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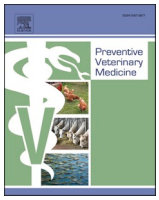

Comparing the transmission of carbapenemase-producing and extended-spectrum beta-lactamase-producing *Escherichia coli* between broiler chickens

Natcha Dankittipong [a,1], Jesse B. Alderliesten [a,1,2,3], Jan Van den Broek [a], M. Anita Dame-Korevaar [b], Michael S.M. Brouwer [b,4], Francisca C. Velkers [a,5], Alex Bossers [a,b,6], Clazien J. de Vos [b,7], Jaap A. Wagenaar [a,b,8], J. Arjan Stegeman [a], Egil A.J. Fischer [a,*,9]

[a] *Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, Utrecht, the Netherlands*
[b] *Wageningen Bioveterinary Research, Wageningen University & Research, Houtribweg 39, Lelystad, the Netherlands*

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ABSTRACT

The emergence of carbapenemase-producing *Enterobacteriaceae* (CPE) is a threat to public health, because of their resistance to clinically important carbapenem antibiotics. The emergence of CPE in meat-producing animals is particularly worrying because consumption of meat contaminated with resistant bacteria comparable to CPE, such as extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, contributed to colonization in humans worldwide. Currently, no data on the transmission of CPE in livestock is available. We performed a transmission experiment to quantify the transmission of CPE between broilers to fill this knowledge gap and to compare the transmission rates of CPE and other antibiotic-resistant *E. coli*. A total of 180 Ross 308 broiler chickens were distributed over 12 pens on the day of hatch (day 0). On day 5, half of the 10 remaining chickens in each pen were orally inoculated with $5{\cdot}10^2$ colony-forming units of CPE, ESBL, or chloramphenicol-resistant *E. coli* (catA1). To evaluate the effect of antibiotic treatment, amoxicillin was given twice daily in drinking water in 6 of the 12 pens from days 2–6. Cloacal swabs of all animals were taken to determine the number of infectious broilers. We used a Bayesian hierarchical model to quantify the transmission of the *E. coli* strains. *E. coli* can survive in the environment and serve as a reservoir. Therefore, the susceptible-infectious transmission model was adapted to account for the transmission of resistant bacteria from the environment. In addition, the caecal microbiome was analyzed on day 5 and at the end of the experiment on day 14 to assess the relationship between the caecal microbiome and the transmission rates. The transmission rates of CPE were 52 – 68 per cent lower compared to ESBL and catA1, but it is not clear if these differences were caused by differences between the resistance genes or by other differences between the *E. coli* strains. Differences between the groups in transmission rates and microbiome diversity did not correspond to each other, indicating that differences in transmission rates were probably not caused by major differences in the community structure in the caecal microbiome. Amoxicillin treatment from day 2–6 increased the transmission rate more than three-fold in all

Abbreviations: ASV, amplicon sequence variant; BSL, Biosafety level; CPE, carbapenemase-producing *Enterobacteriaceae*; *E. coli*, *Escherichia coli*; ESBL, extended-spectrum beta-lactamase; HPDI, highest posterior density interval; MAP, maximum a posteriori estimate; PCoA, Principal coordinate analysis; SI-model, susceptible-infectious model; SIS-model, susceptible-infectious-susceptible model; WBVR, Wageningen Bioveterinary Research.

* Corresponding author.
E-mail address: E.A.J.fischer@uu.nl (E.A.J. Fischer).
[1] These authors contributed equally
[2] Current address: Biosystems Data Analysis, Swammerdam Institute for Life Sciences, University of Amsterdam, Sciencepark 904, Amsterdam, The Netherlands
[3] https://orcid.org/0000-0003-1132-4310
[4] https://orcid.org/0000-0002-1067-7555
[5] https://orcid.org/0000-0002-2923-3233
[6] https://orcid.org/0000-0002-6586-717X
[7] https://orcid.org/0000-0002-9280-7041
[8] https://orcid.org/0000-0002-3506-1490
[9] https://orcid.org/0000-0002-0599-701X

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inoculums. It also increased alpha-diversity compared to untreated animals on day 5, but not on day 14, suggesting only a temporary effect. Future research could incorporate more complex transmission models with different species of resistant bacteria into the Bayesian hierarchical model.

1. Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE; also referred to as carbapenem-resistant *Enterobacteriaceae*) are potentially life-threatening bacteria because of their resistance to clinically important carbapenem antibiotics (Brink, 2019; World Health Organization, 2019; Zhou et al., 2021). CPE are detected worldwide in farm animals, wild animals, companion animals, and the environment (Köck et al., 2018; Bonardi and Pitino, 2019). The emergence of CPE in meat-producing animals is particularly worrying because consumption of meat contaminated with resistant bacteria comparable to CPE, such as extended-spectrum beta-lactamase (ESBL)-producing bacteria or plasmid-encoded AmpC-producing bacteria, contributes to colonization in humans worldwide (Leverstein-van Hall et al., 2011; Rousham et al., 2018; Mughini-Gras et al., 2019). Consequently, it is crucial to assess the transmission dynamics of CPE in livestock farms. We looked at transmission between broilers because the prevalence of ESBL-producing bacteria in broilers is high compared to other livestock (European Food Safety Authority and European Centre for Disease Prevention Control, 2022). Although the prevalence of CPE in animals is much lower than the prevalence of ESBL/plasmid-encoded-AmpC-producing bacteria (European Centre for Disease Prevention and Control, 2018), poultry is at risk of CPE introduction (Dankittipong et al., 2022). Differences in selective pressure caused by historical use in livestock of third-generation cephalosporins that co-select for carbapenemase-producing genes (Ogunrinu et al., 2020) compared to the use of carbapenems having worldwide never been allowed in livestock might contribute to the difference in prevalence. Despite this restriction of carbapenem usage in livestock, cases of CPE have been detected worldwide in livestock since 2009 (Köck et al., 2018; Madec and Haenni, 2018).

Transmission of ESBL-producing *Escherichia coli (E. coli)* in poultry has been investigated extensively (Huijbers et al., 2016; Dame-Korevaar et al., 2019; Robé et al., 2019; Dame-Korevaar et al., 2020a; Dame-Korevaar et al., 2020b), showing among others that 2 strains of beta-lactamase-producing bacteria (carrying $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-2}}$, respectively) colonized broilers at the same rate (Dame-Korevaar et al., 2019). In contrast, no data on the transmission of CPE in livestock is available. The transmission rate parameter β is a key parameter to describe the transmission dynamics in populations and is here defined as the rate of successful transmission per time unit following contact with an infectious source such as bacteria carrying resistance genes (Keeling and Rohani, 2007).

Conventional methods to quantify the transmission of bacteria assume direct transmission between animals (Velthuis et al., 2007). However, *E. coli* can survive for a considerable amount of time in the environment (Table S15) and is commonly transmitted between animals through the faecal-oral route (Lister and Barrow, 2008; van Elsas et al., 2011; van Bunnik et al., 2014). Previous transmission experiments of ESBL-producing bacteria in broilers, nalidixic-resistant *E. coli* in broilers, and *Salmonella* Dublin in young dairy calves highlighted the excretion of these bacteria into the environment and subsequent acquisition of excreted bacteria from the environment as a key mechanism of transmission (Nielsen et al., 2007; van Bunnik et al., 2014; Dame-Korevaar et al., 2017).

Antibiotic usage is a primary driver of resistant bacteria in clinical and non-clinical settings (Knobler et al., 2003; Davies and Davies, 2010; Holmes et al., 2016) and is widespread in livestock worldwide (Mathew et al., 2007; Aarestrup, 2015). Twenty-two per cent of the conventional broiler farms in the Netherlands did not use antibiotics in 2020, but 44% had a persistently high antibiotic usage exceeding the action threshold defined by the Netherlands Veterinary Medicines Institute and 5% had a persistently high antibiotic usage exceeding the sector-negotiated action threshold (Bonten et al., 2021). Treatment with antibiotics generally temporarily decreases the number of bacterial species in the gut microbiome and lowers the abundance of some common taxa, allowing the abundance of some low-abundant taxa or opportunistic pathogens to increase (Kim et al., 2017; Rochegüe et al., 2021). This might affect the transmission of bacteria, because a more diverse gut microbiome hinders colonization by exogenous bacteria (Kim et al., 2017; Sorbara and Pamer, 2019), thereby reducing the excretion of these bacteria (Dame-Korevaar et al., 2020b).

We performed a transmission experiment to quantify the transmission of CPE between broilers and to quantitatively compare the transmission rates of CPE and ESBL-producing *E. coli*. Groups with and without amoxicillin treatment were compared to investigate if and how antibiotic treatment affects the transmission, and relations between differences in transmission rates and the caecal microbiome were assessed.

