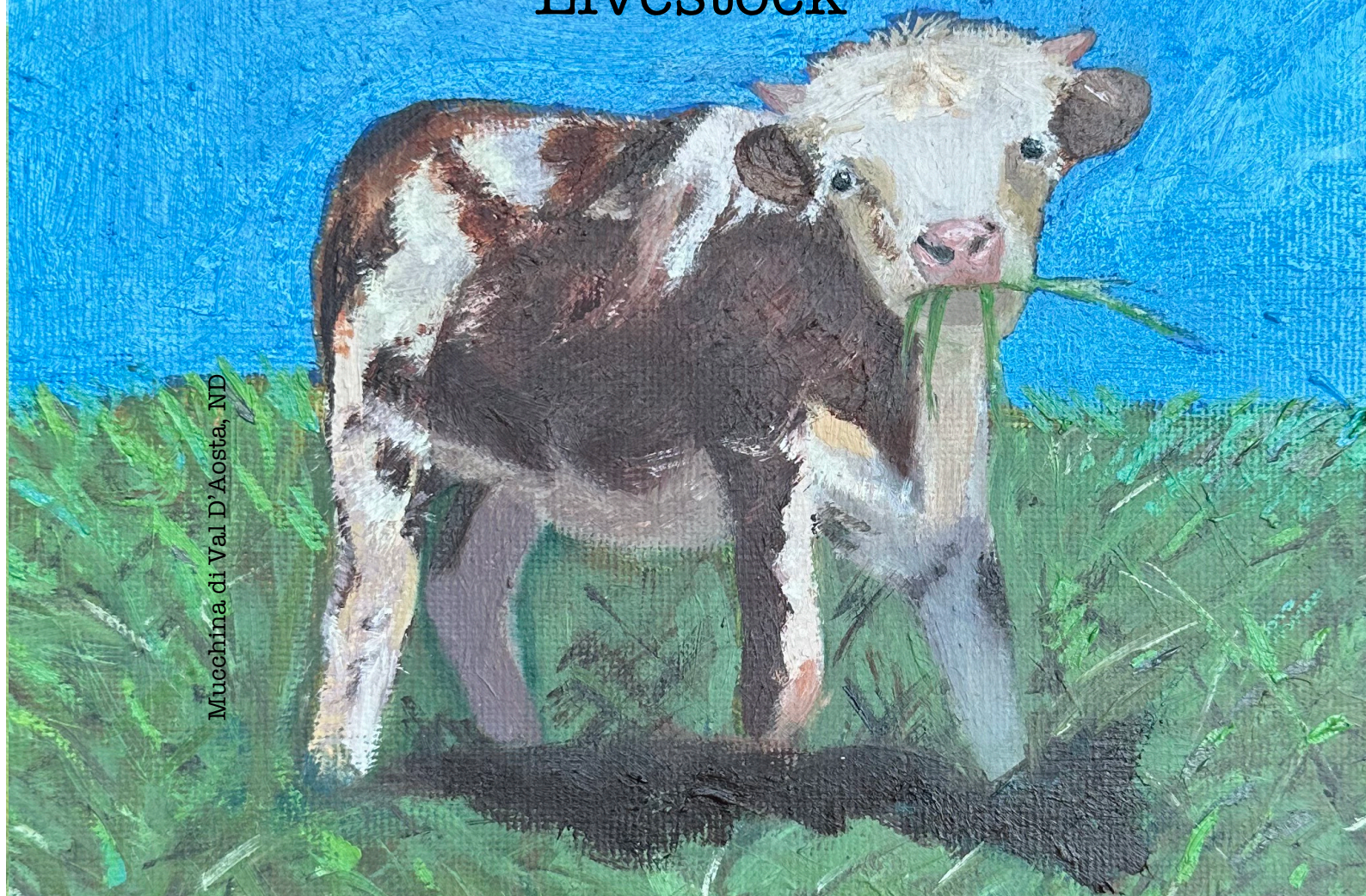
Venturing into the Unknown

A Quest to Detect and Build the Framework for Emerging Resistance Bacteria Surveillance in Dutch Livestock



Mucchina di Val D'Aosta, ND

Natcha Dankittipong

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A Quest to Detect and Build the Framework for Emerging Resistance Bacteria Surveillance in Dutch Livestock

Zich wagen aan het onbekende

Een zoektocht naar het detecteren en bouwen van een raamwerk voor surveillance van opkomende resistentiebacteriën in de Nederlandse veehouderij
(met een samenvatting in het Nederlands)

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Chapter 1

General Introduction

Bacteria are ubiquitous in our environment and exist in various forms. Some are pathogenic, causing illness in their hosts, while others serve as commensal organisms, peacefully coexisting symbiotically with their hosts (Tenaillon et al., 2010; Faust and Raes, 2012). When a host, human or animal, experiences pathogenic bacterial infections, antibiotics will assist the human and animal by weakening or killing these pathogenic bacteria, if antibiotics capable of targeting the pathogenic bacteria concerned are available. This use of antibiotics enables the host's immune system to effectively eliminate these harmful bacteria from the body (Kohanski et al., 2010). However, the distressing issue of antibiotic-resistant bacteria has emerged, which pose a threat to human and animal health. A systematic review conducted by Antimicrobial Resistance Collaborators has estimated that 4.9 million human deaths were associated with antibiotic-resistant bacteria in 2019 (Bush and Bradford, 2016; Antimicrobial Resistance Collaborators, 2019; ECDC, 2022).

Part of the public health risk relates to animals where rampant antibiotic use exists in some parts of the world (Mughini-Gras et al., 2019; Aslam et al., 2021). This may result in bacteria resistant to the antibiotics used and following the interaction between humans and animals, this may jeopardize the effectiveness of these antibiotics in humans (Bottery et al., 2021; De Wit et al., 2022; Munita and Arias, 2016; Reygaert, 2018).

How is AMR disseminated?

Antibiotic resistance (AMR) originates from three main mechanisms: intrinsic resistance, acquisition via horizontal transfer of resistance genes from other bacteria, and development of resistance through "de novo" mutation following exposure to antibiotics (Spagnolo et al., 2021; Woodford and Ellington, 2007; Handel et al., 2014). The consequences of antibiotic resistance are dire. Initially, resistance may only be present in a small fraction of the bacterial population. However, when the bacterial population is exposed to antibiotics, selection occurs, in which bacteria with resistance traits survive and reproduce while susceptible bacteria are eliminated (Santos-Lopez et al., 2021). This process leads to the dissemination of resistance genes within the bacterial population (CDC, 2022; Munita and Arias, 2016).

Horizontal gene transfer (HGT) facilitates the exchange of genetic material, including plasmids and other mobile elements, among various bacterial species, thereby promoting the development of multidrug-resistant (MDR) traits (Vinayamohan et al., 2022; Sun et al.,

2019). In conditions of environmental stress, like those found in the gut or under antibiotic treatment, HGT is significantly heightened, contributing to the resilience of multi-drug resistance in diverse environments and the dissemination of MDR genes in multiple bacteria populations (Vinayamohan et al., 2022; Sun et al., 2019). The persistent transmission of resistant genes through horizontal transfer plays a crucial role in the global spread of antibiotic resistance, even in populations not exposed to selective antibiotmaiic pressure (Alderliesten et al., 2020; Pallecchi et al., 2012; Barret et al., 2019; Bartoloni et al., 2008; EFSA, 2022).

How does bacterial adaptation drive the increased usage of critically important drugs?

With the rise of MDR bacteria in human and animal populations, combination therapies involving multiple antibiotics are employed against bacteria resistant to these drugs (Tangden, 2014; Uddin et al., 2021; Miller et al., 2016; Kerantzas and Jacobs, 2017; Ahmed et al., 2014). This approach creates a selective environment favoring the dissemination of MDR bacteria. (Baran et al., 2021; Aminov, 2010; Anzia and Rabajante, 2018) For example, beta-lactamase-producing bacteria have acquired extended-beta-lactamase enzymes against new antibiotics designed to affect beta-lactamase-producing bacteria (Bradford, 2001; Gniadkowski, 2001; Larsen et al., 2021). The remaining effective antibiotics against these highly resistant bacteria are limited, with drugs like carbapenems and colistin serving as a last resort (European Commission, 2022; Mohapatra et al., 2021). Carbapenems, in particular, play a critical role in treating severe infections caused by Extended Spectrum Beta-lactamase-producing Enterobacteriaceae (ESBL).

The main concern is that humans are at risk of losing the race against the rapid evolution of antibiotic-resistant bacteria, as our ability to develop new antibiotics cannot keep pace with the speed at which antimicrobial resistance arises. Even when antibiotic use is prudent, the emergence and persistence of antibiotic-resistant bacteria remain a concern, driven by Red Queen dynamics – an ongoing coevolution in antagonistic interactions (Anzia and Rabajante, 2018; Joop and Vilcinskas, 2016). While commensal bacterial resistance may not cause direct harm, gene transfer from these resistant commensal bacteria to pathogenic bacteria poses a critical challenge. The rapid generational turnover of bacteria and the slow

development of new antibiotics since 1983 means that our limited arsenal is at risk due to this mechanism (Durand et al., 2019).

How does AMR travel in the interconnected system between humans, animals, and the ecosystem?

Human, animal populations, and plants coexist in shared environments, which fosters the exchange of microorganisms, including antibiotic-resistant bacteria, through various channels such as cohabitation, consumption, and direct (Figure 1.1). Tackling this complex issue requires a collaborative and interdisciplinary approach guided by the One Health principle. This principle directs efforts towards promoting the overall health of humans, animals, and the interconnected ecosystem (OHHLEP, 2023).

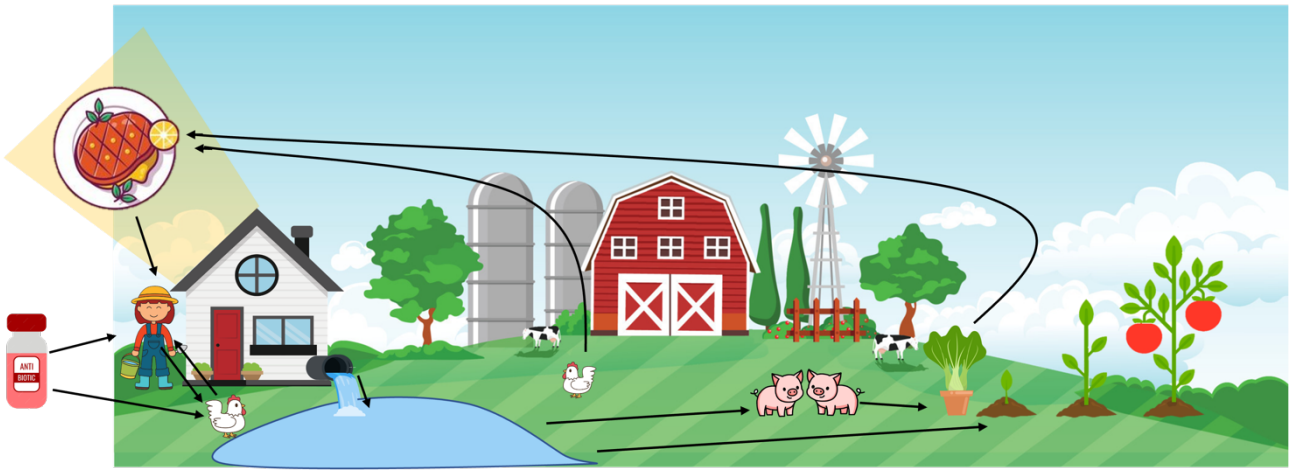

Figure 1.1-The *conceptual movement of resistant bacteria through the environment, where arrows indicate the movement of resistant bacteria (extended from Aslam et al., 2021).*

A clear illustration of this interconnectedness is evident in foodborne illnesses. Globally, an estimated 10 percent of the human population suffers from foodborne and waterborne illnesses annually (WHO, 2015). Some of these illnesses arise from the consumption of food contaminated with pathogenic bacteria, enhanced by factors such as inadequate food storage and preparation, often involving temperature lapses, as well as contamination through unsafe water and cross-contamination during food preparation (WHO, 2023; Bintsis, 2017; Abebe et al., 2020). The bacteria present in contaminated food can establish in the human gut, outlining their potential pathway for transmission between animals, humans, and environmental reservoirs such as water and crops (Cody et al., 2018; Bortolaia et al., 2015; Nadimpalli et al., 2019; Ravel et al., 2017). This intricate dynamic between humans, animals, and the environment demands a comprehensive approach to tackling antibiotic-resistant

bacteria. Keeping this interconnectedness in mind, antibiotic-resistant bacteria in livestock could contribute to the emergence of antibiotic resistance in humans, and vice versa.

The utilization of antibiotics in livestock production and agriculture can be traced back to the synthesis of sulfonamides by Bayer in 1935 (Kirchhelle, 2018). Despite the emergence of antibiotic-resistant bacteria within a decade of this milestone, global antibiotic usage in 1960 witnessed widespread expansion (Saga and Yamaguchi, 2009). The global occurrence of resistant bacteria in livestock currently poses a significant concern, especially in lower-income countries. Van Boeckel et al. (2019) conducted a comprehensive analysis, compiling data from 900 point-prevalence surveys on resistance rates of *Escherichia coli, Campylobacter spp.,* nontyphoidal *Salmonella spp.,* and *Staphylococcus aureus* in lower-income nations. Their findings revealed that 41% of antimicrobial compounds were ineffective (with resistance rates surpassing 50%) in broiler chickens, 21% in cattle, and 34% in pigs (Van Boeckel et al., 2019). Asia and the Americas have up to 40% prevalence of resistance to colistin. Although carbapenem resistance remains low, detected cases of resistance in non-human sources is worrisome (Van Boeckel et al., 2019; Madec et al., 2017). In Europe, the prevalence of E. coli and Salmonella spp. carrying resistance to ampicillin, tetracyclines, and sulfonamides ranged from moderate to very high across most Member States (EFSA, 2023). However, the prevalence of E. coli and Salmonella spp. carrying resistance to other critically important antibiotics, such as ciprofloxacin and cefotaxime, as well as the presence of ESBL genes, was comparatively low (EFSA, 2023).

The direct and indirect transfer of resistant bacteria from animals to humans is a pressing concern. Direct transmission is exemplified by cases of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in farm workers, where isolates of LA-MRSA bacteria are genetically identical in both human and livestock (Ma et al., 2021; Wendlandt et al., 2013). Indirect transmission, as demonstrated in population-based studies in the Netherlands and Denmark, identifies food consumption as a significant risk factor contributing to the incidence of resistant bacteria within communities (Mughini-Gras et al., 2019; Duarte et al., 2021). Numerous studies further elaborate on the transferability of resistant bacteria from meat-producing animals to humans (Ramirez-Castillo et al., 2023; Cao et al., 2022; Cody et al., 2019; Bortolaia et al., 2015; Nadimpalli et al., 2019). These findings underscore the intricate web of antibiotic resistance and emphasize the need for comprehensive strategies to mitigate its impact on both animal and human health.

How do carbapenemase-producing bacteria emerge and spread?

The proliferation of antibiotic resistance in livestock extends beyond singular drug resistance to encompass MDR. In some lower-income nations, MDR bacteria exhibit resistance even to last-resort drugs like colistin and carbapenems (Aslam et al., 2021). Carbapenem-resistant Enterobacteriaceae (CPE), which emerged in the 1990s, have evolved into a global health priority, classified as such by the World Health Organization (WHO). In settings where CPE are endemic, mortality rates among hospitalized patients can range from 22% to 72% (Falagas et al., 2008; Soontaros and Leelakanok, 2019; Jean et al., 2022).

