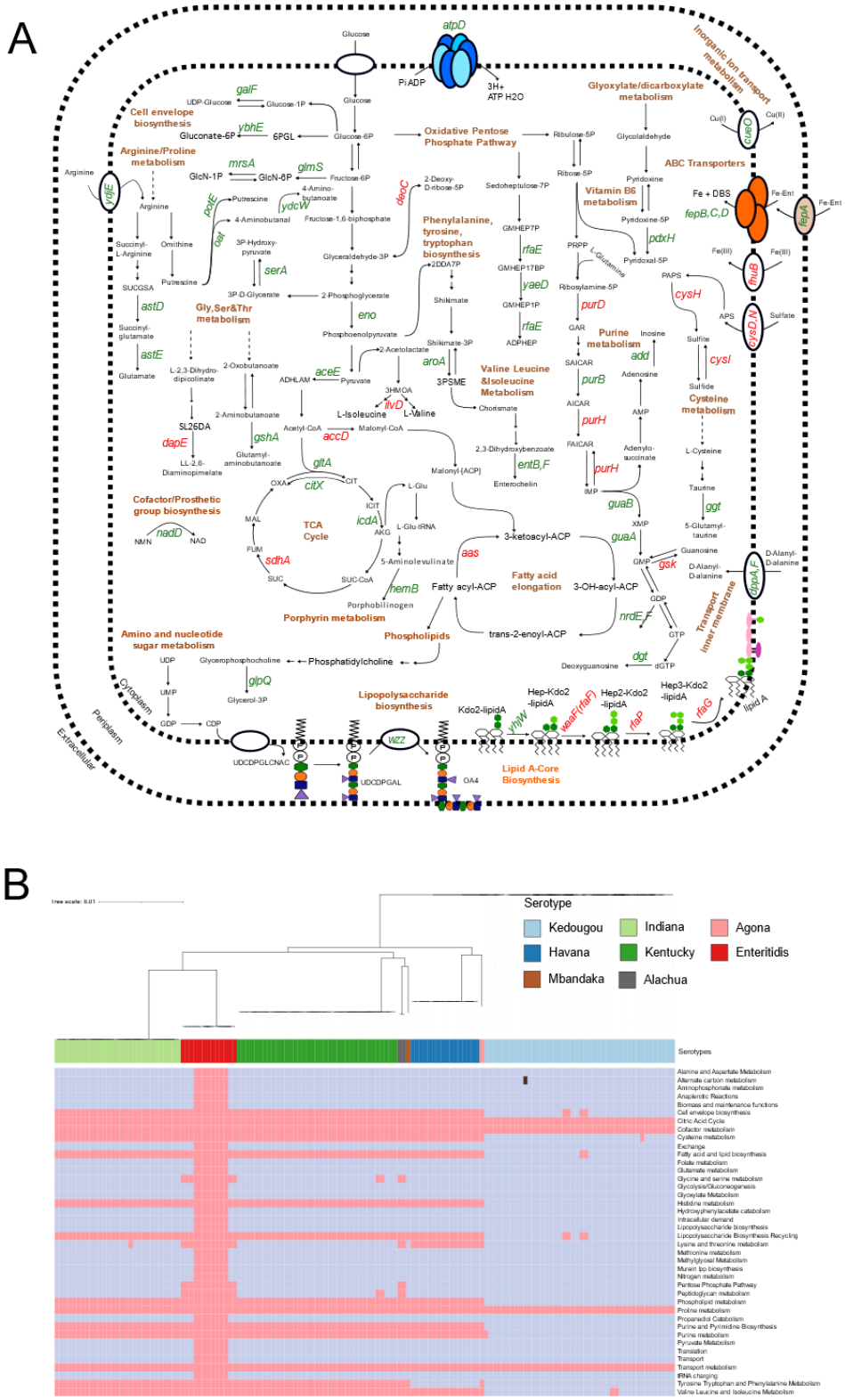

Figure 10. AMR-associated genes have been found to impact numerous metabolic pathways in *S. enterica*, many of them are also considered essential metabolic genes, however, are prevalent across all pathways only in certain serotypes in the *S. enterica* phylogeny. (A) An overview of the metabolic pathways affected by those AMR-associated genes significantly influencing reaction fluxes in *S. enterica*. All genes annotated in figure were found to have reduced the flux span through the metabolic system when knocked out,

six of which were essential genes (i.e. gene knockouts were lethal in rich media). Genes highlighted in brown were selected in the *S. enterica* ML models only, genes in red were selected in both the *E. coli* and *S. enterica* models. (B) Heatmap showing, for each *S. enterica* isolate (columns of the heatmap), whether the isolate contained an AMR-associated gene associated to one of the significantly affected pathways, found via flux variability analysis to reduce the flux span if knocked out. The top part of the heatmap shows the phytogenic relationship between each isolate. The first row of the heatmap indicates the phylogroup to which each isolate belongs. The subsequent rows indicate the metabolic pathways into which the genes were grouped, with only pathways significantly enriched with ML selected genes shown. Pink represents the presence of a mutated gene/accessory gene, blue represent the absence.