2. Material and method

2.1. Transmission experiment

The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands) under registration number AVD108002015314 and all procedures were performed in full compliance with all legislation. All broilers were observed daily, and any abnormality and mortality were recorded.

2.1.1. Inoculums

Three inoculums were prepared for this experiment, referred to as the CPE-strain, ESBL-strain, and catA1-strain throughout the paper (Table 1). The inoculums were three different *E. coli* strains obtained from broilers in conventional farms in Europe. They contained 3 – 6 plasmids and resistance genes from various families (Tables S17 – S19). Before inoculation, all strains were streaked on heart infusion agar with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany), transferred to LB medium, and cultured overnight. The *E. coli* cultures were diluted in phosphate-buffered saline with 0.5 McFarland standards resulting in $1.10{\cdot}10^8$ bacteria suspension per mL. Prepared inoculums were enumerated in duplicate counts and each contained $0.55{\cdot}10^3$ – $1.0{\cdot}10^3$ colony-forming units per mL.

2.1.2. Sampling scheme and experimental design

The experiment was conducted in human Biosafety level 3 (BSL-3) facilities at Wageningen Bioveterinary Research (WBVR), Lelystad. Before the experiment, samples from the parent stock and environmental samples from the incubator (BSL-1) and experimental facilities were taken which confirmed the absence of ESBL-producing *E. coli*. Two hundred and forty eggs were collected from a conventional Ross 308 broiler parent stock, individually disinfected with 3% hydrogen peroxide and incubated for 21 days at BSL-1 experimental facilities of WBVR. On the day of hatch, day 0, 180 hatchlings were transported to the BSL-3 animal facilities of WBVR, where they were weighted, neck tagged with an individual number and randomly distributed over 12 pens, with 15 unsexed broilers per pen (see Table S3 for an overview of the distribution of the sexes in the different groups). Broilers of both sexes were used because a mixed group reflects the practical situation in

terms of group dynamics and the prevalence of ESBL or CPE is not known to differ by gender. Pens had a surface area of 1.35 m^2, with a bedding of sterilized wood shavings, and were separated from each other by fences of 70 – 80 cm high such that no direct contact was possible between pens. Broilers had ad libitum access to feed and water and a standard lighting and temperature scheme for broiler chickens was used. The feed should have been a standard broiler diet without antibiotics or coccidiostats containing 2800 – 2900 kcal of apparent metabolizable energy per kg, but accidentally feed for layer pullets, free of antibiotics and coccidiostats, was provided. The feed was based on wheat, maize, and soybean meal and contained 2563 kcal of apparent metabolizable energy per kg and 20% of crude protein heated to 90 °C. From days 2–6, amoxicillin was provided via drinking water twice a day at the suppliers' recommended dose of 20 mg/kg live weight to the broilers in pens 3, 4, 7, 8, 11, and 12 (Fig. 1). Amoxicillin was used as an example of a broad-spectrum antibiotic commonly used in broilers (Ventola, 2015; Heederik et al., 2017) to compare the transmission of all inoculums in the absence and presence of antibiotic treatment. Amoxicillin is rapidly degraded in the environment (Peng et al., 2016), which ensures antibiotic residues in the environment will not serve as an additional source of antibiotic exposure for the broilers.

On day 5, cloacal swabs were taken from all broilers using sterile dry Eswabs (MW100, Medical Wire & Equipment, England) to confirm the absence of CPE and ESBL-producing *E. coli*. Ten broilers per pen were kept for the transmission experiment and surplus broilers (at most 5 per pen) were euthanized and their caecal content was collected for microbiome analysis. Five broilers randomly chosen out of the 10 remaining broilers per pen were separated from the other broilers and orally inoculated (using a syringe with a crop needle) with 0.5 mL PBS containing approximately 10^3 colony-forming units of *E. coli*, i.e., the CPE-strain (pens 1 – 4), the ESBL-strain (pens 5 – 8), or the catA1-strain (pens 9 – 12) per mL. One hour after inoculation, inoculated broilers were returned to their pen where they resided with contact broilers (i.e., broilers that were not inoculated). Cloacal swabs were taken from all broilers at approximately 8 h after inoculation on day 5, twice on day 6 (8 h apart), and once per day on days 7–10, 12, and 14 (Fig. 1) (Dame-Korevaar et al., 2020a). All broilers were euthanized on day 14 and their caecal content was collected for microbiome analysis.

2.1.3. Phenotypic resistance detection

All cloacal swabs were non-selectively enriched overnight in 3 mL buffered peptone water at 37 °C. Thereafter they were inoculated onto selective MacConkey plates supplemented with 0.5 mg/L ertapenem (swabs from pens 1 – 4), 1 mg/L cefotaxime (swabs from pens 5 – 8), or 64 mg/L chloramphenicol (swabs from pens 9 – 12) using a sterile loop and incubated overnight at 37 °C. A test result was defined as positive when colonies were detected on MacConkey plates after overnight incubation. The pen, used inoculum, antibiotic treatment, and the test results of the cloacal swabs (i.e., positive or negative for CPE-strain, ESBL-strain, or catA1-strain) at each sampling time point were recorded for all inoculated and contact broilers (Table S1).

2.1.4. Microbiome sequencing

Microbial DNA was isolated from 0.2 g caecal content according to the manufacturer's instructions using the PureLink microbial DNA isolation kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Negative controls spiked with a low concentration of microbial community DNA standard (ZymoBIOMICS; Zymo Research Corporation, Irvine, CA) were used in the batches of DNA isolation and amplification thereafter as control of performance and sanity throughout the processing (see Fig. S1 for a comparison of the theoretical and obtained composition of these negative controls). Following extraction, the DNA extracts were quantified with an InvitrogenTM QubitTM 3.0 Fluorometer and stored at − 20 °C for further processing. The hypervariable regions V3 +V4 of the 16 S rRNA gene were amplified in triplicate using a limited-cycles PCR with the primers CVI_V3-forw CCTACGGGAGGCAGCAG and CVI_V4-rev GGACTACHVGGGTWTCT. The following amplification conditions were used as previously described (Jurburg et al., 2019): 98 °C for 2 min, followed by 20 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 10 s, and finally by 72 °C for 7 min. Triplicate PCR products were pooled per sample and checked on a TapeStation (Agilent, USA) and after barcode indexing subsequently sequenced on a MiSeq sequencer (Illumina Inc., San Diego, CA) using a version 3 paired-end 300 bp kit.

2.2. Data analysis

2.2.1. SI- and SIS-models

The transmission of *E. coli* between broilers was modelled using a compartmental susceptible-infectious model (SI-model; Fig. 2) and a compartmental susceptible-infectious-susceptible model (SIS-model). Previous research identified excretion and subsequent acquisition of *E. coli* from the environment as a key mechanism of transmission (Lister and Barrow, 2008; van Bunnik et al., 2014). We incorporated this in our models by assuming excreting broilers (I) excrete viable bacteria into the environment of their pen at a constant rate of ω units per hour from the moment they start to excrete, and these excreted bacteria will decay at a rate δ. The unknown excretion rate (ω) was scaled such that the hazard produced by 1 broiler during 1 time unit is 1 (Chang and de Jong, 2023). The environmental hazard at time t is denoted as E_t. A detailed description of the models including the scaling is given in Section 3.1 'Susceptible-infectious model' of the supplementary material.

Based on the test results of the cloacal swabs (see Section 2.1.3 'Phenotypic resistance detection' above), broilers were considered to be uncolonized or colonized. When uncolonized contact broilers were colonized through contact with bacteria in the environment at rate $\beta S_t \quad E_t$, they were denoted as cases and incorporated in the SI- and SIS-model as excreting from the time point they tested positive. Initially-uncolonized inoculated broilers were assumed to start excreting through inoculation instead of through contact with bacteria in the environment and were therefore not denoted as cases.

In the SI- and SIS-models it is assumed that contact broilers are either susceptible (S) or excreting (I). In the SI-model it is assumed broilers will continue to excrete until the end of the experiment once they start excreting. To adhere to this structure, a negative test result in a broiler that previously tested positive is assumed to be false negative (see section 1.2 'Protocols to adjust raw transmission data' with Table S2 of the supplementary material). In pens 3, 4, 11 and 12, the first positive tests for inoculated and contact broilers occurred at the same time point. However, at least one inoculated broiler must start excreting before

Table 1
Characteristics of the CPE, ESBL, and catA1 isolates used as inoculums. Abbreviations: Inc-group: incompatibility group; MLST: multi-locus sequence type.