Initially confined to hospital environments, CPE have swiftly become a global concern, establishing themselves in Asian and African countries (Duin and Doi, 2017; Logan and Weinstein, 2017; Lovlena and Doi, 2017; Manenzhe et al., 2015). The increased use of carbapenems to treat patients suffering from infections with ESBL-producing bacteria, alongside co-resistance development associated with the use of other antibiotics like aminoglycosides, contributes to the increasing prevalence of CPE (Paterson, 2000; Ye et al., 2022; Rhodes et al., 2019; Zhang et al., 2019; ESAC-Net, 2021; Khurshid et al., 2019; Nowak et al., 2014). Alarmingly, CPE have been detected in wildlife, livestock, and the environment worldwide since 2010, raising concerns about spilling back to the human community (Kock et al., 2018; Ramírez-Castillo et al., 2023; Alexander et al., 2018; Blaak et al., 2015). In Europe, southern European countries consistently report CPE presence, while western European countries, including the Netherlands, experience sporadic cases (ECDC, 2016; ECDC, 2021).

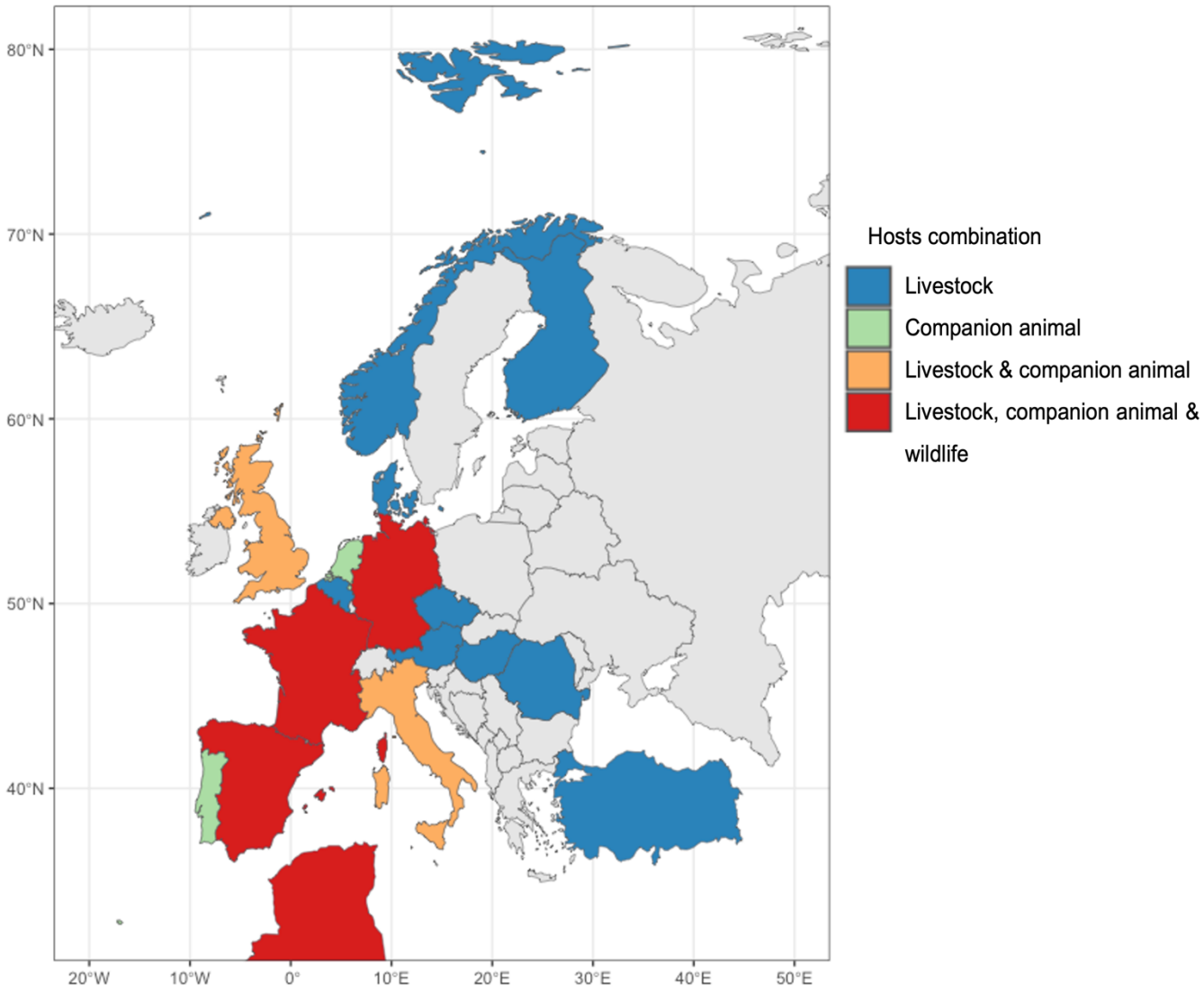

Figure 1.2- The occurrence of Carbapenemase-Producing Enterobacteriaceae (CPE) in various species, including wildlife, livestock, and companion animals within the European Union (EU). The data on CPE presence is primarily derived from the study conducted by Kock et al. in 2018 and has been further supplemented by recent cases reported by Veterinærinstituttet (2023), MARAN (2018), Hendriksen et al. (2023), and Ramírez-Castillo et al. (2023). The majority of these cases were initially identified through research studies, while a limited number of occurrences in livestock and companion animals were detected through national and regional surveillance efforts.

Are there historical cases of resistant bacteria emergence and widespread dissemination in livestock?

The detection of CPE strains in both livestock and human populations emphasizes the need for thorough investigations into potential sources, particularly within the livestock sector, considering the interspecies transferability of resistance in Enterobacteriaceae (San Milan, 2018). Figure 1.2 shows the distribution of reported cases of CPE in livestock, wildlife, and

companion animals across Europe in the period from 1980 to 2023 (Kock et al., 2018; Veterinærinstituttet, 2023; MARAN, 2018; Hendriksen et al., 2023; Ramírez-Castillo et al., 2023). The potential impact of CPE from non-human transferring to humans can be learned from colistin resistance gene *mcr-1.* The plasmid-mediated *mcr-1* genes originating from pigs emerged and swiftly spread within the population to impact humans (Aslam et al., 2021).

In the broader historical context, two other noteworthy forms of resistance, namely LA-MRSA and ESBL *E. coli*, have emerged. The global spread of ESBL *E. coli* in animal reservoirs has been substantial since its initial detection in pet animals in 1989, and its rise in food-producing animals could facilitate transmission from the food chain to humans (Caratotti, 2008). Similarly, LA-MRSA was first detected in pig populations in the 2000s, and within five years, it had rapidly disseminated among other livestock and farm workers worldwide (Harkins et al., 2017; Wendlandt et al., 2013). The low host specificity of LA-MRSA strains has facilitated their spread through animal-to-animal and animal-to-human contact, particularly among veterinarians and farm workers (Crespo-Piazuelo and Lawlor, 2021; Anjum et al., 2019). Therefore, a critical aspect in exploring emerging resistances is understanding the prevalence of antibiotic resistance in the livestock population.

What current measures prevent the emergence of resistant bacteria against critically important antibiotics?

The correlation between antibiotic use and the development of resistant bacteria is a pivotal focus that has garnered significant attention since the 1990s when antibiotic stewardship initiatives commenced in both hospital and community settings (Saga and Yamaguchi, 2009). These initiatives were specifically tailored to counter the emergence of antibiotic-resistant bacterial strains, aiming to guide and promote adherence to guidelines for the appropriate use of antimicrobials while discouraging unnecessary usage (Dyar et al., 2017). Antimicrobial stewardship programs proactively evaluate and enhance the responsible utilization of antimicrobials, such as precise selection of the most suitable drug regimen (Al-Yamani et al., 2016; CDC, 2024). Antimicrobial stewardship now also covers regulating antibiotic use in livestock, with a focus on addressing widespread usage (European Commission, 2011). The latest EU directive emphasizes a significant reduction in antibiotic use in food-producing animals and a complete ban on antibiotics for preventive measures

in addition to the ban of growth promotors in 2006 (Commission Notice, 2015; Dibner and Richards, 2005). Despite the increased attention, antibiotic-resistant bacteria, including CPE, are emerging in multiple EU countries.

What are the limitations of regional and national surveillance systems?

The emergence of antibiotic-resistant bacteria has spurred an initiative within the European Union to monitor this growing concern. Surveillance systems specifically targeting resistant bacteria in humans and livestock began in 2012 (ECDC, 2014; ECDC, 2021). To adhere to the European Union's directives, authorities in the Netherlands conducted national surveillance through programs like Nethmap and Maran, aiming to track the prevalence and patterns of resistant bacteria in both human and livestock populations (MARAN, 2020; Nethmap, 2020).

The current national surveillance system in the Netherlands, follows the European Union's recommended sampling methodology of at least 175 batches of animals per species each year. While Dutch national surveillance excels at monitoring with 300 samples each year, the protocol was designed to monitor the prevalence of existing resistant bacteria in livestock, and not for promptly identifying emerging drug-resistant bacteria (Wit et al., 2017). According to the latest standard for estimating the probability of detecting CPE, with 300 samples per year, cases can be detected with 95% confidence if the prevalence is 1% (Wit et al., 2017). Applying this to the current Dutch broiler livestock population of 45 million, a 1% prevalence implies colonization of approximately half a million birds.

It is crucial to note a significant caveat in this calculation, as it assumes a homogeneous distribution of CPE at the batch level and across the production system, which are likely not the case. The distribution of colonized animals within the flock and the distribution of colonized herds across the production system are unknown. By taking only 10 samples from a single flock at the slaughterhouse and not accounting for different farm types and other factors, there is an even higher risk that small emerging CPE may escape detection.

In summary, the current surveillance system has a low sensitivity at the population level, and, in combination with the continuous sampling throughout the year, is expected to detect CPE emergence only by the time a substantial number of colonized animals are present.

Consequently, the current sampling protocol is primarily suitable to monitor trends in endemic AMR and is not suited for detecting emerging resistance strains in an early stage (MARAN, 2020).

The current EU surveillance system confronts financial constraints that impede its capacity to expand sampling and testing processes (Reist et al., 2016). Originally designed to address prevalent resistant bacteria like ESBL in livestock, it lacks the necessary capacity to effectively detect emerging threats such as CPE due to the substantial costs involved in enhancing its capabilities (EFSA, 2014; Gonzales et al., 2014). Variations in mechanisms of resistance to carbapenems make it challenging to detect all forms of resistance solely through phenotypic tests, bringing technical complexity to the surveillance (Reist et al., 2016). Yet, there is a need for a cost-effective, risk-based surveillance system to early detect new AMR bacteria (Bisdorff et al., 2017; Reist et al., 2016; Gonzales et al., 2014; Rüegg et al., 2018).

What is the aim of this thesis?

Surveillance stands as the cornerstone of disease control, operating with the key objective of detecting emerging and re-emerging diseases (FAO, 2014). The global rise in antibiotic resistance and the emergence of resistant bacteria in livestock necessitate a refocus of the current surveillance efforts. In this thesis, we delved into building basic ingredients to design a surveillance system aimed for early detection of CPE emergence in meat-producing animals.

Risk-based surveillance, targeting high-risk sources linked to emerging resistant bacteria, proves more efficient than random sampling (FAO, 2014; Stark et al., 2006). Implementing this approach successfully requires a comprehensive understanding of resistance presence and risk factors, along with accessible information on populations and their distribution (Doherr and Audige., 2001).

Detecting antimicrobial-resistant commensal bacteria at an early stage is paramount, especially considering that the number of farms involved is still limited. In situations where only a few farms are positive during the initial detection, control measures such as quarantine can be implemented to minimize human exposure. Unlike pathogenic bacteria,

which often exhibit noticeable symptoms in animals, commensal bacteria carrying antibiotic resistance may not manifest observable signs, creating detection and data collection challenges.

The thesis is organized around the objective of designing an active surveillance protocol built on three fundamental research questions, each addressing a crucial aspect of risk-based surveillance (Figure 1.3). Firstly, what is the source of CPE entering different farm types? In Chapter 2, the potential sources of CPE and the farm types most likely to be at risk for the introduction of CPE were identified (Smith and Lewin, 1993; Woodford and Ellington, 2007; San Milan, 2018). We laid the groundwork for a risk-based surveillance by quantifying the probability of introducing CPE into the Dutch meat-producing population (broiler chickens, pigs, and veal calves). Secondly, what is the speed of CPE transmission between animals? This is essential in predicting the course of the within-farm prevalence once CPE is introduced. However, research on CPE prevalence in healthy livestock populations is limited, with case reports being the primary available research, providing valuable yet insufficient information (EFSA, 2022). Thus, in Chapters 3 and 4, we quantified the key parameters influencing the spread of resistant bacteria through a combination of experimental studies and meta-analysis. Lastly, what is the spread of CPE across the animal sector after CPE has been introduced into a single farm? In Chapter 5, we simulate the transmission dynamics of CPE introduced into broiler production pyramids. We further explore the effectiveness of current surveillance based on a dynamic transmission model of CPE in meat-producing animals in terms of the probability of detection and time to detection in the current system.

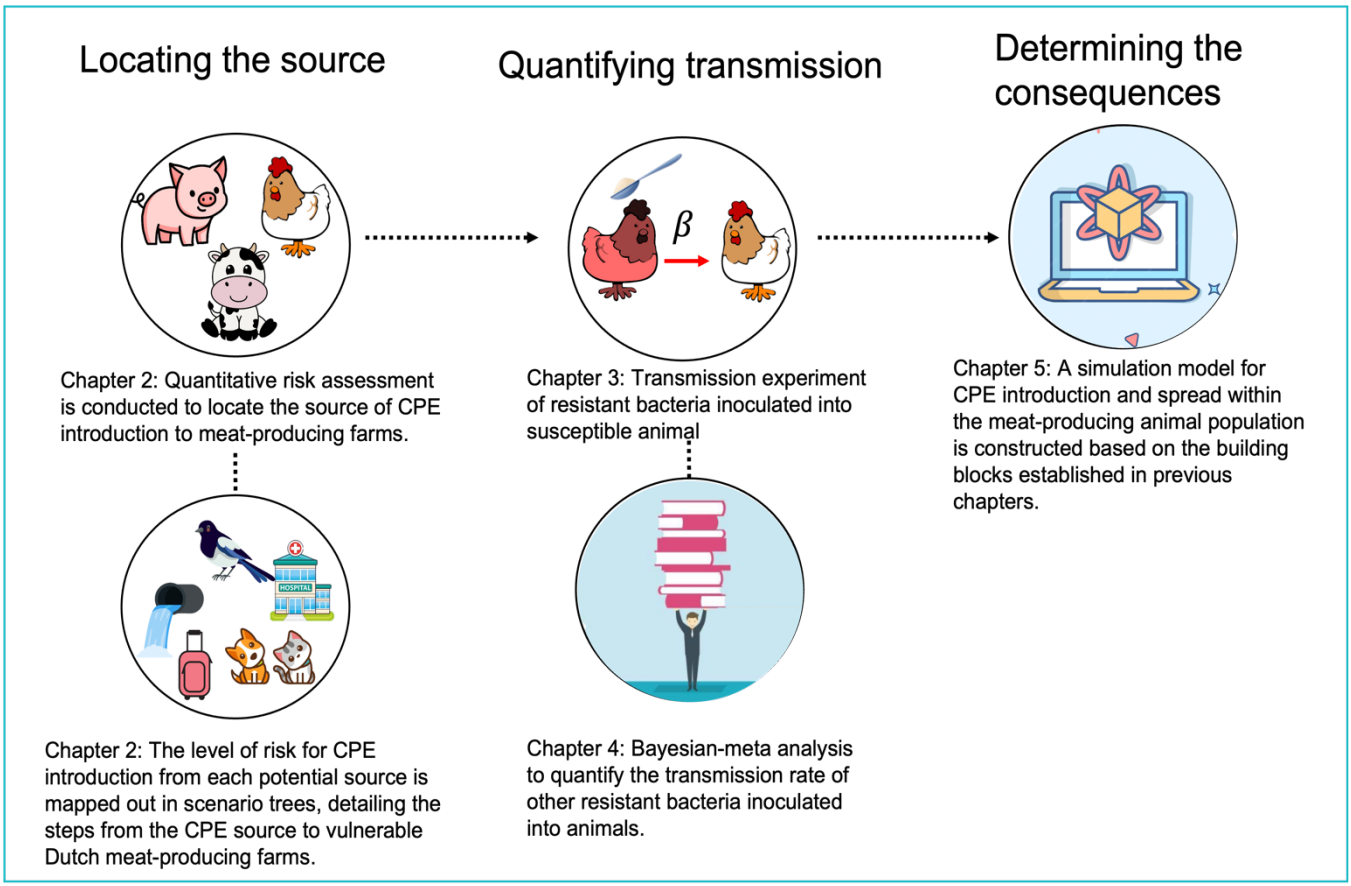

Figure 1.3- Framework to assess the risk of the introduction and spread of CPE in Dutch meat-producing animals.