Discussion

Bacteria in intensive farming environment typically exist within multispecies microbial communities, such as the gut microbiota, and interactions within these communities can impact antimicrobial responses (*12*). Whilst conventional AMR surveillance, testing pathogens in pure cultures is suitable for monomicrobial infections such as many human bloodstream infections, these methods are not sufficient for complex polymicrobial communities[3], such as those within intensive farming. A better understanding of the AMR evolutionary dynamics in microbial communities (*12*) and their impact on opportunistic pathogens such as *E. coli* and *S. enterica* is essential for developing targeted treatments in different ecological settings, where community can impact resistance selection. Understanding how *S. enterica* and *E. coli* can affect the responses to antimicrobials in the other species, in interconnected but difference communities such as the gut of animals and their surrounding environments, and to which extent both species rely on the mobile genetic elements or mutations in the core genome can help to elucidate AMR in a broader range of bacteria than single species studies. This knowledge can ultimately inform on how individuals and communities respond to antibiotics and potentially direct new antibiotic discovery research. A second important consideration is the close relationship between bacterial metabolism and antimicrobial efficacy. However, conventional AMR research typically ignores the role that metabolic adaptation (such as mutations in metabolic genes) has been shown to play in antibiotic resistance (*33, 45*).

In this work we have conducted a large-scale study of *E. coli* and *S. enterica* isolates taken from ten commercial chicken farms and connected slaughterhouses in China. At larger scale, genomic analysis showed differences in the phylogeny and evolution of each species, but, at finer scale, most isolates of each species inhabiting the same host and environment, possessed same plasmids and mobile genetic elements carrying ARGs. Bayesian analysis of IncHI2 plasmids in isolate pairs (pairs of *S. enterica* and *E. coli* isolates from the same sample) showed recent co-evolution of plasmids from these species, whilst gene ontology of the mobile ARGs from isolate pairs showed a conserved gene structure. Machine learning found an overlapping set of genetic features (mutations in core genes and accessory genes) predictive of resistance to antibiotics in both species, many of which AMR-associated features had essential metabolic functions again in both species.

Our *E. coli* isolates from China, showed a diverse phylogeny with phylogroups A and B predominating as well as group C-G appearing at lower prevalence across all provinces; this has been similarly observed in other studies of healthy chickens conducted globally (*46, 47*). There are fewer large scale surveillance studies for *S. enterica*, but a recent study of 91 Salmonella strains isolated from chicken farms, slaughterhouses and abattoirs in the Hubei province of China showed a more diverse phylogeny than ours, with 27 different serotypes isolated (*48*). Similarly another study from a single farm and connected

slaughterhouse in Sichuan Province identified seven serotypes over three production cycles (*49*). In contrast our results are indicative of clonal spread of isolates within farms, also supported by the small SNP distance between many isolates (*50, 51*).

Our study found a IncHI2, a plasmid known to be a very important carrier of AMR genes (*52*) in a number of *S. enterica* and *E. coli* strains isolated from the same samples. This plasmid has been previously associated with cross species horizontal transfer (*53*) , however, to our knowledge direct transfer between strains isolated from these two species has not previously been observed. Here, using Bayesian divergence analysis we found two recently co-evolved clusters of IncHI2 plasmids isolated from both species, indicative of relatively recent HGT between species. To our knowledge this is the first time this has been observed between closely sourced samples of different species. Similarly, we found same mobile ARG sequences (ARGs-transposons) in pairs of *E. coli* and *S. enterica* isolates collected from the same sample. Whilst the same mobile ARGs have been found in different bacterial species (*54*) and different sample sources (*26, 27*), this is the first time that almost all of *E. coli* and *S. enterica* isolates collected from the same sample, showed mobile ARGs including clinically relevant ARGs (*CTX-M*, *APH(3)*, *floR*, *mphA*, and *qnrS1)* with a highly conserved mobile ARG structure.