Inoculum	*E. coli* isolate	MLST	Selected resistance	Gene	Plasmid Inc-group	Host's country of origin	Reference
CPE-strain	CFSAN 083827	4980	Carbapenem	*blaOXA-162*	HI2	Romania	(Bortolaia et al., 2021)
ESBL-strain	SafeFoodEra-230	101	Extended-spectrum beta-lactam	*blaCTX-M-2*	HI2	Germany	(Wu et al., 2013)
catA1-strain	EFFORT 102803008	10	Chloramphenicol	*catA1*	FIB/FII	The Netherlands	(Leekitcharoenphon et al., 2021)

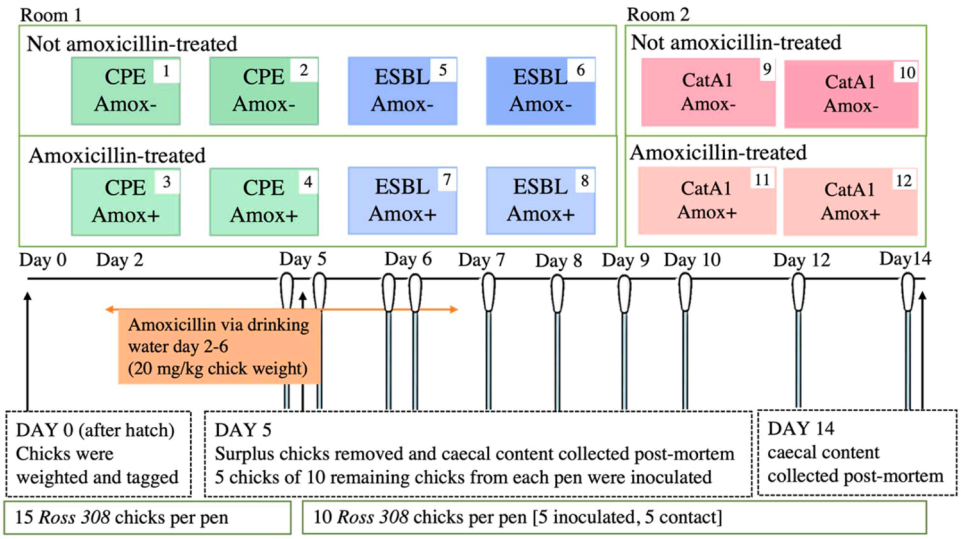

Fig. 1. Setup of the pens (top) and timeline of the experimental design from the moment of hatch (day 0) to the end of the experiment on day 14, with the sampling time points indicated by the swabs (bottom). Abbreviations: Amox-: non-amoxicillin-treated; Amox+ : amoxicillin-treated.

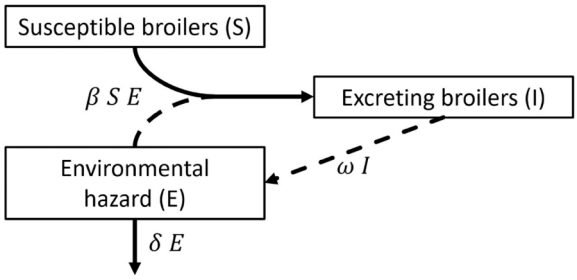

Fig. 2. Compartmental SI-model of indirect transmission of E. coli between broilers. Excreting broilers (I; positive inoculated broilers and positive contact broilers) excrete bacteria into the environment at rate ω. Only negative contact broilers are counted as susceptible broilers (S) because uncolonized inoculated broilers are assumed to start excreting through inoculation instead of through colonization after contact with the environmental hazard (E). Environmental hazard decays at rate δ(h^{-1}). Susceptible contact broilers become colonized through contact with bacteria in the environment at transmission rate parameter β (h^{-1}), thus becoming excreting broilers. The lines connecting the environmental hazard with excreting broilers are dashed to indicate they do not denote flow from one compartment to another.

colonization of contact broilers can occur. Therefore, we assumed inoculated broilers started excreting halfway between the first time point they tested positive and the previous sampling time point, and contact broilers were assumed to start excreting slightly slower, from the time point they tested positive. In the SIS-model it is assumed excreting broilers (*I*) can lose the resistant bacteria and become susceptible (*S*) again if they test negative after a positive test.

It was not possible to estimate the decay rate and the transmission rate parameter simultaneously with the Bayesian model in our study, because a given number of cases can be explained equally well by a higher transmission rate or a lower decay rate. We reviewed the literature on decay rates (Table S15) to find a suitable range of decay rates and ran the hierarchical model with several fixed decay rates ranging from 0.04 to 55 h^{-1} (Table S16). This entire range of decay rates could be fitted well with low Watanabe–Akaike information criterion and divergence transition. Multiple studies in various environments suggest a very low level of *E. coli* decay in the first few days (see Section 3.3 'Decay rate' of the supplementary material), therefore we selected the lowest fixed decay rate (δ) of 0.04 h^{-1} in the final model.

2.2.2. Bayesian hierarchical inference

A Bayesian hierarchical model was used to infer the parameters of the SI-model (see Section 3.2 'Bayesian hierarchical inference' of the supplementary material). Bayesian inference is a statistical method which requires prior probability distributions for the parameters, observed data (i.e., the number of positive and negative broilers at each sampling time point in each pen), and a likelihood function. A hierarchical model was used to simultaneously analyse the data at the group level and at the entire dataset to take the information that is present in the different clusters into account (McElreath, 2020), which fits perfectly into transmission experiment data where animals are grouped in pens. The transmission rate parameter (β), which indicates the infectivity and susceptibility of animals, was estimated separately for each pen i from the number of susceptible broilers and the hazard by estimating the average transmission rate parameter over all pens ($\overline{a}$) and the between-pen variation of the transmission rate parameter (z_i). Consequently, transmission in pen i occurs at rate parameter β_i that is the product of the individual transmission rate parameter in that pen ($\overline{a} + z_i$) and the environmental hazard in that pen (E_t). Posterior distributions of the transmission rate parameter for the different clusters (i.e., inoculum and antibiotic treatment) were obtained by combining the posterior distributions of $\overline{a}+z_i$ of all pens in that specific cluster.

We used results from a previous transmission study in broilers (Dame-Korevaar et al., 2020a) to define prior probability distributions (priors) for the average transmission rate parameter ($\overline{a}$) and its standard deviation (σ). In contrast to (Chang and de Jong, 2023), we fixed the decay rate to 0.04 h^{-1} because the broilers remain excreting until the end of the experiment (Table S1) such that the decay rate could not be estimated from the data.

Using the prior probabilities of the parameters and the likelihood function, parameter values were drawn using the Markov chain Monte Carlo simulated process. Four independent Markov chains (Fig. S11) were initiated in the model. The transmission rate of each inoculum was extracted from the posterior distribution and transmission rates were compared using the 95% highest posterior density interval (HPDI) and the point estimate at the highest density (maximum a posteriori estimate, MAP). Differences in transmission rates between inoculums and antibiotic treatments were compared by calculating the posterior distribution of the ratio of the transmission rates.

2.2.3. Microbiome analysis

The amplicon sequences were demultiplexed using *bcl2fastq*

(Illumina Inc., San Diego, CA) and subsequently filtered, trimmed, error-corrected, dereplicated, chimaera-checked, and merged using R package dada2 1.16.0 (Callahan et al., 2016) with the standard parameters except for TruncLength = (270, 220), trimLeft = (25, 33), maxEE = 2 and minOverlap = 10, using a pseudo-pooling strategy. Reads were classified against the SILVA database version 138 (Quast et al., 2012). The data, the phyloseq object containing the sequence data, and the R code used for the modelling and analyses are provided at https://zenodo.org/record/7766926 (DOI: 10.5281/zenodo.7766926).

The number of reads in the samples (excluding negative controls) ranged from 1363 to 320392 and was standardized to 9071 reads per sample (7th least number of reads; rarefy_even_depth, seed = 314; Fig. S2) before alpha-diversity analysis. The final dataset contained 9540981 reads and 7952 different amplicon sequence variants (ASVs). Sequences are deposited in NCBI's Sequence Read Archive under BioProject accession number PRJNA948179.