The project was structured to address these fundamental questions through three studies:

Stochastic risk assessment: to identify sources and transmission pathways of CPE into Dutch meat-producing animals, a risk assessment was conducted. This process began by pinpointing the potential sources of CPE and farm types at risk. Subsequently, scenario trees were created outlining the sequential steps for the specific CPE source to reach susceptible farms. Finally, the probability of each event was quantified using a stochastic approach.

Parameterization of transmission rates: transmission experiments of CPE and ESBL between broiler chickens were conducted to quantify the transmission rate of CPE and compare it to the transmission rate of ESBL. Bayesian statistics were employed for the statistical analysis of these transmission data. Moving forward, a further exploration of the transmission rate mechanisms of CPE and other resistant bacteria was undertaken by conducting a meta-analysis of non-pathogenic resistant bacteria transmission experiments

in meat-producing animals. This approach extended insights into other factors such as livestock species, resistance gene location, and antibiotic usage.

Simulation model development: all parameters investigated in the previous studies were consolidated and put into a simulation model of CPE transmission between animals and between farms. This aimed to provide a comprehensive understanding of CPE dissemination dynamics within and across farms in livestock production and the likelihood and timeliness of detection by the current surveillance system. The model was parameterized and evaluated for the broiler production chain.

By unifying these research efforts, this thesis endeavored to make a significant contribution to the development of an early detection framework for emerging antimicrobial resistance in livestock, ultimately safeguarding the health of humans.

Chapter 2

Quantitative Risk Assessment for the Introduction of Carbapenem-Resistant Enterobacteriaceae (CPE) into Dutch Livestock Farms

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Abstract

Early detection of emerging carbapenem-resistant Enterobacteriaceae (CPE) in food-producing animals is essential to control the spread of CPE. We assessed the risk of CPE introduction from imported livestock, livestock feed, companion animals, hospital patients, and returning trav- elers into livestock farms in The Netherlands, including (1) broiler, (2) broiler breeder, (3) fattening pig, (4) breeding pig, (5) farrow-to-finish pig, and (6) veal calf farms. The expected annual number of introductions was calculated from the number of farms exposed to each CPE source and the probability that at least one animal in an exposed farm is colonized. The total number of farms with CPE colonization was estimated to be the highest for fattening pig farms, whereas the probability of introduction for an individual farm was the highest for broiler farms. Livestock feed and imported livestock are the most likely sources of CPE introduction into Dutch livestock farms. Sensitivity analysis indicated that the number of fattening pig farms determined the number of high introductions in fattening pigs from feed, and that uncertainty on CPE prevalence impacted the absolute risk estimate for all farm types. The results of this study can be used to inform risk-based surveillance for CPE in livestock farms.

Keywords: carbapenems; CPE; meat-producing animal; companion animal; travelers; feed; risk assessment; introduction risk; stochastic risk model

2. 1 Introduction

Antimicrobial-resistant (AMR) bacteria have been one of the greatest public health challenges since the 1950s (Davies and Davies, 2010). Increased use of broad-spectrum antibiotics has resulted in a race between resistant bacteria and treatments. The lagging development of new antibiotics and the speed at which resistance emerges are propelling the healthcare sector toward using “drugs of last resort”, administered only after other antibiotics have failed. One antimicrobial class of last resort, carbapenems, represents extremely potent, broad-spectrum drugs for treating serious infections, primarily from multidrug-resistant Enterobacteriaceae (EFSA, 2013). Enterobacteriaceae with carbapenem-resistant genes have a 50% mortality rate in humans due to the absence of alternative antibiotic treatments (Jacob et al., 2013). Carbapenemase-producing Enterobacteriaceae (CPE) have spread globally since early 2010 in hospital facilities and have risen at an alarming rate in the human community (Albiger et al., 2015; Kelly et al., 2017). CPE quickly disseminate resistant genes between bacteria through horizontal transfer, specifically plasmid-mediated gene transfer [6]. A plasmid is a mobile circular DNA carrying useful genes for adaptation and moving within and between species of bacteria.

Inter-host transmission of resistant genes via plasmids enables the development of CPE cases in humans, not from using antibiotics directly, but from interacting with environ- ments and hosts colonized with CPE (Köck et al., 2018). As an illustration, plasmid-mediated, extended- spectrum β-lactamase-producing Escherichia coli (ESBL-EC) in the Dutch community is partly attributable to ESBL-EC in food, the environment, and animals (Mughini-Gras et al., 2014).

AMR has rapidly disseminated worldwide in the community and hospitals due to excessive antibiotic usage, international travel, and global trade networks. The multiple sources of the AMR pandemic have prompted the European Union (EU), since 2010, to extend its surveillance of AMR to include food-producing animals. Cecal samples from live fattening pigs, veal calves, and broilers are collected at slaughterhouses and tested for resistant genes. Since 2016, this surveillance also includes CPE (EFSA, 2017; ECDC, 2017). The current compulsory and harmonized AMR surveillance carried out by all EU member states is adequate to detect widespread AMR but will not quickly detect a newly emerging resistant bacterium due to the limited sample sizes and sampling frequency. In the current EU

surveillance protocol, EU member states must annually collect a total of 170–300 samples, depending on the states' production volume, from each species of food-producing animal. This sample size was set to detect CPE with 95% confidence, provided the prevalence is at least 2%. However, because the sampling is conducted only once a year, CPE could be widespread before they are detected. Enhancing EU surveillance to detect emerging CPE is possible through an increased sampling frequency, increased sample sizes, and risk-based surveillance.

This study aimed to inform risk-based surveillance for CPE E. coli (referred to as CPE in the remainder of the text of this paper) by ranking the farm types according to the likelihood of CPE introduction using a quantitative risk assessment model. We based our study on The Netherlands, but it is scalable to the European Union. We included six farm types at risk of CPE introduction: broiler farm, broiler breeder farm, fattening pig farm, breeding pig farm, farrow-to-finish pig farm, and veal calf farm. The reason for this selection was that these farm types are the ones most associated with AMR in The Netherlands (Veldman et al., 2014). Seven potential sources of CPE relevant to the Dutch livestock sector were identified in the literature review (Köck et al., 2018; Blaak et al., 2011; Blaak et al., 2014) Figure 2.6. These potential sources are hospital patients, returning travelers from abroad, companion animals, wild animals, wastewater from hospitals, imported livestock, and animal feed (Supplementary 2.7.1). The results from expert elicitation highlight returning travelers, wastewater from hospitals, and imported veal calves as the most important sources of CPE introduction (Supplementary 2.7.2).

2. 2 Materials and Methods

We quantitatively assessed the risk of CPE introduction to broiler, pig, and veal calf farms from five potential CPE sources, i.e., imported livestock, livestock feed, companion animals, hospital patients, and returning travelers, and ranked farm types by the expected number of farms with CPE introduction and the probability of CPE introduction for an individual farm. This quantitative risk assessment followed the guidelines for import risk assessment provided by the World Organisation for Animal Health (OIE) (World Organisation for Animal Health, 2021; World Organisation for Animal Health, 2010) to assess the risk of exposure of farms, and the guidelines for microbial risk assessment provided by the Codex Alimentarius to assess the risk of infection upon exposure (Haas et al., 2014; WHO, 2014). We conducted

sensitivity analysis to assess the effect of uncertainty surrounding important input parameters toward the output and evaluated alternative biosecurity practices and trade restrictions via scenarios analysis.

Despite being highlighted as an important potential CPE source, wastewater from hospitals was excluded from the model because CPE will be effectively removed in the wastewater treatment facilities. Additionally, although small traces of CPE could be present in surface water due to overflow from rainfall, the vast majority of the meat-producing animals of our concern (veal calf, fattening pig, breeding pig, broiler, and broiler breeder) were raised in a closed system where they drink tap water. This water source undergoes extensive purification, ensuring no traces of resistant bacteria such as CPE (Schmitt et al., 217; Smeets et al., 2009; Vemin et al., 2017). Wild mammals and birds were also excluded from the model. Small mammals such as rodents move locally and thus would not be exposed to CPE from outside the Netherlands. Interactions between local target farms and wild birds are mostly prevented as livestock live in closed barns.

2. 2. 1 Risk Model

2. 2. 1. 1 Model Outline

CPE introduction was defined as the colonization of at least one animal with CPE upon exposure of a farm to any of the sources included in the model. The risk of CPE introduction was modeled with two sub models (Figure 2.1). The first submodel used scenario tree modeling to estimate the number of farms exposed to CPE-colonized sources (N_{col}). The second submodel was a microbial risk assessment model to estimate the probability that at least one animal will be colonized on an exposed farm (P_{col}) given the dose to which the animals on the farm are exposed (CPE_{ing}), using an exponential dose–response model. The outputs of both submodels were combined to calculate the expected annual numbers of farms on which CPE is introduced (N_{intro}). Parameters and values used in the model are presented in Table 2.1.

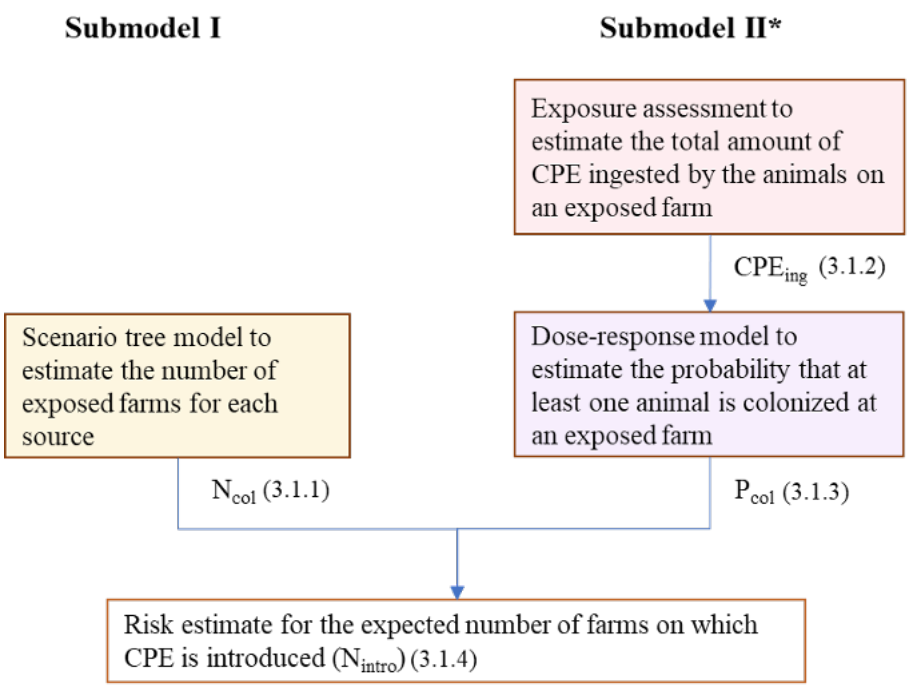

Figure 2.1- *Outline of the risk model to estimate the introduction risk of CPE into Dutch livestock farms from five sources: imported livestock, livestock feed, companion animals (cats and dogs), hospital patients, and returning travelers. * Submodel II is not used for imported livestock because the introduction of a colonized animal into a livestock farm automatically results in colonization of the farm.*

The annual expected number of CPE introductions via each source was calculated using multiple input parameters, some of which are uncertain. Parameters on CPE prevalence, CPE concentration, number of animals in transport, and colonization duration were chosen to be included with a distribution to account for uncertainty and variability. Less variable data, such as total numbers of farms and livestock in the Netherlands, were entered as point estimates. The impact of these parameters on the model results was studied by a sensitivity analysis where the input values were increased and decreased two-fold. We ran 10,000 iterations using Monte Carlo sampling in ModelRisk, an add-on for Microsoft Excel version 1908® (Vose, 2022).

2. 2. 1. 2 Submodel I Scenario Tree Model

The exposure of the following six farm types: broilers, broiler breeders, fattening pigs, breeding pigs, farrow-to-finish, and veal calves, to CPE from sources s (imported livestock (A), livestock feed (F), companion animals (C), farm workers being hospitalized (H), and farm workers traveling abroad (T)) was calculated by multiplying the number of farms in contact with people or animals or receiving feed, N_s, or by the probability that these persons or animals are colonized with CPE, or that the feed is contaminated with CPE, P_{CPE_s}. Mixed

species livestock farms were not considered in the risk assessment because they represented a small proportion of local farms (Statline, 2019).

$$N_{col_s} = N_s \cdot P_{CPE_s} \quad (1)$$

2. 2. 1. 2. 1 Imported Livestock

The number of farms exposed to CPE from imported animals, N_{col_A}, was calculated by multiplying the annual number of batches of animals imported from the source country—among all EU member states in 2017—to six farm types (N_A) by the probability that an imported batch from the source country which is delivered to an individual farm type is colonized with CPE (P_{CPE_A}).

We assumed that CPE colonization is maintained during transport and will reach local farms without detection. Sustained CPE colonization in animals during transportation between EU member states is likely within the maximum 24 h transport time (European Union, 2005), because in livestock, ESBL colonization can be maintained for 30 to 180 days (Dame-Korevaar et al., 2018; Robe et al., 2019; Hansen et al., 2013; Mir et al., 2018). Within the EU, antimicrobial testing in imported animals is not obligatory and not conducted (EFSA et al., 2013). The probability of detecting a CPE-colonized animal is thus negligible and was not accounted for in the calculations.