Antibiotic susceptibility testing done against 26 antimicrobials showed similar proportions of resistant isolates for many antibiotics in our study in line with previous studies (*13*). Using the resistance-susceptibility profiles, our machine learning pipeline selected different genomic features (SNPs in the core genome and accessory genes) from each species that were predictive of the AMR profiles, and comparison between the two species highlighted a core set of genes found in both that were linked to AMR (604 annotated genes, of which 88 were in the top 10% of most important genes correlated to AMR). In both species, accessory gene presence-absence tended to be selected in multiple AMR models, whilst SNP features originating from the core genome tended to be more specific to a single antibiotic. Overall, we observed that whilst for *E. coli* the genes mainly were positively correlated with resistance, for *S. enterica* the genes mainly were correlated with susceptibility. The genes from this core set were found to have a significant level of interaction with each other and with some known AMR genes (e.g. *gyrA*, *gyrB*, *bcr*, *oppC*) (*31, 39*). *OmpC* variants have been linked to multidrug resistance in *E. coli* (*55*) and very recently the mutations in the gene *ompC* have been suggested as being correlated to extensively drug resistant *E. coli* with the appearance of mutations preceding the acquisition of resistance (*56*). Our results suggest that this gene could have similar important functions also in *S. enterica*.

Genome-scale metabolic modelling further indicated a core set of same metabolic pathways which were affected by the AMR-associated genes found in both *E. coli* and *S. enterica*. Whilst several of these genes have been previously correlated to AMR this is the first time, they have been found to be linked to metabolic processes and to functionally overlap in the two species. One such pathway, tRNA charging, is essential to protein translation and recent papers have suggested that bacteria may use mistranslation to control exposure to stress caused by antibiotics (*57*). This response involves mistranslation of methionine (*58*) and so could be linked to methionine metabolism another pathway significantly linked to AMR in both species. In addition, cell envelope biosynthesis, pentose phosphate pathway, histidine metabolism and phospholipid metabolism have all previously been linked to potential antibiotic resistance mechanisms (*33*). Of the AMR-related genes found to affect the metabolic pathways in both species there are several genes from the *cys* operon, as well as several *pur* genes. Both of these gene clusters have been previously highlighted in connection to AMR in *E. coli* (*33*) using machine learning and GSM modelling. In addition, genes involved the TCA cycle were selected in each species with

sdhA selected in both; the TCA cycle has recently been highlighted as a promising target for new antimicrobial therapies (*59*).

Overall, this study has highlighted patterns of differences and similarities in the opportunistic pathogens, *E. coli* and *S. enterica*, that coexist within microbial communities in intensive farming environments. At a large scale, involving classical phylogeny approaches and antimicrobial susceptibility testing major differences were observed, however, at a finer scale analysis when analysing *E. coli* and *S. enterica* isolates collected from the same sample, high similarity among MGEs (plasmids coevolution and highly conserved clinically relevant ARGs-transposons elements), a core resistome underlying AST against 26 antimicrobials, and metabolic pathways affected by the identified core resistome suggest a coevolutionary trajectory. This work has shown the importance, as also suggested by others (*12*), of adopting bespoke analytical methods when studying how bacteria coexisting in multi-species communities respond to antibiotics, focussing not only in individual pathogens but also in the microbial communities within which they reside and evolve.

Materials and Methods

Ethics Statement

This study was performed in accordance with protocols approved by the Ethics Committee of the State Key Laboratory of the China National Centre for Food Safety Risk Assessment (CFSA). The ethical approval number of CFSA is 2018018. Ethical approval has also been obtained from the Research Ethics Committee in the School of Veterinary Medicine and Science at the University of Nottingham, application ID: 2340 180613.

Experimental Design

For the study we selected ten large-scale commercial poultry farms belonging to three different provinces in China (Shandong, Henan and Liaoning), covering an area of 472500 km2, each farm feeding into one of four regional abattoirs (two in Henan, one in Liaoning and one in Shandong). Each farm features multiple barns, each containing between 12000 and 32800 birds, leading to a total production capacity of 110730 to 380000 birds per breeding cycle (depending on farm). Broiler production is based on self-breeding with broilers bred on the farm and moved to the barns in same-aged batches. Of the ten selected farms, three (two in Liaoning and one in Shandong) use net housing systems, whilst the other six use cage housing systems. During collection, the number of birds per barn did not significantly differ between the two housing systems (t-test, p value = 0.07). *E. coli* isolates (n=96) taken from one farm (Shandong 1) were part of a pilot experiment with the results previously published (*17*).