DNA sequences isolated from caecal material obtained on days 5 and 14 were analysed separately. Non-bacterial sequences were discarded. Rarefaction curves on genus- and ASV-level were created to check if all genera and ASVs in the samples were recovered (Fig. S3). Observed richness, Shannon's index and Pielou's evenness were used to measure alpha-diversity (Finotello et al., 2018). Kruskal-Wallis rank sum test and post hoc Dunn's test with Benjamini-Hochberg correction (Dinno, 2017) were used to test for the effects of the inoculums, antibiotic treatment, and their interaction on alpha-diversity, using a significance level of 0.05. Beta-diversity, a measure of dissimilarity between communities regarding shared taxa, was analysed on non-rarefied data using Bray-Curtis dissimilarity (measuring the fraction of the bacteria specific to either group) and Jaccard distance (measuring the fraction of taxa specific to either group, i.e., comparing presence and absence) (Schmidt et al., 2017) and visualized using the first 2 axes of the principal coordinate analysis (PCoA). Permutational multivariate analysis of variance was performed using the adonis2 function from the vegan package in R to test for effects of inoculum, antibiotic, and their interaction on beta-diversity, and the betadisper function from the vegan package was used to test for homogeneity of group dispersions. The simper function from the vegan package was used to determine which genera contribute most to the Bray-Curtis dissimilarity between groups without and with antibiotic treatment.

2.2.4. Used software

Transmission data were analysed with R version 4.1.2 (RCore Team, 2020) with package rstan 2.21.5 (Stan Development Team, 2020) using a tree depth of 14, an acceptance rate of 0.99 and 4 chains with 4000 iterations, and packages rethinking 2.21 (McElreath, 2020), cmdstanr 0.5.2 (Gabry and Cešnovar, 2022), StanHeaders 2.21.0–7 (Stan Development Team, 2018) and bayestestR 0.12.1 (Makowski et al., 2019). Sequence processing and statistical analyses related to the sequencing were performed with R 4.0.2 (R Core Team, 2020) with package dada2 1.16.0 (Callahan et al., 2016). Subsequent analyses of the microbiome data were performed with R 4.1.2 (R Core Team, 2021) with packages phyloseq 1.38.0 (McMurdie and Holmes, 2013), microbiome 1.16.0 (Lahti and Shetty, 2019), vegan 2.6.2 (Oksanen et al., 2022), and dunn.test 1.3.5 (Dinno, 2017), using packages tidyr 1.2.0 (Wickham and Girlich, 2022), dplyr 1.0.9 (Wickham et al., 2021), and Biostrings 2.62.0 (Pagès et al., 2022) for data handling, and ggplot2 3.3.6 (Wickham, 2016) and cowplot 1.1.1 (Wilke, 2020) for plotting.

3. Results

3.1. Transmission experiment

The 111 out of 120 inoculated and contact broilers that survived until the end of the experiment all became colonized by the *E. coli* strain used for inoculation (i.e., CPE-strain, ESBL-strain, or catA1-strain) and were still colonized on day 14 (the last day of the experiment). Four broilers from the CPE-strain group, 4 broilers from the ESBL-strain group, and 1 broiler from the catA1-strain group died (Table S1). The majority of the broilers gained weight slower and reached 20% lower weights at day 14 than typical Ross 308 broilers, probably because they received feed for laying pullets instead of broilers. No other abnormalities were observed.

3.2. Transmission rates

3.2.1. Predicted versus observed cases

The number of cases predicted by the hierarchical model is higher than the number of observed cases in non-antibiotic-treated pens and lower than the number of observed cases in antibiotic-treated pens because of the shrinkage caused by the hierarchical modelling (Fig. 3). Shrinkage is a key feature of a hierarchical model because the measurements of different clusters (i.e., inoculum and antibiotic treatment) inform one another such that the predicted results shrink towards the overall mean (McElreath, 2020).

The number of cases increased over a longer period in non-amoxicillin-treated pens (top rows) than in amoxicillin-treated pens (bottom rows) because the larger transmission rate in amoxicillin-treated pens led to the depletion of susceptible broilers.

3.2.2. Effect of inoculums

The estimated transmission rates for broilers inoculated with the CPE-strain, ESBL-strain, and catA1-strain are shown with their 95% HPDI and the MAP (shaded area and purple vertical line in Fig. 4, Fig. 5). The MAP suggests that CPE-strain has the lowest transmission rate of the 3 inoculums.

The MAP of the estimated transmission rate of the CPE-strain is 46% and 48% of the transmission rate of the ESBL-strain and the catA1-strain in the non-amoxicillin-treated groups, respectively, and 32% and 41% of the transmission rate of ESBL-strain and catA1-strain in the amoxicillin-treated groups, respectively (Fig. 5). HPDIs of the ratio of the transmission rates indicate the probability that transmission of the CPE-strain is faster than the transmission of the ESBL-strain or catA1-strain is 8–10% in non-amoxicillin-treated groups, and 3–6% in amoxicillin-treated groups (Fig. 5). The MAP of the ratio of the ESBL-strain transmission rate to catA1-strain transmission rate is 0.80 without amoxicillin treatment and 0.90 with amoxicillin treatment, and the probability of a ratio equal to or larger than 1 is 0.48 and 0.63 for the groups without and with amoxicillin, respectively. This indicates the transmission rates of the ESBL-strain and the catA1-strain were similar in this experiment.

3.2.3. Effect of amoxicillin

The transmission rates of all inoculums are smaller in the non-amoxicillin-treated groups than in the amoxicillin-treated groups (Fig. 6). The difference between those groups is slightly larger for the ESBL-strain and catA1-strain than for the CPE-strain.

3.3. Microbiome analysis

3.3.1. Alpha-diversity

Observed richness which measures the observed number of taxa, Shannon's index which takes evenness into account (with higher values if more taxa are present or taxa are more evenly distributed), and Pielou's evenness which is not influenced by richness (with a value between 0 and 1, with higher values if taxa are more evenly distributed), were used to measure alpha-diversity. All alpha-diversity measures of the caecal microbiome at genus level on day 5 (i.e., before inoculation) were similar in the groups inoculated with the different inoculums (i.e., CPE-strain, ESBL-strain, catA1-strain; Fig. 7). On day 14 various small differences in observed richness and Pielou's evenness were found at genus level. Repeating these analyses at the level of individual ASVs mostly gave the same results (Fig. S8; Tables S6 – S9).