2. 2. 1. 2. 2 Livestock feed

The number of farms exposed to CPE-colonized feed, N_{col_F}, was calculated as the product of the total number of six farm types in the Netherlands (N_{farm}) and the probability that an individual farm would receive at least one batch of feed contaminated with CPE $\left(P_{CPE_{batch}}\right)$. $P_{CPE_{batch}}$ was calculated from the probability that a batch of feed is contaminated with CPE ($P_{CPE_{feed}}$) and the annual number of feed batches received by a farm (N_{batch}). The estimated value for $P_{CPE_{feed}}$ was used for all farm types because no data were available to estimate $P_{CPE_{feed}}$ separately for each farm type.

$$P_{CPE_{batch}} = 1 - \left(1 - P_{CPE_{feed}}\right)^{N_{batch}} \quad (2)$$

2. 2. 1. 2. 3 Companion Animals

The number of farms exposed to CPE-colonized companion animals (N_{col_C}) was derived by multiplying the number of farms with companion animals (N_C) by the probability that companion animals in the Netherlands are colonized with CPE ($P_{cCPE_{NL}}$). The number of farms having companion animals (N_C) was calculated from the total number of farms (N_{farm}) multiplied by the probability of farms having a companion animal (P_{farmC}).

2. 2. 1. 2. 4 Farm Workers

CPE introduction from humans is possible when farm-related workers k (farmers, veterinarians) acquires CPE during holidays outside the Netherlands or in local hospitals (Figure 2.1). Here, the number of farm workers acquiring CPE in hospital (N_{colH_k}) was calculated by multiplying the number of farm workers hospitalized (N_H) by the probability that patients acquire CPE in Dutch hospitals ($P_{CPE_{NL}}$). The number of farm workers hospitalized (N_H) was estimated by multiplying the number of farm workers and veterinarians in the Netherlands (N_k) by the annual probability of hospital admission in the general population ($P_{admit_{NL}}$).

The number of farms exposed to CPE through infected farm workers returning from travel abroad (N_{colT_k}) was calculated by multiplying the number of farm workers returning from abroad (N_{T_k}) by the probability of travelers acquiring CPE during travel. The probability of traveler-acquired CPE differed according to the 16 regions of destination based on the United Nations geoscheme excluding the Netherlands (United Nation, 2021) (Supplementary 2.7.6), and therefore calculations were performed for each region individually. The number of farmers returning from each of these regions was estimated based on the probability of Dutch travelers visiting each region (P_T). Both the probability of acquiring CPE in the hospital (P_{CPE}) and the probability of acquiring CPE from the community (P_{cCPE}) during travel were considered in the model. The probability of hospital-acquired CPE during holidays (P_{CPE}) was multiplied by the probability of travelers being hospitalized (P_{admit}). The probability of community-acquired CPE (P_{cCPE}) was multiplied by the probability of non-hospitalized travelers ($1 - P_{admit}$) (Figure 2.2). The estimated value for P_{admit} was used for all regions because no data were available to estimate P_{admit} separately for each region.

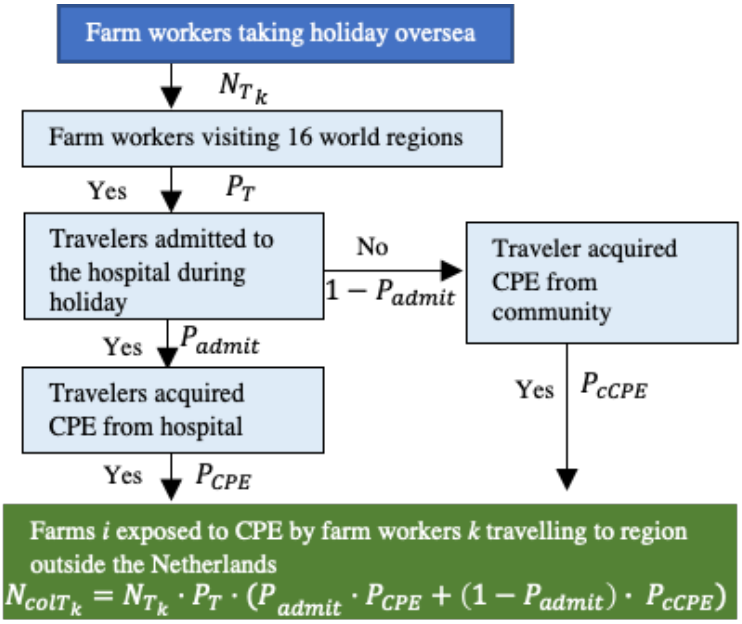

Figure 2.2- *Scenario tree to calculate the number of farms exposed to CPE by farm workers returning from travel abroad.*

2. 2. 1. 3 Submodel II Exposure Assessment

We estimated the numbers of farms where CPE was introduced by multiplying the number of exposed farms (N_{col_s}) by the probability that at least one animal on an exposed farm would become colonized (P_{col_s}). The probability that at least one animal on an exposed farm would become colonized was calculated with an exponential dose–response model using the total number of CPE E. coli bacteria ingested by the animals on the farm (CPE_{ing_s}) as the dose. The ingested dose (CPE_{ing_s}) was calculated separately for each farm type and CPE source s, as described in Equations (3)–(5). These calculations were not performed for the source imported livestock, since the introduction of a colonized animal into a livestock farm directly results in a colonized farm.

2. 2. 1. 3. 1 Animal Feed

The ingested dose of CPE from contaminated feed on a single farm (CPE_{ing_F}) was estimated as the product of the concentration of CPE E. coli (cfu/g) in contaminated animal feed delivered to a farm (CPE_{concF}) and the average weight of one batch of feed in grams (V_{batch}).

$$CPE_{ing_F} = CPE_{concF} \cdot V_{batch} \qquad (3)$$

2. 2. 1. 3. 2 Companion Animals

To estimate the total CPE deposited by companion animals in the farm environment, we multiplied the concentration of CPE in companion animal feces (CPE_{gramC}) (cfu/g) by the average weight (grams) of feces defecated by a companion animal in each defecation (W_{fec}), the daily defecation frequency of companion animals (N_{eli_C}), the length of the colonization period in companion animals in days (T_{CPE_C}), and the proportion of time that a companion animal is present in the barn (P_{barnC_i}). The total CPE ingested by the farm animals (CPE_{ing_C}) was subsequently calculated by multiplying the deposited CPE in the farm environment by the proportion of excreted bacteria taken up by the livestock animals from the farm environment (C_{tranA}) (Table 2.1).

$$CPE_{ing_C} = W_{fec} \cdot N_{eli_C} \cdot T_{CPE_C} \cdot CPE_{gramC} \cdot P_{barnC} \cdot C_{tranA} \qquad (4)$$

2. 2. 1. 3. 3 Farm Workers

The number of CPE bacteria ingested by colonized farm workers (CPE_{ing_H}) was calculated in a similar manner to the ingested dose from companion animals (CPE_{ing_C}), albeit with different inputs. The transmission event started after the colonized farm worker (farmer or veterinarian) used the toilet for defecation. We assumed CPE contaminated their hands after toilet usage and that not all would be removed by hand washing. Thus, CPE_{hand} was the number of CPE (cfu) remaining on a farm worker's hands after hand washing. The number of CPE deposited in the farm environment was then calculated by multiplying this number by the daily defecating frequency of humans (N_{eli_H}), the length of the colonization period of CPE in humans in days (T_{CPE_H}), the proportion of bacteria transferred from the farm worker's hand to the farm environment (C_{tran_E}), and the proportion of the day that a worker is in the barn (P_{barnH}). The last parameter is different between farm workers and veterinarians, assuming that a farmer spends much more time in the barn of a single farm than a vet. The total CPE ingested by the farm animals (CPE_{ing_H}) was subsequently calculated by multiplying the deposited CPE in the farm environment by the proportion of bacteria taken up by the livestock animals from the farm environment (C_{tranA}).

$$CPE_{ing_H} = CPE_{hand} \cdot N_{eli_H} \cdot T_{CPE_H} \cdot C_{tran_E} \cdot P_{barnH} \cdot C_{tran_A} \qquad (5)$$

2. 2. 1. 4 Submodel II Dose-Response Model

The probability that at least one animal at farm type i is colonized with CPE (P_{col_s}) is a function of the CPE ingested dose from a source s (CPE_{ing_s}) and the dose–response parameter. The dose–response parameter gives the probability of a single CPE bacterium colonizing an animal's gut (P) and is calculated from the ID50 (the dose at which 50% of the animals are expected to be colonized). An exponential dose–response model was used, and P was calculated as $\frac{ln\,2}{ID50}$. The probability that at least one animal is colonized with CPE was then calculated as

$$P_{col_s} = 1 - e^{-(P \cdot CPE_{ing_s})} \qquad (6)$$

2. 2. 1. 5 Risk Estimate Combining Submodel I and Submodel II

The expected number of introductions to each farm type from each source s (N_{intro_s}) was calculated by multiplying the number of farms exposed to each source s (N_{col_s}) by the probability that at least one animal on an exposed farm is colonized (P_{col_s}).

$$N_{intro_s} = N_{col_s} \cdot P_{col_s} \qquad (7)$$

The absolute risk of CPE introduction into local Dutch farms was given as the expected annual number of introductions per farm type (N_{intro}) from all CPE sources considered in the model. The probability of CPE introduction for an individual farm was estimated by dividing the number of expected introductions per farm type by the total number of farms of this type in the Netherlands.

2. 2. 2 Input Parameters

2. 2. 2. 1 Imported Livestock

Data on the number of livestock imported into the Netherlands from EU member states (N_{imp}) were available for the period 2016 to 2020 and fluctuated slightly. Import data for the year 2017 were used in the baseline model to be consistent with the data used for the number of farms and veterinarians. The livestock import records were derived from two publicly available sources, namely, Statistics Netherlands (CBS) and the Netherlands

Enterprise Agency (RVO) (Supplementary 2.7.6 and Table 2.1) (RVO, 2021). To estimate the number of imported batches (N_A), the annual number of imported animals was divided by the average number of livestock per shipment (N_{size}). In estimating the number of animal batches delivered to each farm type annually (N_{batch}), we assumed that all imported one-day-old broilers would go to broiler farms, all imported parent broilers would go to broiler breeder farms, all imported veal calves would go to veal calf farms, all imported piglets would go to fattening pig farms, and all imported breeding pigs would go to breeding pig farms and farrow-to-finish pig farms in a ratio of 2:1, representing the ratio of these farms in the Netherlands.

The probability that imported animals from EU member states are colonized with CPE (P_{CPE_A}) was directly inferred from national surveillance data provided by the European Antimicrobial Resistance Surveillance Network (EFSA, 2017; European Food Safety , 2016). CPE surveillance in livestock consisted of random sampling of fecal samples from live animals at slaughter, the results of which were used as a proxy for herd prevalence in the risk model. Data on surveillance in pigs and broilers were available for all EU member states, EFTA countries, and the UK, whereas only 9 EU member states and 2 EFTA countries (Norway and Switzerland) reported on CPE surveillance in calves. For countries that had no data on surveillance in calves, the probability of CPE colonization was inferred from the surveillance in bovine meat (Supplementary 2.7.3, Table 2.5 & 2.6). The probability that imported animals are colonized with CPE (P_{CPE_A}) was estimated using a beta distribution based on the number of animals sampled (n), the number of animals that tested positive (s), and test sensitivity (se) (Table 4).

2. 2. 2. 2 Animal Feed

The average number of batches of feed received by individual farms (N_{batch}) was calculated as

$$N_{batch} = \frac{n_a \cdot c_a \cdot 365}{V_{batch}} \qquad (8)$$

where n_a is the average number of animals on a farm of type *i*, c_a is the average consumption of feed per day per animal on each farm type (in grams), and V_{batch} is the average size of a batch of feed delivered to a farm (in grams). The average number of animals on farm type *i* (n_a) was calculated by dividing the total number of animals in the

Netherlands present at each farm type (N_{animal}) by the total number of farms at each farm type in the Netherlands (N_{farm}). The number of Dutch farms (N_{farm}) and livestock heads (N_{animal}) was based on 2017 data provided by Statistics Netherlands. Due to a lack of farm-specific data, V_{batch} was set equal for all farm types.

Since feed ingredients are heat-treated, CPE contamination was expected to result from cross-contamination during processing and storage in a local feed mill. The probability of feed colonized with CPE was therefore based on Dutch data. As there is no CPE surveillance conducted on animal feed at all, the probability of batches of feed contaminated with CPE ($P_{CPE_{feed}}$) was inferred from the ratio between E. coli prevalence in feed ($P_{ec_{feed}}$) and in humans ($P_{ec_{NL}}$) under the presumption that the ratio of E.coli in the two aforementioned sources is the same as the CPE ratio (Equation (9)). $P_{ec_{feed}}$ was based on the prevalence of compound feed for cattle contaminated with E. coli in the EU (Da Costa et a., 2007), and $P_{ec_{NL}}$ was based on the prevalence of E. coli in Dutch residents reported in the national surveillance of antimicrobial resistance (Veldman et al., 2017). No data were available for the CPE prevalence in the Dutch community ($P_{cCPE_{NL}}$). However, we had data on CPE prevalence in Dutch hospitals ($P_{CPE_{NL}}$). Therefore, $P_{cCPE_{NL}}$ was inferred from the ratio between ESBL *E .coli* in the community and in clinical settings ($C_{com:cli}$), under the presumption that the CPE correlation between the community and the clinical setting is similar to the ESBL E.coli correlation in European countries. The CPE prevalence in Dutch hospitals ($P_{CPE_{NL}}$) was therefore multiplied by the ratio of ESBL E.coli in the community versus ESBL in a clinical setting, $C_{com:cli}$. This ratio was estimated to be 0.79 based on the Pearson correlation between ESBL prevalence in the community and in the clinical setting in the EU, as observed in five studies (Husickova et al., 2012; Stapleton et al., 2017; Smet et al., 2010; Schoevaerdts et al., 2011; Olesen et al., 2013). The derived value of $P_{CPE_{feed}}$ was used for all farm types owing to the lack of data on E. coli in feed for other animal species.

$$P_{CPE_{feed}} = \frac{P_{cCPE_{NL}}}{P_{ec_{NL}}} \cdot P_{ec_{feed}} \qquad (9)$$

No data were available on the concentration of CPE in feed if it was contaminated. The concentration of CPE in feed (CPE_{concF}) was estimated by multiplying the strict

concentrations of E. coli allowed (minimum rejection limit) in feed components ($Ecoli_{concF}$) as given by GMP+ (GMP+, 2019) by the ratio of E. coli carrying CPE genes to non-resistant E. coli ($P_{CPE:EC}$), as observed in samples from 100 Dutch wastewater treatment facilities (Schmitt et al., 2017).

2. 2. 2. 3 Companion Animals

The number of farms with a companion animal (N_C) was calculated by multiplying the total number of farms in the Netherlands (N_{farm}) by the proportion of farms with companion animals (P_{farmC}). No data were available on the proportion of farms with companion animals in the Netherlands. Assuming that farmers' behavior in the Netherlands does not greatly deviate from other Western regions, we used surveillance data of farmers' behavior in the United States of America to estimate P_{farmC}.

The probability of companion animals colonized with CPE in the Netherlands was set equal to the CPE prevalence in the Dutch community ($P_{cCPE_{NL}}$). Although some information on numbers of colonized companion animals in the Netherlands was available from the Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands report (Veldman et al., 2020), these numbers were not considered representative as these were cases from animals visiting a veterinary clinic only (Supplementary file 2.7.5). The concentration of CPE (cfu/g) in feces (CPE_{gramC}) was estimated from the concentration of ESBL *E. coli* (cfu/g) in animal feces ($ESBL_{gramFec}$) measured in an observational study of healthy dogs in the United States (Moran et al., 2017) and the proportion of ESBL *E. coli* carrying CPE genes ($P_{CPE:ESBL}$) (Schmitt et al., 2017).