In total 977 samples were collected from: chicken faeces (n=372), chicken cecal (n=52), chicken anal swabs (n=10), chicken feathers (n=65), chicken carcass (n=193), Feed (n=64), barn environment swabs (n=32), drinking water (n=58), abattoir environment swabs (n=15), wastewater (n=32), external soil (n=84). Details on the collection methods are as follows. For faecal samples and cecal, each sample consisted of approximately 10g fresh sample of mixed chicken faeces (2-3 chickens) or an approximately 2g mixed fresh sample of 2–3 chicken caecal droppings, collected from the bottom of the chicken cage/net using a sterilized spoon. Chicken anal swab samples were collected using cotton-tipped swabs. Feather samples were collected from the birds and swabbed using cotton tipped swabs. Barn floor samples were taken using a sterilized spoon. Not less than 20mL each of chicken drinking water from farm and waste effluent water from the slaughterhouse were collected from the water pipe or by pipettes. About 10g each feed sample was collected using a sterilized spoon. Pooled carcass samples were collected in the abattoirs using a sponge swab

(SS100NB, Hygiena International, Watford, UK) on the surface of the carcass. Abattoir environment samples were taken from the processing and collected from multiple surfaces, e.g., the cutting table and transfer belt of the cutting and deboning house. Soil samples consisted of about 10g soil, collected outdoors at depth of 1-3cm, 5m from the external barn walls, to ensure sufficient separation from areas of human use. All biological samples were collected using aseptic techniques, and then stored in secure containers at 4℃ during transportation to the laboratory and extracted within 24h.

Bacterial isolation and identification

A quantity (volume) of 1 g (mL) sample of faeces, soil, feed, water each was vortexed with 9 mL of sterile buffered peptone water tube (BPW; Luqiao Inc., Beijing, China) for 1 min; chicken carcass sponge samples were homogenised with 10 mL BPW for 1 min in a stomacher bag. For isolation of *E. coli*, approximately 1mL dilution (of any of the above samples) was then added to 9 mL *E. coli* (EC) broth (Luqiao Inc.) and incubated at 37°C for 16-20h in order to enumerate presumptive *E. coli* populations. A loopful of these solutions was then streaked onto an eosin-methylene blue (EMB) agar and MacConkey (MAC) Agar (Luqiao Inc.) and incubated at 37°C for 18-24h. Typical *E. coli* colonies were screened and subsequently characterized by a Bruker MALDI Biotyper (Germany). The positive isolates identified were further confirmed by PCR using *E. coli*-specific primers ITS-F (5'-CAATTTTCGTGTCCCCTTCG-3') and ITS-R (5'- GTTAATGATAGTGTGTCGAAAC-3'). Thermal amplification conditions were as follows: pre-incubation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, elongation at 72°C for 30s, and a final extension of 72°C for 5 min. Out of all isolates analysed, 518 were identified as *E. coli* positive and included: chicken faeces (n = 261), chicken carcasses (n = 67), chicken feather (n=53), chicken cecal droppings (n=45), chicken feed (n=30) external soil (n=16), barn environment (n=16), waste water (n=12), anal swabs (n = 9), abattoir environment (n=7) and drinking water (n=2). Similarly, for isolation of *S. enterica*, approximately 1 mL enriched dilution was added to 9 mL RV broth and SC broth (Luqiao Inc.) and incubated at 42 ℃ and 37 ℃ for 16-20 h, respectively, in order to enumerate presumptive *Salmonella* populations. A loopful of these solutions was then streaked onto a Chromogenic Salmonella agar (Luqiao Inc.) and incubated at 37°C for 24-48 h. Typical *Salmonella* colonies were screened and subsequently characterized by Bruker MALDI Biotyper (Germany). The positive isolates identified were further confirmed by PCR using *Salmonella* specific primers invA-F (5'-GTGAAATTATCGCCACGTTCGGGCAA-3') and invA-R (5'- TCATCGCACCGTCAAAGGAACC-3'). Thermal amplification conditions were as follows: pre-incubation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, and a final extension of 72 °C for 5 min. In total, 143 were identified as *S. enterica* positive and included: chicken faeces (n=59), chicken cecal droppings (n=23), chicken carcass (n=20), chicken feather (n=13), barn environment (n=8), wastewater (n=6), external soil (n=5), chicken feed (n=4), abattoir environment (n=4), drinking water (n=1). The identified *E. coli* and *S. enterica* isolates were kept in brain heart infusion broth (BHI) medium with 20% glycerol at -80°C freezer for further characterization.