Amoxicillin treatment affected the microbiome composition at class

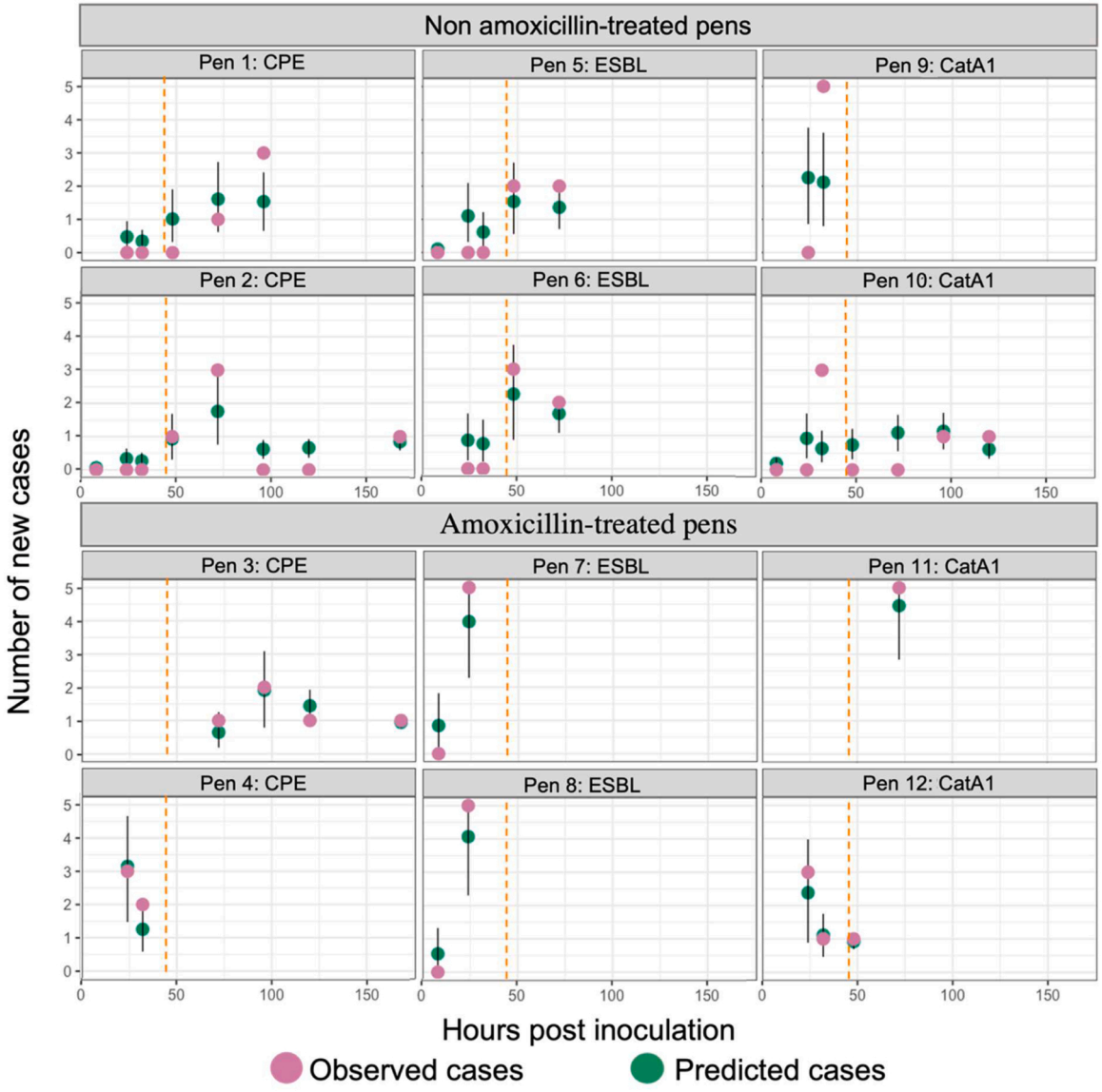

Fig. 3. Number of cases over time. Observed (violet) and predicted (green) number of new cases among the 5 susceptible broilers (i.e., susceptible contact broilers that became colonized) (vertical axis) in each of the 12 pens until the sampling time point in hours after inoculation (horizontal axis). Dashed yellow vertical lines indicate the time point antibiotic administration stopped. For the predicted numbers the maximum a posteriori estimates are given, with the whiskers indicating 95% highest posterior density intervals. Transmission cannot occur when none of the broilers is excreting yet or when all broilers are excreting. No data is shown at those time points.

level, family level, and genus level (Fig. S4; Fig. S5; Fig. S6). Observed richness and Shannon's index at genus level on day 5 were lower in the non-amoxicillin-treated groups than in the amoxicillin-treated groups, but Pielou's evenness was not different (Fig. 7), indicating fewer genera were present in the non-amoxicillin-treated groups but the distribution of their abundances was similar to the distribution of their abundances in the amoxicillin-treated groups. By day 14, 8 days after finishing amoxicillin treatment, alpha-diversity was similar in the amoxicillin-treated and non-amoxicillin-treated groups. Repeating these analyses at the level of individual ASVs mostly gave the same results (Fig. S8; Tables S6 – S9).

3.3.2. *Beta-diversity*

The inoculums explained 6% and 3% of the variation between the groups in Bray-Curtis dissimilarity and Jaccard distance at genus level on day 5, antibiotic treatment explained 27% and 50% of the variation, and their interaction explained 5% and 3% of the variation (Table S10). Only groups without and with antibiotics were separated in the PCoA-plot (Fig. 8). Repeating these analyses at the level of individual ASVs mostly gave the same results (see sections 2.5 'Beta-diversity at ASV level' and 2.6 'Beta-diversity: tables' of the supplementary material). Similarity percentage analyses showed the Bray-Curtis dissimilarities on day 5 between groups without and with antibiotic treatment are driven by the same genera in the groups inoculated with the different inoculums. Most of these genera belonged to the classes Bacilli and Clostridia, and some to the class Gammaproteobacteria (Tables S12 – S14).

The inoculums explained 16% and 17% of the variation between the groups in Bray-Curtis dissimilarity and Jaccard distance at genus level on day 14, antibiotic treatment explained 9% of the variation for both measures, and their interaction explained 4% and 6% of the variation (Table S11). For both beta-diversity measures, CPE-strain and ESBL-strain overlapped much with each other in the PCoA-plots, whereas catA1 without antibiotics separated from CPE-strain and ESBL-strain without antibiotics. Groups without and with antibiotics were not separate from each other on genus level (Fig. 8) but separated on ASV level with Bray-Curtis dissimilarity (Fig. S9).

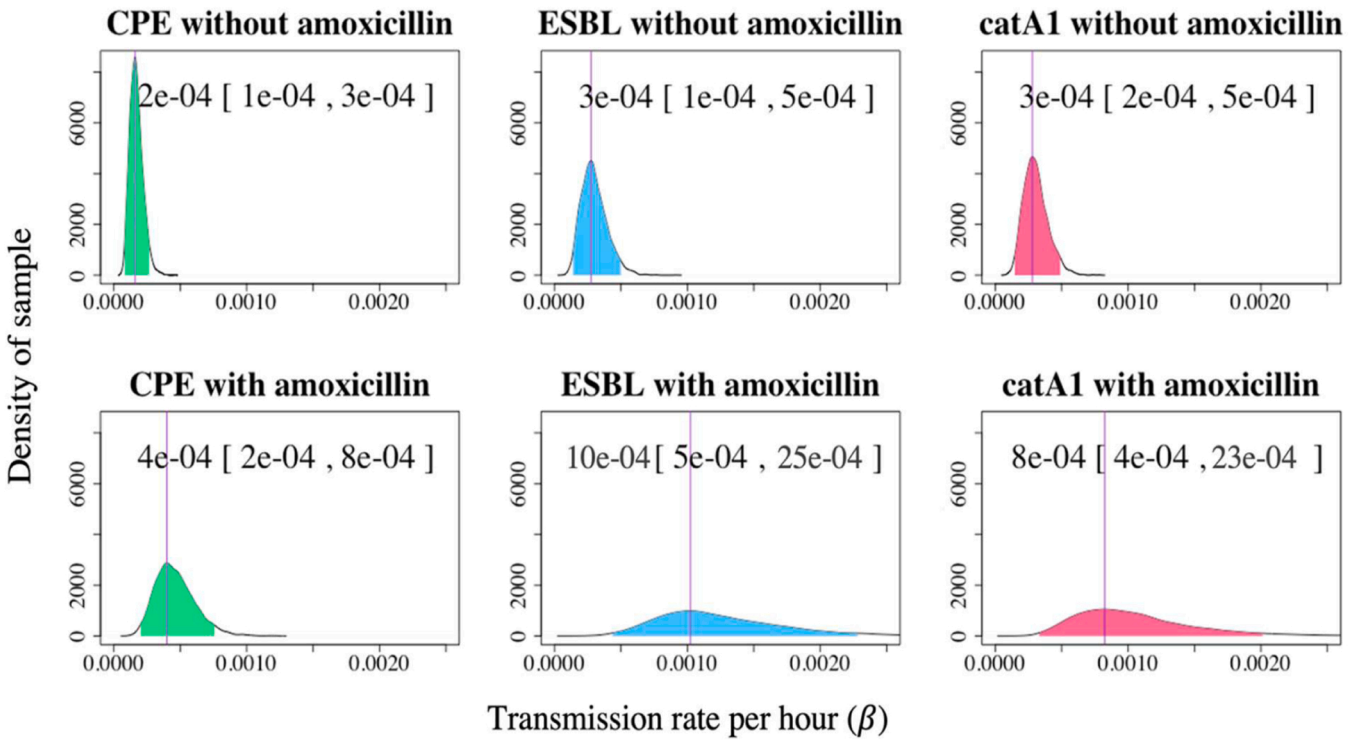

Fig. 4. Density (vertical axis) of the posterior distribution of the transmission rate per hour (horizontal axis) for the CPE-strain, ESBL-strain and catA1-strain. The top and bottom row show plots for the pens without and with amoxicillin treatment, respectively. Purple vertical lines indicate the point estimate at the highest density and shaded areas are the 95% highest posterior density intervals of the posterior distribution; the estimated values of both are shown at the top of the plot.