The frequency of defecating (N_{eli_C}) was based on a report from a commercial feed company in the United Kingdom (Scrumbles, 2021). The weight (grams) of feces defecated by a companion animal was based on a study in healthy medium-sized dogs in the United States (W_{fec}) (Wright et al., 2009). Time spent in the livestock area (P_{barnC}) was set to zero for all farm types in the default calculations, assuming compliance with biosecurity protocols in the Netherlands. However, we explored non-zero P_{barnC} reflecting farms with a lower biosecurity standard in a what-if analysis (Section 2.2.3.3 & Table 2.4). The proportions of CPE transfer from the environment to animal (C_{tranA}) were based on a study that measured the proportion of *Acinobacter* transferred from fomite to finger (Greene et al., 2015). The CPE colonization

period in companion animals (T_{CPE_C}) was set equal to the ESBL E. coli colonization period in healthy dogs in the Netherlands (Baede et al., 2015).

2. 2. 2. 4 Farm Workers

The total number of farms in the Netherlands (N_{farm}) was multiplied by the average number of employees per farm (Avg_{farmer}) to parameterize the number of farmers ($N_{farmers}$). Each farm is typically visited by a single veterinarian, and therefore the number of veterinarians (N_{vet}) in the model was set equal to the total number of farms in the Netherlands (N_{farm}). The number of farm-related workers spending their holiday abroad (N_{T_k}) was calculated by multiplying the number of farm workers (N_{farmer}) and veterinarians (N_{vet}) by the probability of farm workers and veterinarians traveling abroad for their holidays ($P_{holiday}$). The probability of farmers taking a holiday abroad was derived from an online survey among 300 Dutch farmers conducted by a farm-oriented magazine, Boerderij (Farm) (Welink, 2020). The probability of veterinarians taking a holiday abroad was based on data from Statistics Netherlands (Statline, 2019) for the general Dutch population. The proportion of Dutch travelers visiting each UN region (P_T) was based on Statistics Netherlands data from 2013, where the number of holidays to each region was divided by the total number of holidays taken by Dutch citizens (Supplementary 2.7.6). To estimate the probability of hospital admission for farm workers ($P_{admit_{NL}}$), the number of Dutch inpatients in 2017 was divided by the total population of the Netherlands in 2017. The prevalence of CPE in hospital ($P_{CPE_{NL}}$) was based on data provided by EARS-Net (ECDC, 2017). The probability of hospital admission during holidays outside of the Netherlands (P_{admit}) was derived from a study among 2000 Dutch travelers. The probability of acquiring CPE during hospitalization (P_{CPE}) in non-European countries was parameterized from national surveillance on CPE prevalence from multiple countries around the world reported in the WHO's global report of surveillance [62] and independent academic publications (Iregui et al., 2018; Patel et al., 2008). The probability of non-hospitalized travelers acquiring CPE from the community in a foreign country (P_{cCPE}) was inferred by multiplying the hospital CPE prevalence (P_{CPE}) by the ratio of ESBL in the community versus ESBL in the clinical setting ($C_{com:\,cli}$) (Supplementary file 2.7.4). The number of CPE (cfu) remaining on a farm worker's hands after hand washing (CPE_{hand}) was estimated from an observational study in Mexico among tomato farmers, in which the number of E. coli on hands after toilet use followed by

hand washing ($Ecoli_{hand}$) was measured. $Ecoli_{hand}$ was multiplied by the probability of *E. coli* carrying CPE genes ($P_{CPE:EC}$) to calculate CPE (cfu) on farm workers' hands. The number of defecations per day (N_{eli_H}) was retrieved from an observational study of 2000 returning Dutch travelers (Arcilla et al., 2016). Proportion of time spent in the livestock area (P_{barnH}) was estimated at eight hours a day for farmers and one hour per week for veterinarians. The proportions of CPE transfer from the hands to the environment (C_{tranE}) were based on the same study used to estimate the proportions of CPE transfer from the environment to the animal (C_{tranA}) (Greene et al., 2015).

2. 2. 2. 5 Dose-Response Parameter

The median infectious dose ($ID50$) was used to calculate the dose–response parameter (P). The median infectious dose ($ID50$) was based on experimental studies
for ESBL in broilers and pigs. No data were available to estimate the $ID50$ for veal calves, and, therefore, it was set equal to the median infectious dose of pigs.

Table 2.1- *Input parameters for the model to assess the risk of CPE introduction into Dutch livestock farms.*

Input *	Description	Value Distribution **	Value in Sensitivity Analysis	References
N_{intro}	Expected annual number of farms on which *CPE* is introduced			
N_{col_s}	Number of farms exposed to *CPE*-colonized sources *s* (imported livestock (A), livestock feed (F), companion animals (C), farm workers being hospitalized (H), and farm workers traveling abroad (T))			
N_s	Number of farms in contact with people, import animals, companion animals, and livestock feed			
P_{CPE_s}	Probability of sources exposed to farm are colonized/contaminated with *CPE*			
$P_{CPE_{batch}}$	Probability that an individual farm receives at least one batch of feed contaminated with *CPE*			
N_{batch}	Annual number of feed batches received by a farm			
$P_{CPE_{feed}}$	Probability that a batch of feed is contaminated with *CPE*			
N_C	Number of farms with companion animals			
N_H	Number of farm workers/vets hospitalized			
N_{T_k}	Number of farm workers/vets returning from abroad			
CPE_{ing_s}	Total number of CPE *E. coli* bacteria ingested by the animals on an exposed farm			
CPE_{concF}	Total number of *CPE E. coli* (cfu/g) in contaminated animal feed			
P	Probability of a single CPE bacterium colonizing an animal's gut			

Input *	Description	Value Distribution **	Value in Sensitivity Analysis	References
CPE_{gramC}	Total number of *CPE E. coli* (cfu/g) in companion animal feces			
CPE_{hand}	Total number of *CPE E. coli* (cfu) remaining on a farm worker's hands after hand washing			
N_{imp}	Annual number of imported broilers, parent broilers, piglets, breeding pigs, and veal calves from EU member states *j* to farm type *i* in the Netherlands	Supplementary 2.7.9	Yes	(Statline, 2019; RVO, 2021)
se	CPE surveillance sensitivity	0.85	Yes	(Wit et al., 2017)
$C_{com:cli}$	Ratio of ESBL in the community versus ESBL in a clinical setting	0.79	N	Table 2.7
$P_{ec_{feed}}$	Prevalence of *E. coli*-contaminated feed in compound cattle feed	Beta (59, 46)	Yes	(Da Costa et al., 2006)
$P_{ec_{NL}}$	Prevalence of *E. coli* in Dutch residents	Beta (159,620, 280,677)	Yes	(Veldman et al., 2020)
N_{size}: broiler N_{size}: piglet N_{size}: breeding pig N_{size}: veal calf	Number of livestock *i* per shipment	Pert (45,00,47,000, 55,000) Pert (100, 260,300) Pert (65, 80, 95) Pert (30, 150, 200)	Yes	(Van Dijk, 2020)
c_{a}	The average grams of feed consumed by livestock *i* per day	Table 2.9	Yes	(Turner et al., 2005; Rönnqvist et al., 2018; Bussel, 2020)
V_{batch}	The average grams of feed delivered to a farm derived from the volume of a standard transport truck	Pert (3×10^6, 16×10^6, 3×10^7)	Yes	(Van Dijk, 2020)
N_{farm} and N_{animal}	Total number of farm types *i* and total number of animals *i* in the Netherlands	Table 2.9	Yes	(Statline, 2019)

Input *	Description	Value Distribution **	Value in Sensitivity Analysis	References
N_K	Total number of farm workers and veterinarians in the Netherlands	Table 2.9	Yes	(Statline, 2019)
P_{CPE}	CPE prevalence in hospital patients in region *m*	Beta (α/se, β) (values of beta distribution are in Table 2.9)	Yes	(Iregui et al., 2018; Patel et al., 2008; Khan et al., 2010; Castanheira et al., 2011; Mohanty et al., 2010; Ben-David et al., 2012; Liu et al., 2012; Rimrang et al., 2012; Balm et al., 2013; Koh et al., 2013; Khajuria et al., 2014; Alagesan et al., 2015; Tran et al., 2015; Hsu et al., 2017; Liu et al., 2017; CPE Thailand, 2018; Singh-Moodley et al., 2016; Correa et al., 2013; Schwaber et al., 2008; Johani et al., 2010; Nahid et al., 2013)
$Ecoli_{hand}$	The amount of *E. coli* remaining on a farm worker's hands after toilet use and subsequent hand washing (cfu)	Log-normal (63, 5.02)	Yes	(De Aceituno et al., 2015)
$ESBL_{gramFec}$ (cfu/g)	Number of *E. coli* (cfu) in a gram of healthy companion animal's feces	Normal (70, 35)	Yes	(Espinosa-Gongora et al., 2015)
$P_{CPE:EC}$ $P_{CPE:ESBL}$	Proportion of *E. coli* carrying CPE genes and proportion of ESBL *E. coli* carrying CPE genes	0.00004 0.00424	No	(Schmitt et al., 2017)
$ID50$: broiler $ID50$: pig and veal calf	Infectious dose of ESBL *E. coli* at which, on average, 50% of livestock species *i* are colonized (cfu)	Log-normal (5, 5) Log-normal (4695, 9187)	Yes	(Dame-Korevaar et al., 2019; Cornick et al., 2004; Moran et al., 2017)

Input *	Description	Value Distribution **	Value in Sensitivity Analysis	References
P_{farm_C}	Proportion of farms that have companion animals	Beta (298, 148)	Yes	(Moran et al., 2017)
W_{fec} (grams)	Grams of feces defecated by a companion animal in one defecation	Normal (70, 35)	Yes	(Wright et al., 2009)
N_{eli_C} N_{eli_H}	The average number of defecations by companion animals and humans per day	Pert (1, 2, 5) Uniform (1,3)	Yes	(Scrumbles, 2021) Assumption
T_{CPE_C} T_{CPE_H}	Colonization duration of CPE in companion animals and humans (days)	Pert (0, 120, 180) Pert (1, 30, 365)	Yes	(Baede et al., 2015; Arcilla et al., 2017)
P_{barnC} P_{barnH}: farm worker P_{barnH}: veterinarian	Proportion of day a companion animal, farm worker, and veterinarian spent in the barns	0 0.33 0.005	Yes	Assumption
C_{tranA} C_{tranE}	Proportion of *Acinobacter* transferred from fomite to finger (A) and from finger to fomite (E)	Log-normal (0.24, 0.14) Log-normal (0.06, 0.06)	Yes	(Greene et al., 2015)
$Ecoli_{concF}$: broiler $Ecoli_{concF}$: fattening pig $Ecoli_{concF}$: breeding pig $Ecoli_{concF}$: veal calf	Concentrations of *E. coli* in feed components following minimum rejection limit by GMP+ (cfu/g)	11.8 11.8 14.3 7.3	Yes	(GMP+, 2019)
P_T	The probability of Dutch travelers visiting 16 world regions in 2013	Table 2.9	Yes	(Statline, 2019)
$P_{holiday}$: broiler and pig farm worker $P_{holiday}$: veal calf farm worker $P_{holiday}$: veterinarian	Probability of farm worker on farm *i* taking holiday abroad annually	0.53 0.33 0.64	Yes	(Statline, 2019; Welink, 2020; Molder, 2019)

Input *	Description	Value Distribution **	Value in Sensitivity Analysis	References
$Avg_{farmers}$	The average number of farm workers in all farm types	Pert (1, 2, 4)	Yes	Assumption
P_{admit} $P_{admit_{NL}}$	Probability of hospital admission while traveling overseas and in the Netherlands	0.04 0.054	Yes	(Statline, 2019; Arcilla et al., 2017; Ministerie van Volksgezondheid et al., 2022)

Footnotes: * Type of farm is indicated by subscript *i* and source country by *j*. ** Parameters for input distributions given in brackets: beta (α,β), where α equals the number of positives plus one, and β the number of negatives plus one; log-normal (mean, SD); normal (mean, SD); pert (minimum, most likely, maximum); uniform (minimum, maximum). Parameters with an empty *Value Distribution* are parameters calculated from the raw input.

2. 2. 3 Sensitivity Analysis

2. 2. 3. 1 Spearman Rank Correlation on Baseline Simulations

Sensitivity analysis was applied to the risk model to assess the impact of uncertain and highly variable input parameters that were inputted as probability distributions on the estimated number of CPE introductions (N_{intro_s}). Spearman rank correlation was used to analyze the impact of these input parameters. Only input parameters with a correlation coefficient > |0.1| with N_{intro_s} were included in the result.

2. 2. 3. 2 One-at-a-Time Sensitivity Analysis

In an additional one-at-a-time (OAT) sensitivity analysis, the most input parameters (non-inferred) (Table 2.1) were either decreased or increased by 50%. The result of each input adjustment was compared to the baseline result to determine which parameter had the most effect on the expected number of colonized farms. Results were calculated per CPE source (imported livestock, livestock feed, companion animals, hospital patients, and returning travelers). To analyze the effect of changes in input parameters on the ranking of sources for the expected number of farms with CPE introduction, outcomes of each input adjustment were compared to the outcomes of all other input adjustments, including the baseline model, and the frequency of changes in the ranking were counted.

2. 2. 3. 3 What-If Analysis

Three what-if scenarios were analyzed for their impact on the estimated number of CPE introductions (N_{intro_s}). The first scenario simulated the effect of less sanitary measures in livestock feed production by increasing the bacteria number in feed ($Ecoli_{concF}$) to the maximum limit for rejecting feed according to GMP+. The second scenario modeled the effect of banning livestock importation from EU member states with insufficient CPE surveillance. In the calculations for this scenario, livestock imports from countries that sampled less than 100 animals for CPE surveillance were excluded from the model calculations. The third scenario evaluated weak compliance with biosecurity protocols on farms. This affected both the risk of introduction from humans and companion animals. The lower biosecurity was mimicked by assuming farm workers did not wash their hands after toilet use, resulting in a higher number of CPE on their hands, and by adjusting the proportion of time a companion animal was

present in the animal area P_{barnC}. This parameter was set to 0.1 in broiler and pig farms and 0.3 in veal calf farms. All other input parameters were kept at their baseline values in the what-if scenarios.

2. 3 Results

To estimate the risk of introduction, first, the number of farms exposed to CPE sources (Section 2.3.1) and the probability of colonization after exposure (Section 2.3.2) were estimated. These were combined into the risk of introduction by calculating the number of expected introductions (Section 2.3.3). The sensitivity of model output to model input parameters was determined by two methods of sensitivity analysis (Section 2.3.4). First, Spearman correlation coefficients were used to identify important uncertain parameters. Second, one-at-a-time sensitivity analysis was used to investigate the robustness of the ranking of risks to changes in each of the input parameters. Finally, different scenarios with respect to contamination of feed, restrictions on imports, and biosecurity were studied (Section 2.3.5).

2. 3. 1 Number of Farms Exposed to CPE

Based on our model calculations, fattening pig farms have the highest risk of CPE exposure, with over 600 farms in The Netherlands being exposed to at least one CPE source annually (Figure 2.3). The results indicate that 22% of the 2652 fattening pig farms and 12% of the 4513 pig farms (all farm types) in The Netherlands would be exposed to CPE. The numbers of broiler, breeding pig, and veal calf farms exposed to CPE is lower, though still considerable, with more than 100 farms exposed annually. The risk of CPE exposure is the lowest for broiler breeder farms with only 18 CPE expected exposures annually (Figure 2.3).