Antimicrobial susceptibility testing

Antimicrobial susceptibility to a panel of agents was determined by broth microdilution and interpreted according to the criteria based on the Clinical & Laboratory Standards Institute (CLSI) interpretive criteria (CLSI 2009). The resistance/susceptibility of 28 and 26 antimicrobial compounds were measured for each of the *E. coli* and *S. enterica* isolates, respectively, and these included ampicillin (AMP), ampicillin/sulbactam (AMS),

tetracycline (TET), chloramphenicol (CHL), trimethoprim/sulfamethoxazole (SXT), cefazolin (CFZ), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (CFX), gentamicin (GEN), imipenem (IMI), nalidixic acid (NAL – *E. coli* only), azithromycin (AZI – *S. enterica* only), sulfisoxazole (SUL), ciprofloxacin (CIP), amoxycillin/clavulanic acid (AMC), polymyxin E (CT), polymyxin B (PB), minocycline (MIN), amikacin (AMI), aztreonam (AZM), cefepime (FEP), meropenem (MEM), levofloxacin (LEV), doxycycline (DOX), kanamycin (KAN) and streptomycin (STR). *E. coli* ATCC 25922 was used as a control for the antimicrobial susceptibility testing.

DNA purification and extraction

All the *E. coli* and *S. enterica* isolates were subjected to genomic DNA extraction in accordance with the manufacturer's protocol of E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA, USA) followed DNA sequencing on an Illumina Hiseq 2500 PE150 platform (Illumina, San Diego, CA, USA).

Library construction and whole genome sequencing

The template genomic DNA was fragmented by sonication to an insert size of 350 bp using NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample and sequenced using an Illumina Hiseq 2500 PE150.

Genome assembly and annotation

All sequences were pre-processed through readfq v10. To clean the data, reads containing low-quality bases (mean quality value ≤ 20) over 40% were removed. Reads with greater than 10% unidentified bases (N) were removed as well as the adapters. The whole-genome shotgun sequencing produced high-quality reconstructed genomes with a N50 larger than 50,000 and less than 250 contigs. Cleaned data were processed for genome assembly with SPAdes v3.13, and QUAST v4.5 was used for assessing the assembly. The contigs with length shorter than 500 nucleotides were filtered out. The completeness and contamination of genomes were assessed through checkM with the lineage_wf pipeline. Genomes were annotated with Prokka v1.14.5 (*60*) using default parameters with—addgenes–usegenus.

Screening of annotated genes against ABR databases, plasmid databases and in silico subtyping

The whole genome sequences were screened against the CARD (*31*) database with a minimum coverage and identity of 80% to identify known AMR-associated genes in the isolate cohort. In addition the annotated genes obtained from Prokka v1.14.5 (*60*) were screened against the CARD (*31*), ARG-Annot (*61*) and Resfinder (*62*) databases using Abricate (*63*) and the NCBI AMRfinder (*64*) database; all the comparative analyses have been done with a minimum coverage of 70% and identity of 80%. Plasmids screening was conducted using the PlasmidFinder(*65*) database in Abricate(*63*). Sequence types for both species were identified through MLST (*66*) which mapped the sequences to the PubMLST (*67*) database. For *E. coli* Phylogroups were identified using in silico Clermont typing (*68*). Serotypes were identified through the EcOH database (*69*) using Abricate (*63*).

To compare plasmid presence with the level observed previously we downloaded from BV-BRC, all the good quality *E. coli* (n= 705) and *S. enterica* (n=265) isolates collected from chicken in China and conducted plasmid screening on these using the PlasmidFinder (*65*) database in Abricate (*63*).

Population structure analysis

Linkage disequilibrium of the 518 *E. coli* and 143 *S. enterica* isolates in our cohort was evaluated using the standardized index of association (I^S_A) (*16*), which estimates the homologous recombination for the
cohorts by assessing the linkage disequilibrium among the seven MLST loci. For each species separately the LIAN Ver. 3.7 program was used to calculate the I^S_A for all the isolates and for a subset of them (one isolate for each ST type) from the ratio of the variance of observed mismatches in the test set (V_D) to the variance expected for a state of linkage equilibrium (V_e), scaled by the number of loci used in the analysis (L) (*16*),

$$I_A^S = \frac{1}{L-1}\left(\frac{V_D}{V_e} - 1\right)$$

The significance of I^S_A was determined by a Monte Carlo simulation with 10^5 resamplings.