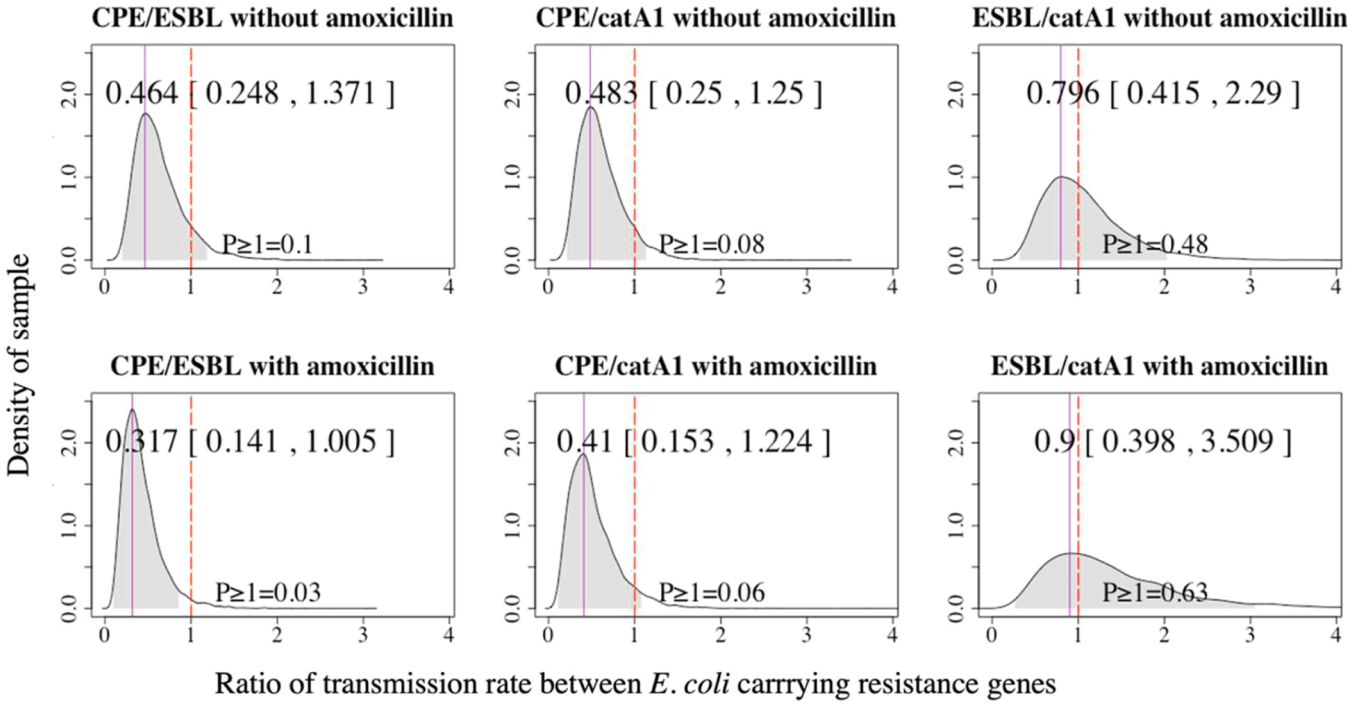

Fig. 5. Density (vertical axis) of the posterior distribution of the ratio of the transmission rates (horizontal axis) for different inoculums: CPE-strain to ESBL-strain, CPE-strain to catA1-strain, and ESBL-strain to catA1-strain. The top and bottom row show plots for the pens without and with amoxicillin treatment, respectively. Purple vertical lines indicate the point estimate at the highest density and shaded areas are the 95% highest posterior density intervals of the posterior distribution; the estimated values of both are shown at the top of the plot. Dotted vertical red lines indicate a ratio of 1 and the probability of a ratio equal to or larger than 1 ($P \geq 1$) is shown at the bottom of the plot.

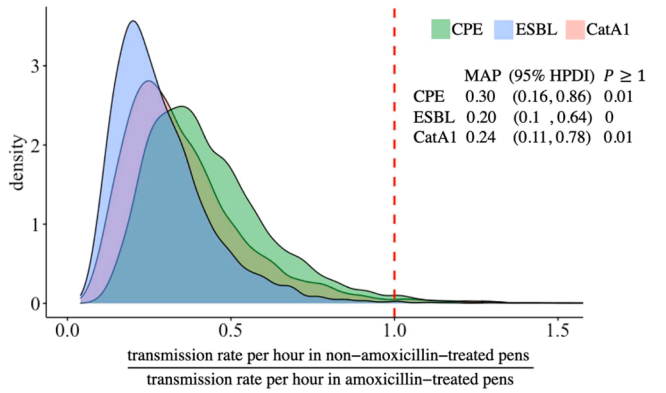

Fig. 6. Density (vertical axis) of the ratio of the transmission rates in non-amoxicillin-treated pens over amoxicillin-treated pens (horizontal axis) for the CPE-strain (green), ESBL-strain (blue) and catA1-strain (pink) in the SI-model. The dotted red vertical line indicates a ratio of 1 (i.e., the transmission rates of amoxicillin-treated and non-amoxicillin groups are the same). The point estimate at the highest density (MAP), 95% highest posterior density intervals (95% HPDI), and the probability of a ratio equal to or larger than 1 ($P \geq 1$) are also shown in the plot.

4. Discussion

To our knowledge, this is the first transmission experiment with CPE *E. coli* in livestock. In addition, although the use of a Bayesian hierarchical model as presented in this study is well-recognized in epidemiology, its use in analysing animal transmission experiments is not common (Hu et al., 2017). Furthermore, we extended previous work on the relationship between the microbiome and the transmission of intestinal antibiotic-resistant bacteria (Dame-Korevaar et al., 2020b).

4.1. Indirect environmental transmission

E. coli is an enteric bacterium that is excreted with the faeces in the environment (Conway and Cohen, 2015; Ramos et al., 2020), from where it can spread to other animals and humans (Rwego et al., 2008; Hussain et al., 2017; Rousham et al., 2018; Lepper et al., 2022). The environment can serve as a reservoir for the transmission of resistant bacteria when no excreting animals are present anymore (Dame-Korevaar et al., 2017). Therefore, we adapted the likelihood function to reflect environmental transmission with its prolonged possibility of transmission from accumulated bacteria in the environment.

The transmission rates of $3{\cdot}10^{-4}$ h^{-1} and $1{\cdot}10^{-3}$ h^{-1} for the ESBL-strain derived from our model assuming indirect environmental transmission are much lower than the transmission rate of $5.5{\cdot}10^{-2}$ ($4.5{\cdot}10^{-2}$ – $6.6{\cdot}10^{-2}$) h^{-1} calculated from a direct model (Dame-Korevaar et al., 2020b). A lower transmission rate is expected because resistant bacteria excreted into the environment were the only source of transmission considered in our SI-model and they decayed at a low rate because we selected the lowest fixed decay rate (δ)of 0.04 h^{-1} based on the available literature (see Section 2.2.1 'SI- and SIS-models' above and Tables S15 and S16). Using a higher decay rate would result in higher estimates for the transmission rates (Table S16), with a decay rate of 7.4 h^{-1} giving a transmission rate of 0.04 h^{-1} for the ESBL-strain without antibiotics, comparable to the value obtained by (Dame-Korevaar et al., 2020b). Using higher decay rates still results in transmission rates of the CPE-strain being lower than the transmission rates of the other inoculums.

The transmission rates calculated from the SIS-model (see section 3.6 'SIS-model result' of the supplementary material) are similar to the transmission rates calculated from the SI-model, showing that relaxing our assumption in the SI-model that broilers are excreting until the end of the experiment once they test positive would not change our conclusions. The robustness of the model can be extended to other transmission experiments in which the inference is dependent on the available information more than the assumed transmission models.

4.2. Bayesian hierarchical inference

The actual moment of transmission is rarely observed in transmission experiments because of logistic and ethical limitations to the number of animals and the sampling frequency (Cauchemez et al., 2004). A Bayesian approach in the analysis of transmission experiments can be used to incorporate the uncertainty that is inherent to the data in the statistical model and to clearly present the uncertainty in the outcomes in the form of the posterior distribution (Hiura et al., 2021). These characteristics make Bayesian hierarchical modelling very suitable to quantify transmission between animals.

The Bayesian hierarchical model quantifies the transmission rate parameter of each pen using the mean transmission rate parameter and its variation simultaneously, instead of conventionally averaging the variation of all pens. This improves the estimates for each pen, especially when transmission events occur between sampling time points such that some pens have less information (McElreath, 2020). This was relevant for pens 7, 8, and 11 in which new cases were only observed at a very limited number of time points, because multiple transmission events occurred within the first few days (Fig. 3), leading to wide HPDIs indicating a wide range of possible transmission rates. The hierarchical structure of the model led to shrinkage of the predicted cases towards the overall mean. Thus, we did not expect the predicted cases to be equivalent to the observed data but instead expected systematic differences between the predicted and observed data (Fig. 3).

4.3. Effect of antibiotic resistance and E. coli strains on transmission

Resistance genes carried on plasmids generally impose fewer fitness costs on their bacterial hosts than chromosomal mutations resulting in resistance (Vogwill and MacLean, 2015). Fitness costs imposed by plasmids are influenced by the number of plasmids within bacteria, by the number of resistance gene families on a plasmid, and by host factors (Vogwill and MacLean, 2015; Lee et al., 2020; Rajer and Sandegren, 2022). The inoculums used in the animal experiment contained 3 – 6 plasmids and resistance genes from various families (Tables S17 – S19). Fitness costs lead to lower population growth of resistant bacteria which might thereby lower the transmission rates of the resistant bacteria. However, the transmission rate of the CPE-strain was also lower than the transmission rates of the other strains in the presence of amoxicillin (Fig. 5) when fitness costs are not expected to limit the growth and transmission rates (see Section 4.4 'Effect of amoxicillin on transmission' below). This suggests the lower transmission rate of the CPE-strain is more likely caused by differences between the used *E. coli*-strains than by differences in plasmids and resistance genes.