The main sources of exposure are livestock feed, imported livestock, and returning travelers, while the small number of farms exposed to companion animals (four) and Hospitalized patients is negligible (one).

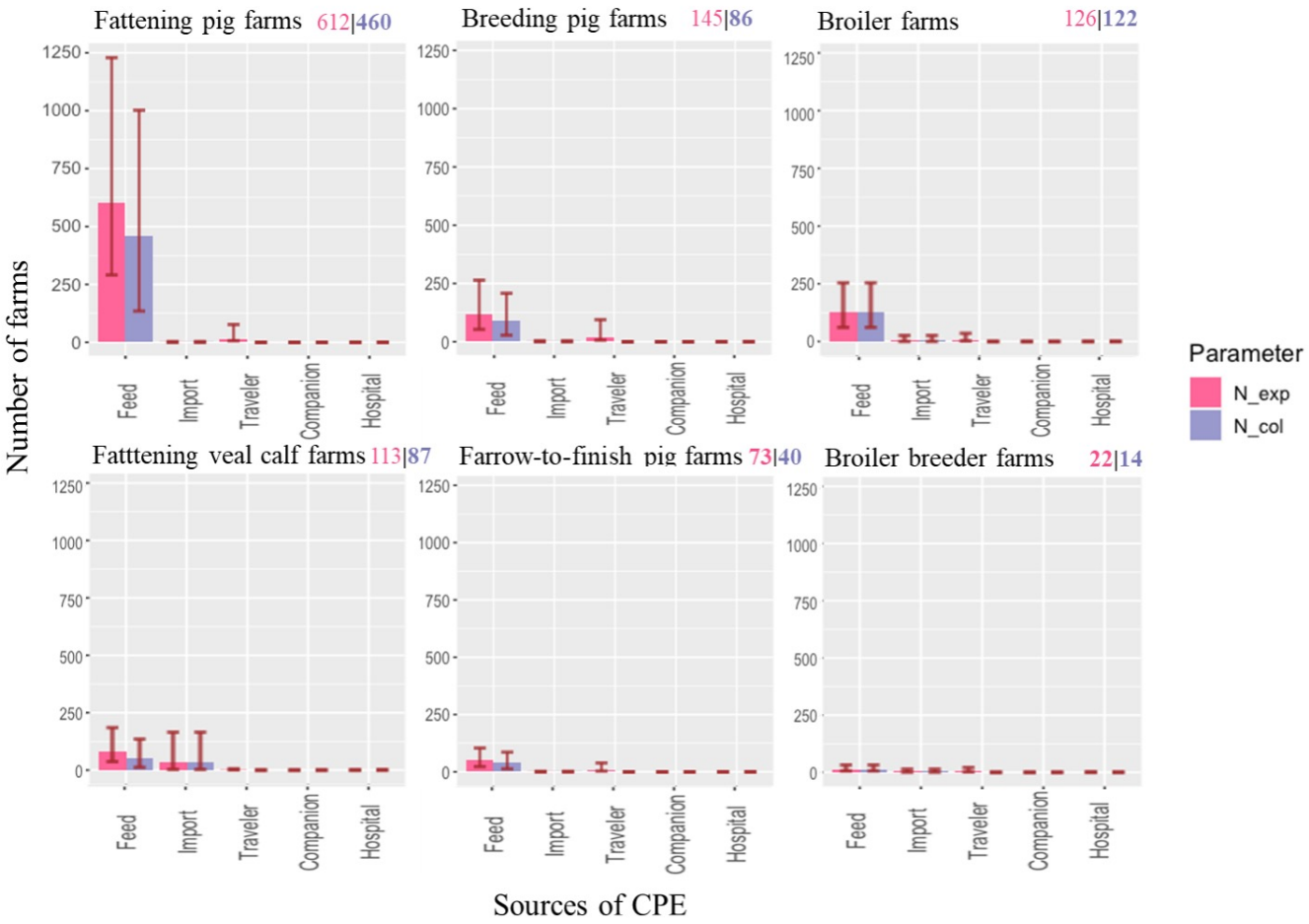

Figure 2.3- *Baseline result: median (whisker: 5th and 95th percentiles) annual number of farms exposed to (red) and colonized by (blue) CPE in each farm type from five sources (feed, imported livestock, returning travelers, companion animals, and hospital patients). The color-coded numbers in the right upper corner of each plot are the total number of farms exposed to CPE and the total number of farms in which CPE has been introduced.*

2. 3. 2 Probability of Colonization Given Exposure to CPE

This probability was not calculated for imported livestock, since introduction of a colonized animal on the farm immediately results in colonization of the farm (where colonization of a farm was defined as the presence of at least one colonized animal on the farm). Livestock feed had the highest probability of colonization in the exposed farms (Table 2.2). Farm workers and veterinarians posed a very low probability of colonization to the exposed farms. The probability of colonization by exposure to companion animals was not calculated for the baseline scenario because we assumed that companion animals would not enter the barns, resulting in zero introduction to the small number of exposed farms. In the farm type comparison, exposed broiler and broiler breeder farms had the highest probability of colonization if exposed. The probability of colonization on a veal calf farm exposed to contaminated feed was the lowest of all farm types. The probabilities of colonization in veal calf and all three pig

farm types exposed to CPE-colonized humans were equivalent. The probability of colonization was the lowest in all three pig farm types and veal calf fattening farms exposed to colonized returning veterinarians from overseas travel and hospital.

Table 2.2- *Probability of at least one animal colonized on a farm given exposure of the farm to CPE. The companion animal source resulted in zero probability, and there was no calculation for im-ported livestock.*

Farms at risk	Median Probability of at Least One Animal Being Colonized Given Exposure by a Specific CPE Source (5th and 95th Percentiles).		
Farm Types	Feed	Farm Workers Returning from Travel and Hospital	
		Farm Workers	Veterinarians
Broiler	1.00 (1.00, 1.00)	1×10^{-4}(1×10^{-5}, 8×10^{-4})	2×10^{-6} (2×10^{-7}, 2×10^{-5})
Broiler breeder	1.00 (1.00, 1.00)	1×10^{-4} (1×10^{-5}, 8×10^{-4})	2×10^{-6} (2×10^{-7}, 2×10^{-5})
Fattening pig	0.88 (0.22, 1.00)	2×10^{-7} (1×10^{-8}, 5×10^{-6})	4×10^{-9} (2×10^{-10}, 9×10^{-8})
Breeding pig	0.92 (0.26, 1.00)	2×10^{-7} (1×10^{-8}, 5×10^{-6})	4×10^{-9} (2×10^{-10}, 9×10^{-8})
Farrow-to-finish	0.92 (0.26, 1.00)	2×10^{-7} (1×10^{-8}, 5×10^{-6})	4×10^{-9} (2×10^{-10}, 9×10^{-8})
Veal calf	0.73 (0.15, 1.00)	2×10^{-7} (1×10^{-8}, 5×10^{-6})	4×10^{-9} (2×10^{-10}, 9×10^{-8})

2. 3. 3 Ranking the Risk of Introduction: Combining Exposure and Colonization

The estimated number of fattening pig farms with CPE introduction was the highest, followed by broiler, fattening veal calf, and breeding pig farms (Figure 2.3). Farrow-to-finish farms and broiler breeder farms ranked lowest in terms of numbers of introductions. Exposure to contaminated feed was most likely to result in CPE introduction, with probabilities of colonization varying between 73% and 100% (Table 2.2). Exposure to hospitalized farm workers and returning travelers, on the contrary, was estimated to hardly ever result in CPE introduction to the farm due to a very low probability of colonization in exposed farms (Table 2.2). The expected annual number of CPE introductions to livestock farms in the Netherlands due to returning travelers was 5 × 10−5, which equals an introduction once every 20,000 years. For an individual farm, the estimated probability of colonization was highest on broiler farms (0.23, Table 2.3). Probabilities of colonization in fattening pig and farrow-to-finish farms were slightly lower (between 0.16 and 0.17). The probabilities of colonization in other farm types were lower than 0.1

***Table 2.3**- Expected number of farms exposed and colonized combined with the total number of farms to calculate the probability of exposure and colonization for an individual farm of a specific type.*

			Broiler	Fattening Pig	Farrow-to-Finish	Veal Calf	Broiler Breeder	Breeding Pig	Total
Expected number	Total number of farms in the Netherlands		524	2652	260	1298	255	1601	6590
	Farms exposed		126	612	73	113	22	145	1091
	Farms colonized		122	460	40	87	14	86	810
Probability per individual farm	Exposure		0.24	0.23	0.28	0.09	0.09	0.09	0.17
	Colonization		0.23	0.17	0.16	0.07	0.05	0.05	0.13
	Probability of exposure due to	Feed	0.229	0.228	0.196	0.059	0.051	0.067	0.148
		Imported livestock	0.004	3×10^{-4}	0.002	0.025	0.004	0.001	0.007
		Returning traveler	0.008	0.006	0.040	0.006	0.015	0.069	0.143
		Companion animal	0.001	0.004	3×10^{-4}	0.002	3×10^{-4}	0.002	0.009
		Hospital patient	1.8×10^{-4}	0.001	2×10^{-4}	4×10^{-4}	8×10^{-5}	5×10^{-4}	0.003

2. 3. 4 Result from Sensitivity Analysis

First, the Spearman rank correlation, a non-parametric metric between −1 and 1, was calculated for all input parameters with an uncertainty distribution to estimate the extent to which these input parameters determined the model results for each source (Section 2.3.1). Secondly, one-at-a-time (OAT) sensitivity analysis was performed (Section 2.3.2). In this additional sensitivity analysis, the value of a single input parameter was either increased or decreased. The outcome of each adjustment was compared to the baseline scenario to investigate the impact of all input parameters on the estimated number of introductions. OAT sensitivity analysis was performed separately for each source. Then, to evaluate if changes in input parameters would affect the ranking of sources, we compared the results of the OAT sensitivity analysis across sources (Section 2.4.3).

2. 3. 4. 1 Result from Spearman Rank Correlation

Based on the model results, feed is indicated as the main contributor of CPE introduction for all livestock farm types (Table 2.3). The Spearman rank correlation for this source revealed that the prevalence of CPE-colonized patients in Dutch hospitals ($P_{CPE_{NL}}$), which was combined with E. coli prevalence to infer the prevalence of CPE in feed ($P_{CPE_{feed}}$), 50% infectious dose (ID50), and the average batch size of feed (V_{batch}) are inputs that are strongly correlated with the expected number of introductions from feed (Figure 2.4). However, these parameters are not expected to affect the ranking of farm types for their introduction risk because these inputs are identical for all farm types apart from 50% infectious dose (ID50), which differs between farm types. CPE prevalence in livestock i in country j (P_{CPE_A}) is highly correlated with the expected number of CPE introductions from imported animals to all farm types. Though CPE prevalence in humans ($P_{CPE_{NL}}\ and\ P_{CPE}$) is correlated with the number of introductions from both hospitalized patients and returning travelers, the average number of farmers per farm ($AVG_{farmers}$) and the probability of admission to hospital during travel (P_{admit}) were more correlated with pig and veal calf farm introductions than CPE prevalence in the returning traveler source. Introductions from returning travelers and hospitalized patients were also correlated with input parameters for probability of colonization given exposure such as infectious dose at 50% colonization (ID50) and proportion of CPE transferred from fomite to finger and vice versa ($C_{tran_E}\ and\ C_{tran_A}$).

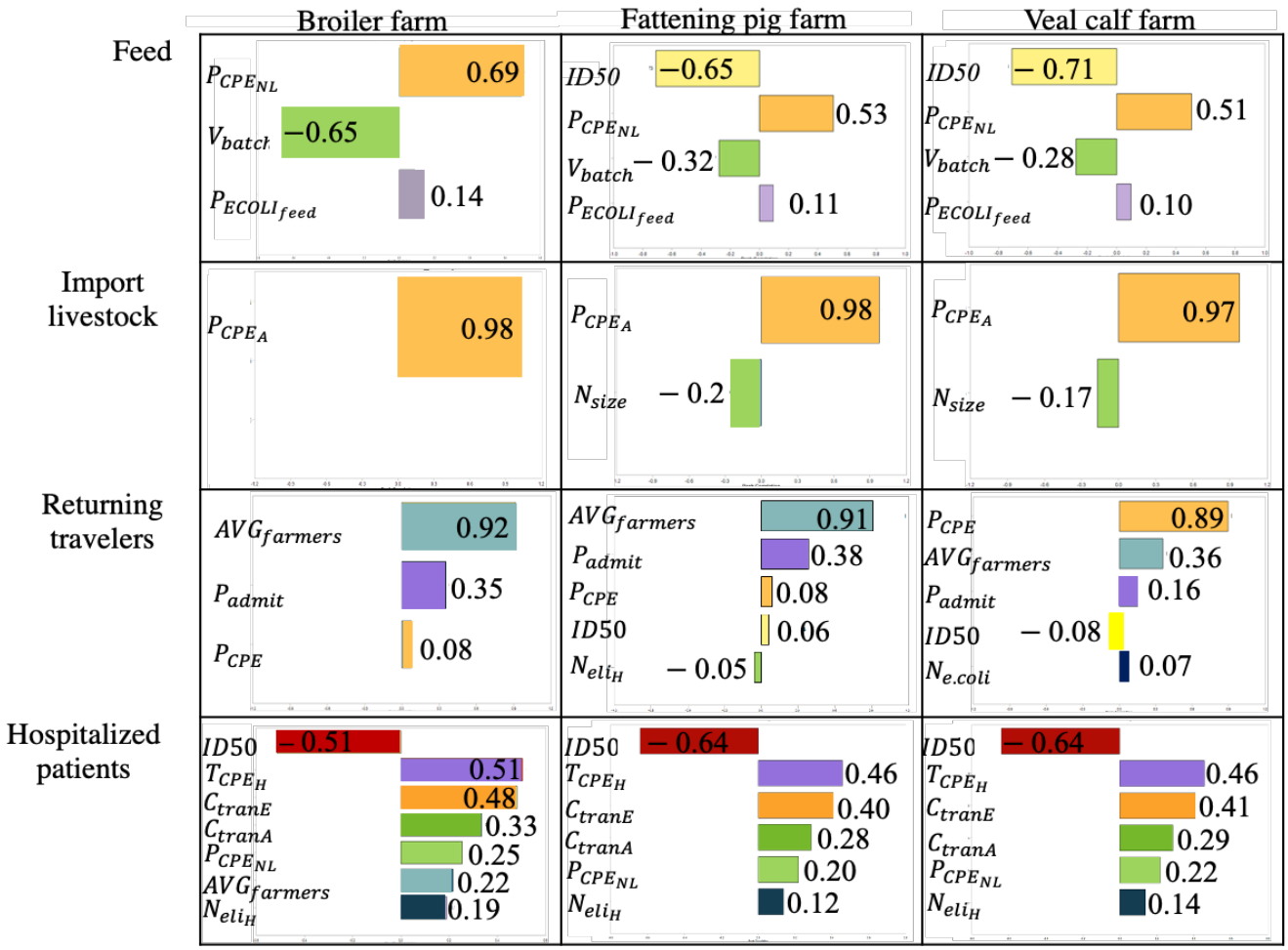

Figure 2.4- *Results of Spearman rank correlation for broiler farm, fattening pig farm, and veal calf farm. Each row shows rank correlation of input parameters with the expected number of CPE colonizations from feed, imported livestock, returning travelers, and hospitalized patients. Only input parameters with a Spearman rank correlation coefficient > |0.1| are included in the plots. Spearman rank correlation of companion animals is excluded from the figure because the introduction is zero.*

2. 3. 4. 2 One-at-a-Time Sensitivity Analysis per Source

One-at-a-time sensitivity analysis of the input parameters for introduction by feed unveiled two parameters that had a huge impact on the estimated number of introductions in different farm types: the total number of animals in the Netherlands (N_{animal}) and the amount of feed consumed per animal per day (C_a) (Figure 2.5). The total number of farms (N_{farm}) was used twice in the model, i.e., to obtain the number of animals per farm and the number of farms exposed, which compiled into a lower effect toward introductions than the total number of animals in the Netherlands (N_{animal}) and the amount of feed consumed per animal per day (C_a). Parameters with the least impact on introduction in all farm types were the number of bacteria in contaminated feed ($Ecoli_{concF}$) and the median infectious dose (ID50). These two

parameters were involved in calculating the probability of colonization in an exposed farm (P_{col_s}), while other parameters were involved in calculating the number of exposed farms (N_{col_s}).