Generation of genetic features input files

For each species, all annotated genomes were taken as input for pan-genome analysis with core gene alignments through Roary v3.13 (*70*). The core genome alignment for each species was taken as input to produce a file of core gene SNPs present in the cohort using snp-sites (*71*). A presence-absence table of accessory genes was generated from the gene presence-absence Rtab file produced in Roary (*70*), with genes considered to be accessory genes if present in less than 99% of isolates (and hence not included the core genome). To map intergenic SNPs, for each species an alignment of core intergenic regions was created using Piggy v1.5(*72*) (the files output from Roary (*70*) were used as input for Piggy (*72*)), and the variable SNP sites in this alignment called using SNP-sites.

Network analysis based on pairwise SNP alignment

Networks of *E. coli* and *S. enterica* isolates collected from different samples sources in the farms and different regions of China were created using a pairwise hamming distance comparison based on SNPs in the core genomes. Each node represents an isolate while the edge represents the hamming distance between two isolates multiplied by the total number of SNPs found in our cohorts (215,224 SNPs in *E. coli*; 96,135 SNPs in *S. enterica*). A threshold of 15 or less SNPs difference was used to filter the edges in the network as suggested by Ludden et al (2019) (*18*) and used by us previously (*17*). To consider intra- and intercluster variability across geography and time a threshold of <100 SNPs was applied as done previously (*19*) and this subset of isolate pairs was plotted using Python. Statistical comparisons were made using the SciPy package (*73*) implementing ANOVA tests with *post hoc* Tukey comparisons.

Whole genome phylogenetic analysis and Bayesian evolutionary analysis

IQTree v2.1.4-beta (*74*) was used to construct the maximum-likelihood phylogenetic
trees from the core genome alignment. For both *E. coli* and *S. enterica* different nucleotide replacement models were tested automatically with the GTR (+F+R3) replacement model selected as the best model. The Ultrafast bootstrap algorithm was used with 10000 replicates to assess branch support. The phylogenetic trees were subsequently visualised through iTOLv5 (*75*). Subsets of sequences from this study from individual phylogroups (*E. coli*) or serotypes (*S. enterica*), were selected for Bayesian evolutionary analysis using BEAST v 1.10.4 (*76*). Analysis was conducted on a core genome alignment of each lineage by using Roary v3.13 (*70*). For each species, all combinations of three clock models (strict, uncorrelated log normal, and uncorrelated exponential) and four tree priors (constant coalescent, logistic growth, Bayesian skyline, and birth-death model) were tested using steppingstone sampling on a subset of the isolates to identify the best model. The GTR-gamma nucleotide substitution model was used, as selected for the maximum likelihood

tree. The analysis was run for 3 independent chains until the effective sample size (ESS), that is, the effective number of independent draws from the posterior distribution, for all parameters was greater than 200 per chain. Convergence was assessed in Tracer v1.7.1 (*77*), and chains were subsequently combined using LogCombiner v1.10.4 (*78*). The maximum clade credibility tree was selected using TreeAnnotator v1.10.4 (*78*) and then visualized in iTOL v5 (*75*).

Plasmid Reconstruction and Evolutionary Phylogeny

Plasmids were reconstructed using the MOB-recon algorithm in the MOB-suite package v 3.1.2 (*21*), using default settings. For a subset of IncH2 plasmids, found in both species in a single sample, variants were called against the plasmid reference R478 (GenBank accession no. BX664015) and a core SNP alignment was generated in snippy v.4.4.5 (*79*). An *E. coli* poultry-sourced IncHI2 plasmid sequence, pAPEC-o1-R (Genbank DQ517526.1) was also included as an outgroup for the phylogeny. The core SNP alignment was used as input for Bayesian evolutionary analysis using BEAST v 1.10.4 (*76*).

To select the best BEAST model all combinations of three clock models (strict, uncorrelated log normal, and uncorrelated exponential) and three tree priors (constant coalescent, logistic growth, and Bayesian skyline) were tested using path sampling to identify the best model. Log marginal likelihood values were in the range of -411,744 to -403,646. The best model was a random uncorrelated exponential clock model, with a constant coalescent growth model. The GTR-gamma nucleotide substitution model was used, for nucleotide substitution. Using the chosen model, the analysis was run for 3 independent chains of 100 million steps. Convergence was assessed in Tracer v1.7.1 (*77*), and chains were subsequently combined using LogCombiner v1.10.4 (*78*). The maximum clade credibility tree was selected using TreeAnnotator v1.10.4 (*78*) and then visualized in iTOL v5 (*75*).