The *blaOXA-162*, *blaCTX-M-2*, and *catA1* resistance genes used in the animal experiment were carried by different *E. coli* strains isolated from healthy chickens between 2004 and 2009 (see Table 1), so we cannot separate the effect of the different plasmids and the resistance genes they carried from the effect of the different *E. coli* strains. In addition, the resistance genes were located on conjugative plasmids and resistant colonies were not tested to identify the *E. coli* type. As such, part of the transmission might also be explained by plasmid transfer between *E. coli*, rather than by colonization of the chicken gut by the *E. coli* strains that were present in the inoculums.

4.4. Effect of amoxicillin on transmission

Antibiotic treatment selects for resistant bacteria in the animal gut (see e.g., (Rochegüe et al., 2021)) because bacteriostatic and bactericidal effects on susceptible bacteria lead to resistant bacteria having a higher growth rate than susceptible bacteria, such that resistant bacteria

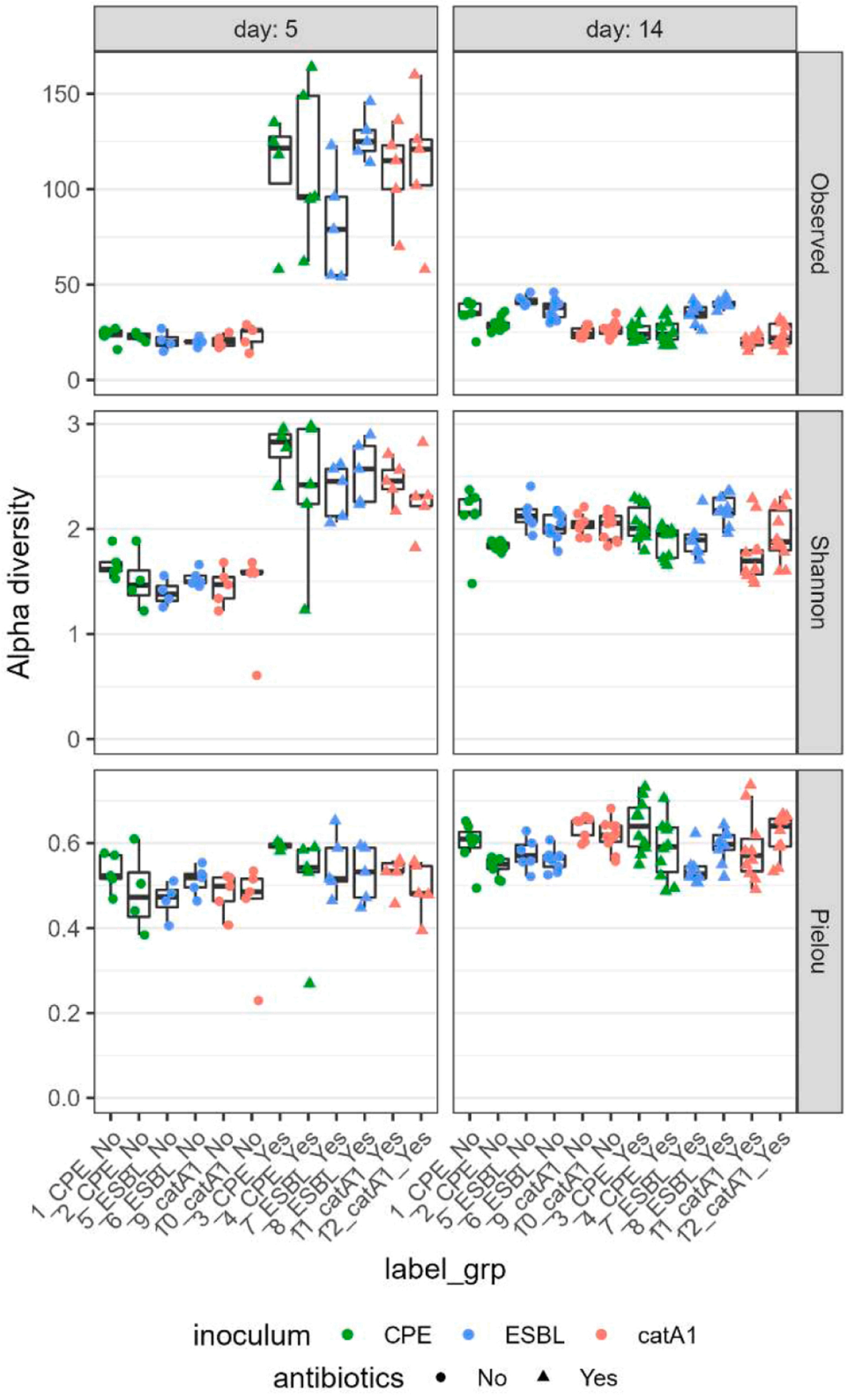

Fig. 7. Boxplots of alpha-diversity (vertical axis) by inoculum and antibiotic treatment (horizontal axis) at genus level. The box indicates the first and third quantiles and the whiskers extend to the smallest and largest values at most 1.5 times the interquartile range from the hinges. Colours indicate different inoculums (CPE-strain: green; ESBL-strain: blue; catA1-strain: red) and symbols indicate the absence (circles) or presence (triangles) of antibiotic treatment. The panels show the different alpha-diversity measures (rows) and different days (columns).

would be expected to colonize the gut more easily and be transmitted faster in the amoxicillin-treated groups. Indeed, the transmission rates of all inoculums were higher in the amoxicillin-treated groups than in the non-amoxicillin-treated groups (Fig. 6). Similarly, the relative abundance of the *E.coli/Shigella* genus was lower in amoxicillin-treated pens than in non-amoxicillin-treated pens on day 5 (i.e., before inoculation) but similar on day 14 (Fig. S7), suggesting the antibiotic treatment decreased the abundance of the susceptible population, giving the inoculum more ability to grow in antibiotic-treated pens. Nevertheless, the differences in transmission rates observed between the CPE-strain versus the ESBL-strain and the catA1-strain were also observed in amoxicillin-treated pens. This suggests intrinsic differences in the