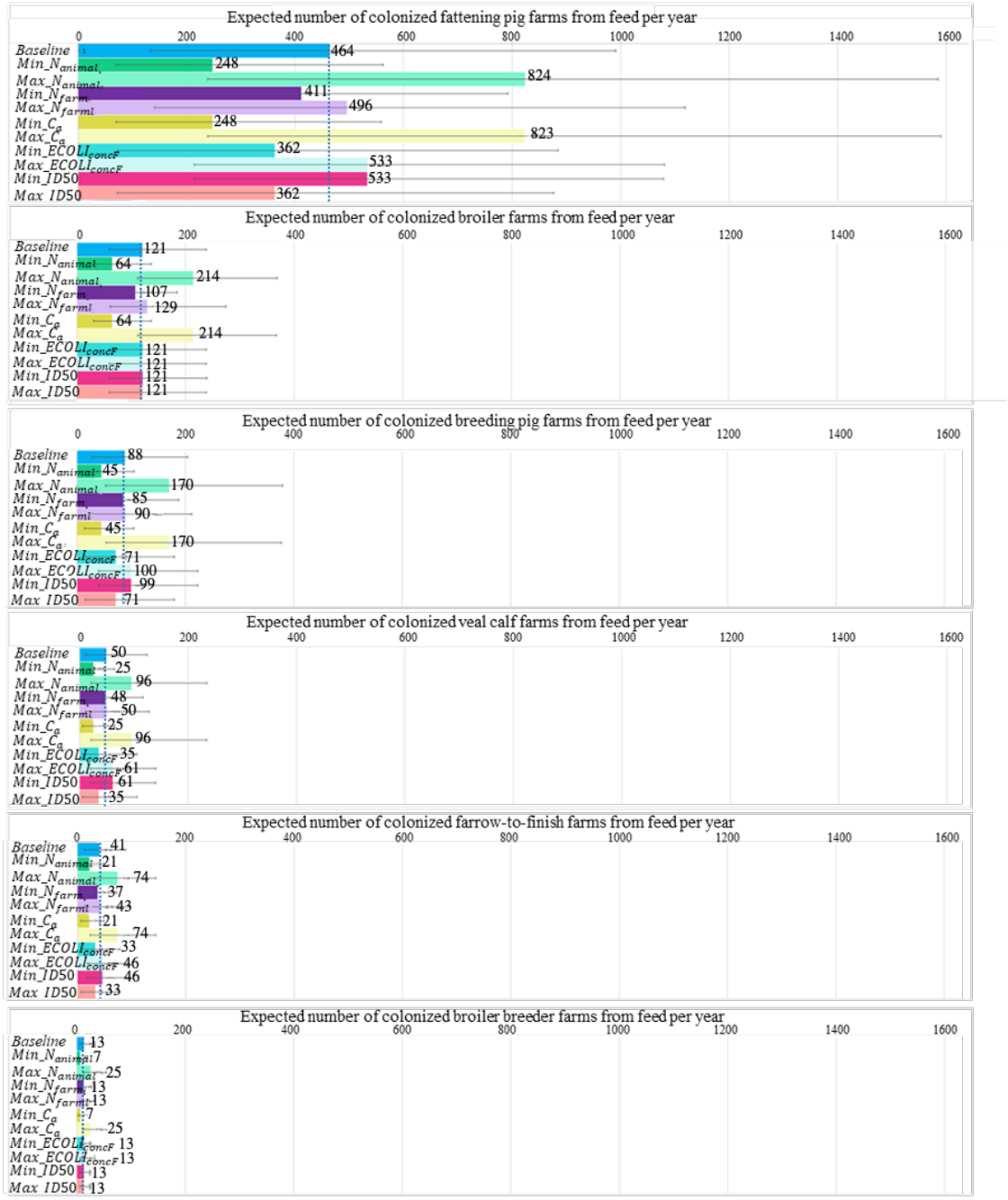

Figure 2.5- *One-at-a-time sensitivity analysis of the number of introductions from feed to six farm types calculated in which one parameter either increases or decreases two-fold. Farm types are ordered according to the highest to lowest number of introductions in the baseline model. Dotted blue line indicates the estimated number of introductions in the baseline model. Only parameters that differed between farm types are included in this figure.*

Input values of three impactful parameters, namely, the total number of animals (N_{animal}), total number of local farms (N_{farm}), and grams of feed ingested per livestock per day (C_a), in the baseline model were compared across all farm types (Supplementary 2.7.6). Fattening pig farms had the highest total number of farms (N_{farm}) but a moderate total number of fattening pigs (N_{animal}) and grams of feed ingested per fattening pig per day (C_a) compared to other farm types. The high number of introductions to veal calf farms arose from imported livestock. Two essential parameters that directly facilitate introduction to fattening veal calf farms are CPE prevalence in the source country (P_{CPE_A}) and the number of livestock i per shipment (N_{size}). When the number of livestock i per shipment was enhanced two-fold, the number of farms exposed was also enhanced two-fold (Supplementary 2.7.8). It should be noted that the number of livestock per shipment is directly correlated with the annual number of animals imported (N_{imp}). However, a two-fold increase in the CPE prevalence in livestock in source countries (P_{CPE_A}) increases the number of introductions only slightly because of the very low prevalence estimates based on the zero CPE cases in livestock (as reported by most source countries).

Fattening pig farms and veal calf farms remained the highest in farm types with introductions from livestock feed and imported livestock in the OAT sensitivity analysis. None of the OAT analysis resulted in increased introduction from human sources. However, one scenario of the OAT analysis indicated introduction to fattening pig farms from the companion animal source.

2. 3. 4. 3 One-at-a-Time Sensitivity Analysis between Sources

To evaluate if changes in input parameters would affect the ranking of sources, we performed a pairwise comparison of the results of the OAT sensitivity analysis of individual sources (Table 2.10). For example, for the comparison of feed and imported livestock, we compared 15 outcomes (7 parameters that were both increased and decreased, and the baseline) of the feed source to 7 outcomes of the imported livestock source (3 parameters that were both increased and decreased, and the baseline). This resulted in a total of 105 combinations of outcomes including 1 combination of baseline parameters for both sources (Table 2.11). Of all the other 104 outcome combinations, we recorded if the ranking of the sources was different from

the comparison of the baseline parameters in both sources. Feed consistently ranked as the source with the highest expected number of CPE introductions in all farm types, except for veal calf farms, when comparing sensitivity tests across all sources (Supplementary 2.7.9). Forty-four percent of the adjusted input parameters resulted in a higher introduction from imported livestock to veal calf farms than feed. In the baseline model, the colonization risk of imported livestock and feed for veal calf farms was on the same order of magnitude, with the risk of feed being slightly higher, whereas for all other farm types, the risk of imported livestock was very low compared to feed (Figure 2.3). On the other hand, all sensitivity tests produced non-zero introduction from feed, while a small proportion of sensitivity tests (19%) resulted in negligible introduction from imported livestock to most farm types except fattening pig and veal calf farms. Imported livestock always had a higher introduction risk than returning travelers, hospitalized patients, and companion animals (Supplementary 2.7.9).

2. 3. 5 Result from What-If Analysis

The effects of higher contamination levels in feed, less strict biosecurity at the farm level, and a ban on livestock imports from countries sampling less than 100 animals for CPE surveillance were explored by adjusting input parameters and evaluating the model outcome (number of introductions) in what-if scenario analysis.

CPE was introduced into eight (one breeding, five fattening pig, and two veal calf) additional farms when the number of *E. coli* contaminations increased to the maximum limit for rejecting feed as given by GMP+. This addition is small compared to the 767 expected introductions in the baseline model (Table 2.4). Interestingly, banning imports from countries with a low surveillance level (less than 100 animals sampled) reduced the risk of introduction from imported livestock by 71%. Following a minor increase in introduction from companion animals in a flexible biosecurity scenario, companion animals would be reclassified from no risk to a low-risk source. Conversely, introduction from returning travelers and hospitalized patients remained negligible when the number of bacteria on a person’s palms increased four times due to non-compliance with hand hygiene protocols.

Table 2.4- *What-if analysis related to probability of colonization in feed, restriction on import of animals from countries with weak surveillance for CPE, and less strict biosecurity practice in local farms.*

Scenario	CPE Source Affected	Parameter Changed	Baseline Number of Introductions from Affected Source (95% Range)	Changed Number of Introductions from Affected Source (95% Range)
Contamination of *E. coli* in feed reaches concentration of maximum rejection limit according to GMP+	Feed	$Ecoli_{concF}$	767 (244, 1679)	775 (246, 1668)
The Netherlands only allows import of livestock from EU member states that sample ≥100 animals in CPE surveillance	Imported livestock	P_{CPE_A}	48 (4, 214)	14 (0, 58)
Lower biosecurity: companion animals have full access to livestock areas in broiler, pig, and veal calf farms	Companion animals	P_{barnC}	0 (0, 0)	2 (1, 7)
Lower biosecurity: non-compliance with hand hygiene	Travelers and hospitalized patients	$Ecoli_{hand}$	1×10^{-4} (9×10^{-6}, 8×10^{-4})	4×10^{-3} (3×10^{-4}, 3×10^{-2})

2. 4 Discussion

This is the first risk assessment that quantifies the risk of CPE introduction into livestock farms in the Netherlands. The results indicate that fattening pig farms ranked the highest with respect to the expected annual number of CPE-colonized farms. However, when considering the probability of CPE introduction per individual farm, broiler farms have the highest introduction risk. Our model indicates that feed is a major potential source of CPE introduction, but this risk estimate has a high uncertainty. Imported livestock is indicated as an important CPE source specifically for veal calf farms. Other sources (companion animals, hospital patients, and returning travelers) were assessed to be of minor or negligible importance.

The number of exposed farms was most important in determining the introduction risk expressed as the expected number of colonized farms for high-rank sources (feed and imported livestock), due to the high probability of colonization upon exposure (P_{col_s}) in both sources (probability varying between 0.73 and 1 for feed (Table 2.2), probability of 1 for livestock imports). The probability of an individual farm exposed to CPE due to feed was similar in broiler, fattening pig, and farrow-to-finish farms (Table 2.3). This probability equaled the probability of receiving at least one CPE-contaminated batch of feed ($P_{CPE_{batch}}$). Although broilers require much less feed per animal than pigs due to their relatively small size, the number of broilers kept per farm is higher, resulting in a similar amount of feed delivered to all farm types.

The overall probability of introduction for an individual farm resulting from all sources was the highest in the broiler sector. If exposed to CPE, broilers have a higher probability of colonization than pigs and veal calves due to the very low median infectious dose ($ID50$) in broilers. This parameter mainly affected the colonization probabilities of farms exposed to CPE-colonized humans because, for this source, the dose to which the animals are exposed is low. With high exposure doses, as was the case with feed, the probabilities of colonization are high, even when the ID50 is high. The total number of CPE introductions is thus mainly determined by the total number of farms exposed to CPE given the high probability of colonization upon exposure by the two major sources (0.73–1 probability). Consequently, the effect of changing the probability of colonization is much smaller than that of changing the number of exposed farms.

According to our model, thirteen percent of Dutch farms are estimated to be colonized by CPE each year, mainly via feed, which is clearly an overestimation as such a percentage of farms being colonized would be detectable under the current national surveillance protocol (Wit et al., 2017; Biedenbach et al., 2014). Still, an undetected CPE presence in Dutch livestock is possible, as the current national surveillance protocol was designed to detect at least one colonized animal with 95% certainty, provided the prevalence is 1% (Wit et al., 2017). However, this surveillance protocol does not take into account clustering of colonization at the farm level, which decreases the sensitivity of the surveillance. Furthermore, introductions could have escaped

detection because most farms for meat production (broiler, fattening pig, and veal calf) apply an all-in-all-out system that produces more than one batch of livestock annually, while the national surveillance collects samples only once a year from a single animal per batch at slaughter from part of the farms. Thus, for each farm unit, multiple samples distributed over time are necessary to calculate an accurate prevalence (Cameron and Baldock, 1998).

In our calculation, a major source of CPE introduction is feed, although no carbapenemase-producing bacteria have been found thus far in feed. The probability that batches are CPE-contaminated and the concentration of CPE in contaminated batches were both inferred from the CPE prevalence among humans, E. coli prevalence in feed, and the ratios of CPE, ESBL, and other E. coli in water sources. Using these proxy measures introduces uncertainty in the calculations. Multiple studies, however, indicated the presence of E. coli in feed to be as prominent as Salmonella, which is a major hazard in animal feed (Davies and Davies, 2010; Dodd et al., 2003; Sargeant et al., 2004; Dargatz et al., 2005; Hancock et al., 1997; Andreoletti et al., 2008). Despite no CPE detection in livestock feed, a small percentage of E. coli from feed collected in Portugal and the United States carried resistant genes against ampicillin and cefotaxime (Dargatz et al., 2005; GE et al., 2012; Da Costa et al., 2007). It is, therefore, reasonable to assume that CPE contamination of feed is possible. Although halving the CPE prevalence in feed lowered the risk of feed considerably (Supplementary 2.7.8), feed still remained an important source of CPE introduction, still being higher than the risk of imported animals. It is therefore recommended to investigate this source of CPE in more detail to either discard this source as a risk or to enable mitigation strategies.

The probability of batches of feed contaminated with CPE ($P_{CPE_{feed}}$), the number of batches delivered to a farm each year (N_{batch}), the median infectious dose ($ID50$), and the concentration of CPE E. coli (cfu/g) in contaminated animal feed (CPE_{concF}) are four parameters worth further examination because they had a large impact on the introduction risk and are surrounded by considerable uncertainty. Uncertainty in the probability of batches of feed contaminated with CPE ($P_{CPE_{feed}}$) and the concentration of CPE E. coli (cfu/g) in contaminated animal feed were due to lack of data for CPE,

and these parameters were therefore inferred from the prevalence and concentration of E. coli in feed and other sources. Equally, no data were available on the median infectious dose ($ID50$) for CPE in livestock, and therefore estimates from studies on ESBL in broilers and pigs were used. Uncertainty in the number of batches delivered to a farm each year (N_{batch}) stems from generalizing highly variable parameters into an average value. The impact of overestimating these parameters was assessed in a sensitivity analysis, where the number of introductions from feed was reduced by, at most, 47% (Table 2.10- 2.12). Still, the 47% reduction in the number of introductions from feed remains higher than other sources (Supplementary 2.7.9).