Mobile ARG analysis

To look for the presence of shared mobile ARG content across different sources, ARGs carried by both species were considered for samples where both species were found in a single sample. Filtered contigs (> 500bp) in each assembly were searched for ARGs and MGEs using a BLASTn (*80*) search against the CARD (*31*) and ISfinder (*81*) databases using a high identity (90%) and coverage (90%) to prevent false positives and variant uncertainty (*82*). The distance between them ARG and MGE was calculated based on the position of the ARG and MGE in the contig (*26*). ARG carrying contigs with a distance between ARG and MGE of greater than 5kb were discarded (*26, 28*), with the remaining contigs classed as mobile ARGs. Contigs were annotated using Prokka 1.14.6 (*60*). ARGs were further classified as clinically important if the ARG was included in the Risk I (clinically important ARGs dataset) according to Zhang *et al* (*32*). The structure for the mobile ARG patterns (the MGE type, ARG carried, MGE carried, sample source, farm, and distance) was summarised. For the ARG *QnrS1*, the gene structure was visualised using EasyFig (*83*).

Machine Learning analysis

Machine learning methods were used to search for the features in the genome sequence of *S. enterica* and *E. coli* isolates which could strongly correlate to resistance to each one of the of the 26 and 28 selected antimicrobials, respectively. The AMR phenotype (resistant, susceptible) of each sample was used as the class label with intermediate phenotypes neglected. One ML pipeline was proposed for each one of the studied species. In both cases, to correct for bias, the clonal the population structure was filtered based on weighted pairwise chi-squared tests between each feature and the phenotype class, as suggested by

Aun et al. 2018 (*84*) using each feature class (accessory genes, core genome SNPs and intergenic region SNPs) individually. The weights of each genome were calculated using the method of Gerstein, Sonnhammer, and Chothia (*85*). As the classes were unbalanced, we oversampled the minority class as a pre-processing step for *E. coli* using a Synthetic Minority Over-sampling Technique approach (SMOTE) (*35*) and during the training phase for *S. enterica* using a SMOTE and Edited Nearest Neighbours (SMOTEENN) approach (*86*) to balance the proportion of classes in the data set. The Python package Scikit-learn version 1.2.1 (*87*) was used to make the classification and to select the most important features.

The overall data analysis pipeline consisted of two phases:

- Phase I – WGS features preselection: For each antibiotic, isolation of a first set of WGS features (i.e. presence/absence of accessory genes, core genome SNPs and intergenic region SNPs) was conducted. For the *E. coli* pipeline the features were selected based on the correlation with the resistance/susceptibility profiles of *E. coli* using a chi-square test (p-value $< 10^{-5}$) and followed by further selection based on the Gini feature importance of an ExtraTree Classifier with 50 estimators. For the *S. enterica* pipeline, the features were selected based on the Gini feature importance of an ExtraTree Classifier with 50 estimators. In both cases the features were selected if their Gini importance was higher than the overall mean value.
- Phase II - Assessment of feature predicting-power through the development of ML-powered predictive functions: a panel of machine learning methods (logistic regression (LR), linear support vector machine (L-SVM), radial basis function support vector machine (RBF-SVM), extra tree classifier, random forest, adaboost and xgboost) were then run using as input the pre-selected features uncovered on the first step and their performances were evaluated based on a nested cross validation.

Nested Cross-validation (NCV) (*88*) was employed to assess the performance and select the hyper-parameters of the proposed classifiers. NCV consists of an outer loop dedicated to randomly split the data into new training and testing sets, and an inner loop where different configurations (sets of hyperparameters) for the predictive function are tested with the outer loop training and testing sets. In our analysis the inner loop of the NCV used a stratified 3-fold cross-validation; while the outer loop measured the ROC-AUC (receiver operating characteristic area under the curve) accuracy, sensitivity, specificity and Cohen's kappa of the test data set (unseen in the inner loop for the training) using 5-fold stratified cross-validation. Thirty iterations were carried out, wherein each iteration an NCV was employed. To compare the results obtained by the seven different classifiers used, a Friedman Statistical F-test (F_F) with Iman-Davenport correction was employed for statistical comparison of multiple (*89*). The classifiers for each dataset were ranked separately based on their AUC performance, i.e., the classifier with the highest AUC gets ranking 1, the second highest AUC gets ranking 2, and so on. In case of ties, average ranks are assigned. The F_F test was applied and the null hypothesis was rejected. The post-hoc Nemenyi test (*89*) was used to find if there is a single classifier or a group of classifiers that performs statistically better in terms of their average rank after the F_F test has rejected the null hypothesis that the performance of the comparisons on the individual classifiers over the different datasets is similar.