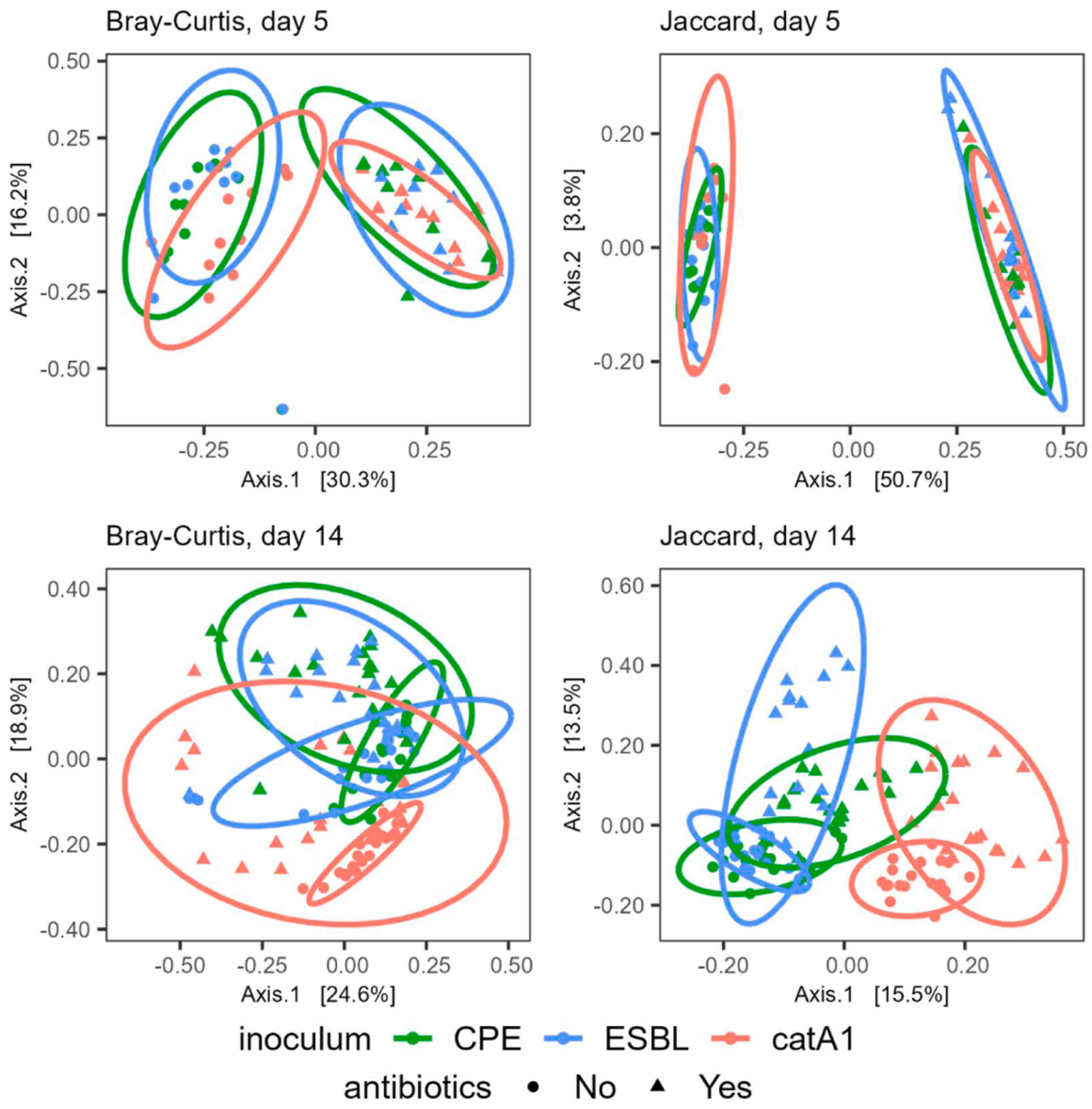

Fig. 8. Principal coordinate plots based on Bray-Curtis dissimilarity (left) and Jaccard distance (right) for day 5 (top) and day 14 (bottom) at genus level. Colours indicate different inoculums (CPE-strain: green; ESBL-strain: blue; catA1-strain: red) and symbols indicate the absence (circles) or presence (triangles) of antibiotic treatment. Ellipses represent 95% confidence regions assuming a multivariate t-distribution.

capability for transmission were present in these bacterial strains, which are independent of the antibiotic resistance itself, as we already stated above.

4.5. Microbiome analysis

The differences in alpha-diversity and beta-diversity between the different inoculums do not correspond to the differences in the transmission rates between the inoculums. This indicates the differences in transmission between the inoculums are most likely not caused by differences in the caecal microbiome.

The separation between the catA1-strain versus the CPE-strain and the ESBL-strain in beta-diversity on day 14 can be explained by broilers inoculated with the catA1-strain being housed in a room separate from broilers inoculated with the CPE-strain and ESBL-strain (Kers et al., 2018), in addition to the effect of being inoculated with a different *E. coli* strain. This room effect was also reflected in the caecal composition of the non-amoxicillin-treated catA1 groups being more similar to the composition of the amoxicillin-treated catA1 groups than to the composition of the non-amoxicillin-treated CPE-groups and ESBL-groups at family level (Fig. S5).

The lower alpha-diversity in non-amoxicillin-treated pens than in amoxicillin-treated pens on day 5 (Fig. 7) was the opposite of the higher alpha-diversity expected based on the literature mentioned in the introduction, which might have been caused by the depletion of some major abundant taxa by the amoxicillin treatment, leaving more room for rare taxa to be detected by the sequencing depth that became available. Similarity percentage analyses indicated the effects of antibiotic treatment on Bray-Curtis dissimilarity on day 5 were driven by the same genera in the groups that would be inoculated with the different inoculums (Tables S12 – S14). Amoxicillin treatment explained less variation in beta-diversity on day 14 than on day 5, and the non-amoxicillin-treated and amoxicillin-treated groups did not separate clearly in the PCoA plot at genus level on day 14. This indicates differences in the genera present in the caecal microbiome on day 5 caused by antibiotic treatment did not last until day 14. Amoxicillin is cleared quickly from chickens when administration ceases and decays quickly in the environment (Peng et al., 2016), such that the effect of amoxicillin

might have been reduced by day 14 because it was last administered on day 6. Although other clinically important antibiotics such as cephalosporins are cleared slower and could last longer in the environment such that they could have an effect on day 14, we did not incorporate them in our study because their use in livestock is subject to legal restrictions (Bonten et al., 2021). The higher alpha-diversity in amoxicillin-treated groups observed on day 5 would still be present in the next few days when most of the transmission events occurred and might be related to the higher transmission rates in amoxicillin-treated groups (Fig. 3). The microbiome of broilers evolves in steps to a more or less stable state in 35 days (Jurburg et al., 2019; Kers et al., 2022). We hypothesize that the dysbiosis of the microbiome caused by antibiotic treatment allows for easier colonization and more rapid growth of new *E. coli* strains such as the inoculums, which is reflected in a more rapid transmission. The opposite, e.g., quicker maturation of the gut microbiome by applying a probiotic, has been shown to slow down transmission (Ceccarelli et al., 2017; Dame-Korevaar et al., 2020b).

4.6. Suggestions for further research

All broilers excreted resistant bacteria until the end of the experiment, showing the successful introduction of CPE. The uncertainty and variability of the transmission rates of the three *E. coli* strains would provide a good range of transmission rates needed for simulations with stochastic models of the transmission of resistance genes carried by commensal bacteria in poultry (Lessler et al., 2016). Future research could expand the Bayesian hierarchical framework adopted in this study by incorporating data from other experiments on bacterial transmission between broilers to capture the influence of differences in environments, chicken feed, and different species of resistant bacteria. This would result in a transmission model that reflects the situation on broiler farms more closely.

In a clean environment, inoculated broilers should start excreting before contact broilers can be colonized. However, in some pens in this experiment, the first excretion of resistant bacteria by both inoculated and contact broilers was detected at the same sampling time point. This is caused by limitations to the sampling frequency. We could use the model by assuming that inoculated broilers started excreting half a time interval earlier. This assumption has previously been used in the analysis of a transmission experiment in broilers where the moment of excretion was similar for inoculated and contact animals (Dame-Korevaar et al., 2020a). In future research, estimation of the exact time point of colonization could be incorporated, e.g., by applying the Bayesian approach described for a model of direct transmission (Hu et al., 2017) to a model of environmental transmission. Taking more frequent samples could also help, although that is limited by logistic and ethical considerations.

Although the presence of multiple plasmids in a bacterium reflects a situation that is common in nature (Davies and Davies, 2010; MacLean and San Millan, 2015), future research should compare the transmission rates of different resistance genes using a single *E. coli* strain that only contains the plasmid of interest for the different inoculums. We were not able to use that approach because of a lack of the necessary permits to work with genetically modified organisms in animal experiments, but here we showed the difference in transmission rates between strains could be substantial (up to 68%) and is thus relevant. Using that same *E. coli* strain with chromosomal resistance instead of plasmids as inoculum would allow for the comparison of the transmission of plasmid-mediated and chromosomal resistance. Such research can build on this paper by determining sampling schemes based on our results and by applying the same methodology.

5. Conclusion

From our study, we conclude early amoxicillin treatment increases the transmission rate of *E. coli* strains carrying different resistance genes between broilers up to five-fold and has a temporary effect on the caecal microbiome: amoxicillin treatment increased alpha-diversity of the caecal microbiome on day 5, but no effects of amoxicillin treatment on the caecal microbiome were found on day 14. The effects of amoxicillin on the transmission rates were most likely not caused by differences in the caecal microbiome because differences in the microbiomes of the different inoculums did not correspond to the differences in the transmission rates of the different inoculums. The transmission rates of $2{\cdot}10^{-4}$ h^{-1} and $4{\cdot}10^{-4}$ h^{-1} for the CPE-strain were 54 – 68 per cent lower than the transmission rates of the ESBL-strain and 52 – 59 per cent lower than the transmission rates of the catA1-strain. This was reflected in the longer time needed for the CPE-strain to colonize all broilers than for the ESBL-strain and catA1-strain. Such delays might be relevant in the field, especially if competition between different antibiotic-resistant strains occurs. The consistent difference in transmission rates with and without antibiotic treatment indicates the differences in transmission rates were more likely caused by differences between the used *E. coli* strains than by differences in plasmids and resistance genes. The Bayesian methodology applied in this experiment can be used to improve the accuracy and reliability of transmission models of resistant bacteria between broilers by making effective use of the available data and reducing the reliance on assumptions about the underlying populations, and the obtained transmission rates can be used in mathematical models of transmission.

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Declaration of Competing Interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2023.105998.

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