Undirected graphs were created using NetworkX (*90*) to visualize the interconnected WGS features (accessory genes, core genome SNPs and intergenic region SNPs) with the antibiotic models where they were selected. In addition, undirected graphs were created to visualize the interconnected ARGs (based on the selected features) and the antibiotics where they were selected.

Genome scale model

The cobra toolbox in python was used for all simulations. The models iML1515 (*41*) of *E. coli* K-12 MG1655 strain and STM v1_0 (*42*) of *S. enterica* subsp. enterica serovar Typhimurium str. LT2 were downloaded from the BiGG database (*91*) using the cameo python toolbox (*92*).

Flux variability analysis (FVA) was applied to the wild-type model and each knockout model using the cobra toolbox in python (*93*). FVA calculates the minimum and maximum flux through each reaction in the model, given a set of constraints, resulting in the range of possible fluxes for each reaction (flux span). FVA was simulated using glucose as the only carbon source in aerobic minimal M9 medium conditions. Note that reaction loops in the solution were not allowed. Networkx's greedy modularity algorithm (*90*) was applied to assign genes and reactions to a cluster in order to identify groups of genes that have a similar impact on the metabolic fluxes. We identified metabolic pathways that were enriched in each cluster using hypergeometric enrichment tests using the scipy function hypergeom. We considered a pathway as significantly enriched in a cluster if the false discovery rate (FDR) was less than 1% and used the Benjamini-Hochberg method for correction against multiple testing. We considered two sets of pathway lists for the enrichment. The first used the 40 subsystems as defined in the GSM models. A second list of pathways was downloaded from the BioCyc database using the SMART tables for *E. coli* and *S. enterica* (*94*), which provided a more extensive list of specific metabolic pathways. Significant pathways from each model were visualised in Gephi v0.9.7 (*95*). To create metabolic system diagrams the KEGG (Kyoto Encyclopaedia of Genes and Genomes) was used to create the pathway for each species, *Salmonella enterica subsp. enterica serovar Typhimurium LT2*: STM0194 (stm) for *S. enterica* and *Escherichia coli K-12 MG1655*: b0153 (eco) for *E. coli*. Additionally, MetaCyc and BIGG models web services were used to check each reaction's metabolites and responsible genes, and genes were also manually curated using literature.

Statistical Analysis

Statistical comparisons were made using the SciPy package implementing: 1. ANOVA tests with *post hoc* Tukey comparisons to consider intra- and intercluster variability across geography and time for the network analysis (p-value <0.001); 2. a chi-squared test with Bonferroni correction to evaluate the similarities between *E. coli* and *S. enterica* AMR patterns over different antibiotics (p-value <0.001); and 3. a Friedman Statistical F-test (FF) with Iman-Davenport correction for statistical comparison of multiple datasets over the seven different classifiers used (p-value <0.05). The post-hoc Nemenyi test was used to find if there is a single classifier or a group of classifiers that performs statistically better in terms of their average rank after the FF test has rejected the null hypothesis that the performance of the comparisons on the individual classifiers over the different datasets is similar. For the GSM models, we considered a pathway as significantly enriched in a cluster if the false discovery rate (FDR) was less than 1% and used the Benjamini-Hochberg method for correction against multiple testing.

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Author contributions:
Designed and supervised the study: JC, FL, ZP, XZ, LL and TD
Planned the methodology: ZP, XZ, LL, FL, JC and TD
Writing—original draft: MB, AMG, and TD
Writing—review & editing: ZP, JC, FL, MB, AMG and TD
Carried out the experiments and collected the animal and environmental samples: ZP, WW, YD, YH, HL, ZT, MZ, YG, LZ, ZH and XZ
Data analysis and visualization: AMG, MB, KB and NP
Acquired funding: ZP, DR and TD

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PRJNA841811 (E. coli WGS from other farms) available at:

https://www.ncbi.nlm.nih.gov/bioproject/PRJNA841811

PRJNA841813 (S. enterica from all farms):
https://www.ncbi.nlm.nih.gov/bioproject/PRJNA841813

The code is available on Github: https://github.com/tan0101/Commercial_WGS2023