Whereas most farm types have a low risk of introduction via routes other than feed, veal calf farms have a high risk of introduction by imported animals. Farms received a higher number of batches of imported veal calves than other animal types due to a high number of imported animals and small batch sizes. Furthermore, the inferred CPE prevalence in veal calves in source countries (P_{CPE_A}) is higher than the estimated CPE prevalence in pigs and broilers (European Food Safety, 2016; EFSA, 2019). Eighteen EU member states did not collect any samples from veal calves for CPE surveillance (Supplementary 2.7.10; Figure 2.9). Therefore, the CPE prevalence in veal calves in these member states was inferred from ESBL surveillance in bovine meat (Supplementary 2.7.3 & Table 2.5 & Table 2.6), resulting in a higher CPE prevalence in our calculations for veal calves. Both countries from which a high number of veal calves are imported (N_A) and countries with a high inferred probability that imported veal calf batches are colonized with CPE (P_{CPE_A}) (Supplementary 2.7.10: Table 2.14) have a high risk of CPE introduction. This outcome resembles a risk assessment by EFSA, which concluded that EU member states with higher volumes of livestock trading have a higher risk of disseminating AMR-ESBL bacteria (EFSA, 2013; EFSA, 2011). We believe that the high-risk level expected for veal calves from the model could be an overestimation given the lack of CPE detection in veal calves in EU surveillance (EARS-net). The high prevalence estimates for source countries were thus not based on reported detections but resulted from uncertainty due to low sample sizes. However, CPE cases in cows were detected in European countries (Ibrahim et al., 2016), and imported veal calves were ranked first for risk of CPE in our expert elicitation (Supplementary 2.7.2). The scenario of reducing risk by only allowing

countries that sample more than 100 animals annually to export to the Netherlands was shown to be an effective mitigation strategy in the what-if analysis. The expected number of introductions was reduced by 71%. It should, however, be kept in mind that this strategy reduces the potential CPE introductions resulting from uncertainty in CPE prevalence in veal calves in source countries. Countries with an effective surveillance program in calves that do find CPE in calves might, in reality, pose a higher risk to the Dutch veal calf sector. A more reliable estimate of the CPE introduction risk via imported livestock can be obtained via enacting EU-wide mandatory surveillance with enough samples in all countries exporting veal calves to EU member states.

Humans were initially thought to be a high-risk source because of high numbers of overseas travel and CPE presence in hospitals (Albiger et al., 2015), but the risk of these sources was found to be very low. In spite of a non-zero number of farms exposed to returning travelers and hospitalized patients (the probability of exposure of an individual farm is as high as for imported livestock (Table 2.3)), the extremely small calculated dose of CPE ingested by livestock leads to a very low number of expected colonizations in the exposed farms (Table 2.2). The prevalence of the clinically relevant CPE Klebsiella pneumoniae in humans is slightly higher than CPE E. coli [10]. Only the latter was considered in this risk assessment. Including CPE Klebsiella pneumoniae is, however, not expected to result in a change in the ranking of sources given the huge difference in the estimated risk between feed and imported livestock, on the one hand, and travelers and hospitalized patients, on the other. Likewise, CPE introduction from the companion animal source was assessed to be negligible because there is no exposure of farm animals to colonized companion animals if strict biosecurity is applied. What-if analysis evaluated the effect of reduced biosecurity in farms, where hand hygiene and exclusion of companion animals from the barns were not complied with (Pickering et al., 2010; De Aceituno et al., 2015; Van Dijk, 2020; WIN/Gallup, 2015). This scenario still resulted in a very low number of expected introductions from human and companion animal sources. This is explained by the low number of humans and companion animals attributed per farm and the very low probability of colonization of the farm if exposed to CPE-colonized humans or companion animals.

The outcome of this introduction risk assessment was used to rank farm types and sources of their CPE introduction risk. The results for the absolute numbers of exposures and introductions have a large uncertainty and cannot be viewed as accurate quantitative risk estimates. The results of the sensitivity analysis provide good indications of the uncertain input parameters that have the largest impact on the model results. Parameters with both a large uncertainty and a large impact are important knowledge gaps that can be targeted in future studies. Despite these uncertainties, the ranking of farm types and sources was robust and the outcome of this risk assessment can thus be used for targeted CPE surveillance (World Organisation for Animal Health, 2010; OIE, 2010; De Vos et al., 2004).

2. 5 Conclusions

Feed and imported livestock are expected to pose the highest risk of CPE introduction to pig, broiler, and veal calf farms. Our risk assessment shows that CPE surveillance should focus on broiler and fattening pig farms, given the highest probability of introduction per farm and the highest total number of introductions, respectively. Our model clearly indicates that we currently do not have sufficient information on the CPE presence in sources, i.e., CPE prevalence in humans, animals, and feed, and the CPE concentration in feed, and that this information is essential for the reliability of this risk estimate and for effective risk mitigation. Therefore, the calculated numbers of exposure and introduction cannot be considered as accurate quantitative estimates of the risk. The ranking of farm types for the total number of introductions in each farm type and for the probability of introduction in individual farm types is, however, robust despite the huge uncertainties in input parameters. More surveillance of CPE prevalence in feed and imported animals, especially veal calves, is essential to improve the certainty of the risk assessment. Banning livestock importation from countries that put little effort into CPE surveillance could reduce the risk from imported livestock.

2. 6 Acknowledgement

We would like to thank feed expert Arjan van Dijk (Nevedi) and veal calf expert Peter Mölder (Denkavit) for providing information to estimate input parameters of the model. We highly appreciate the contribution from Nedzib Tafro (NVWA), Heike Schmidt (RIVM), Engeline van Duijkeren (RIVM), Arjan van Dijk (Nevedi), Alex Spieker (Avined), and Dik Mevius (WBVR) in the expert elicitation on *CPE* sources.

2. 7 Supplementary Information

2. 7. 1 Literature review

Publications related to Carbapenemase-producing Enterobacteriaceae were searched through Pubmed with the following search strings:

carbapenem OR carbapenems OR carbapenemase OR carbapenemase-producing OR carbapenemase producing OR carbapenem resistance OR carbapenem-resistant OR carbapenem resistant OR carbapenemase-positive OR VIM OR KPC OR OXA OR NDM

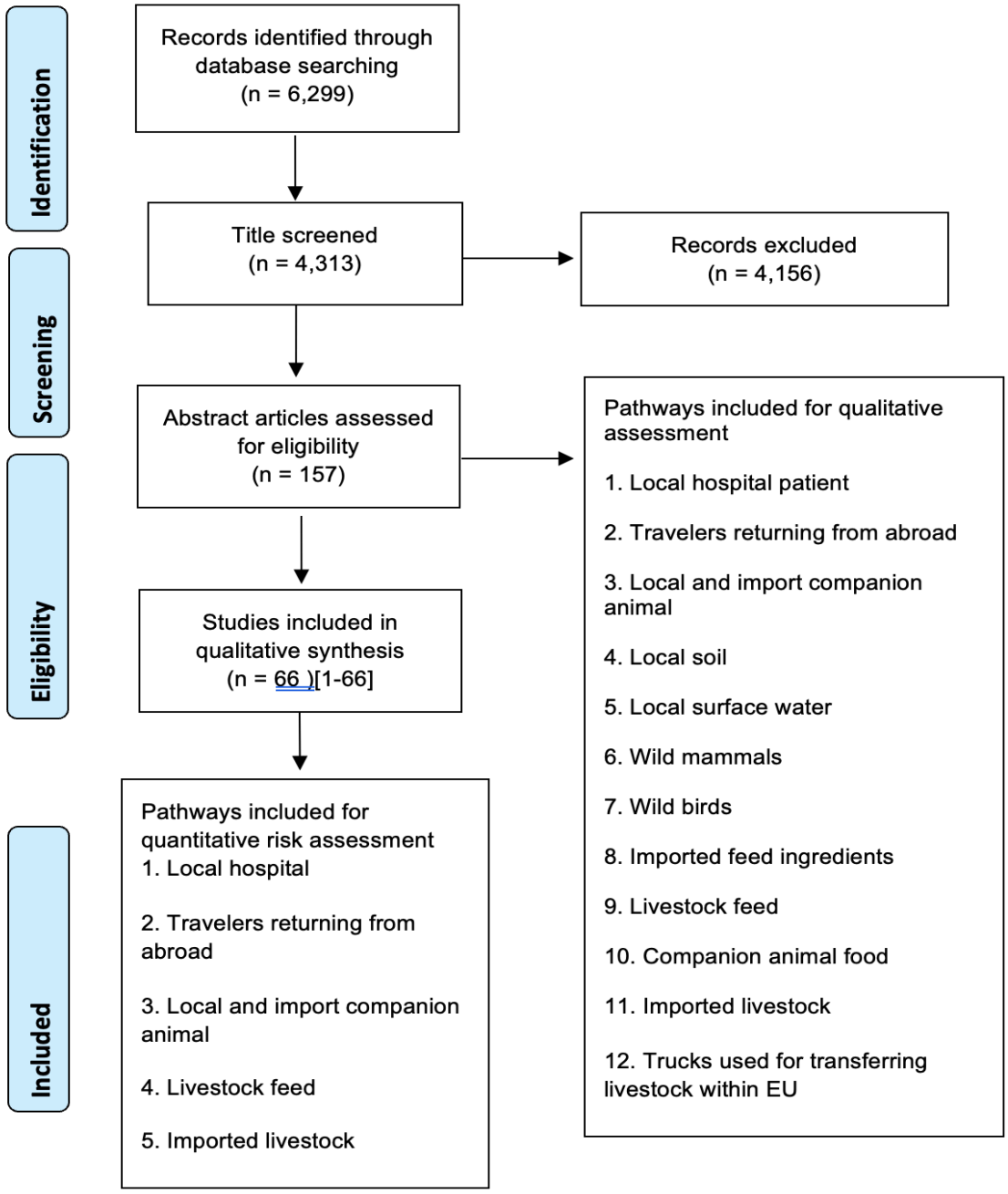

***Figure 2.6**- PRISMA chart indicates the literature review of potential CPE sources*

2. 7. 2 Report expert elicitation in projects “Risk assessment CPE” and “BEWARE”.

Expert elicitation in 3 rounds:

1. Open questions about “reservoirs” of CPE for exposure of Dutch livestock and companion animals
2. Conjoint analyses going more detailed into reservoirs and different regions of origin
3. Workshop to work out pathways in more detail

1. Open questions about “reservoirs” of CPE for exposure of Dutch livestock and companion animals

Aim: Make an inventory of possible reservoirs of CPE (a reservoir is a

Method: Expert names were provided by Dik Mevius and other MRA experts from WBVR. Experts were approached by email. Two questions were asked:
In the first question a list of possible CPE reservoirs, described in literature, was given. In this question the experts were asked if the list was complete. If they thought the list was not complete, they were asked to mention additional possible reservoirs.
The second question was “Which 3 reservoirs do you consider the most important for introduction of CPE in (Dutch*) livestock/companion animals?”. Experts could give an explanation (but were not obliged to do so).
Results: Ten experts sent their answers.

Question 1:
The original list of possible reservoirs in the question:
- Sewage water
- Waste water from waste water treatment plants
- Waste (water) from hospitals,
- Waste (water) from industry
- Animal feed
- Travellers
- Manure
- Imported livestock
- Imported pets
- Imported animal products
- Imported fish, seafood, shellfish

Additional reservoirs mentioned by the experts were (in random order):
- Wild birds, fresh surface water, imported products (vegetables, fruits, spices),
- humans in The Netherlands (not only travellers), animals (not only imported ones)
- pets, animal feed, humans,
- human population, not only imported animal sources, wildlife
-immigrants’ workers and foreign guest workers, wildlife and other environmental sources, environmental bacteria
- you forgot the humans!!
- Dutch residents
- tentatively, treated domestic pigs, human carriers of CPE who work with livestock and poultry

Question 2:
Counting which reservoirs were mentioned as most important by the experts resulted in the following table.

Most important routes by the experts	How many times in top 3?	
Animals		
imported livestock	3	
imported animals	3	
imported animals if not screened	1	
imported animal products	2	
imported pets	1	
pets	1	
		11
Humans		
travellers	3	
humans	1	
humans, including travellers	1	
human carriers	2	
travellers including guest workers	1	
human population (including inhabitants and travellers/visitors)	1	
humans (working in livestock production)	1	
		10
Waste water		
waste water from hospitals	3	
waste water	1	
sewage water or sewage related such as aerosols	1	
waste water from WWTPs	1	
		6
wildlife		1
animal feed		1
raw feed derived from risk countries (the expert means pet food)		1
Total		**30**

Discussion:

It seems that not all experts had understood that, in question 2, they had to think of reservoirs for animals to become infected. Perhaps it was not completely clear to all experts (although it was explained) that we asked for the introduction risk for exposure of animals (and not for The Netherlands or humans).

In the list of reservoirs that we sent to the experts, we only mentioned humans in the form of travellers. The experts added many different human categories as a possible reservoir.

Conclusion “Open questions about CPE reservoirs”:
The experts estimated imported livestock and other animals as most important reservoir, followed by all kind of humans and waste water.
The most important reservoir added by the experts to the original list was humans. These were people having been hospitalized in The Netherlands, and immigrant workers.

2. Conjoint analyses going more detailed into reservoirs and different regions of origin
Aim: to get more insight in the most important reservoirs and regions of origin for possible exposure of Dutch animals with CPE. As the results of part 1 indicated importance of humans, and imported animals, we tried to differentiate more within these categories.
Method:
A questionnaire was sent by the participating experts. This consisted of 3 parts.
In the first part the experts were asked to look at 20 comparisons of 3 combinations of a possible reservoir for CPE’s and a region in the world from which this reservoir originates. For each comparison, experts had to **choose the combination** of reservoir and region which, in their opinion, **leads to the highest probability of exposure of Dutch animals** (livestock, pets) to CPE.
In the second part, the experts were shown two times 8 comparisons. In 2A they had to compare different animals as reservoir, and in 2B different types of humans. Each comparisons showed 3 types of animals (2A) or humans (2B). Experts were asked to choose the reservoir (animal or “type of human”) which they considered the **most important** for introduction of CPE, leading to exposure of Dutch livestock/companion animals, ánd the animal or human that is **least important**.
In the third part experts had to divide 100 points over reservoirs (3A) and regions (3B), so that relative importance of each reservoir or region is shown (more points when more important).
The results of part 1 and 2 were analysed with XLStat in Excel (part 1 in choice based conjoint analysis; part 2 in Maxxdiff analysis). The results of part 3 were put in Excel and average number of added points per reservoir and region were calculated. Reservoirs and regions were ranked, based on averages of points given, but also based on the average of rank numbers per expert.

Results:
Eight experts participated in this step of the expert elicitation.

Part 1: The results showed that regions were considered as more important than reservoirs (68% vs 32%). The experts considered Asia as the region with the highest risk, followed by Africa and southern Europe. The reservoir leading to the highest risk was “waste water from hospitals”, according to the experts, followed by “humans travelling from abroad to The Netherlands” and “water from waste water treatment plants”.

Part 2: The comparison of “different types of humans” as a reservoir resulted in the following ranking:

1. People from abroad immigrating to the NL
2. People from abroad coming to work for a period in the NL

3. People from the NL returning from travel abroad
4. Dutch residents that have recently been or are in hospital or other healthcare institutions
5. People from abroad visiting the NL for holiday/business

The comparison of different imported animals as a reservoir resulted in the following ranking:

1. Veal calves
2. Dogs
3. Pigs
4. Poultry
5. Horses
6. Cats

Part 3: This part was a “check” for consistency of the experts with the answers given in part 1 and 2. Here, 100 points were divided over reservoirs and regions.

We looked at the results in 2 ways: First we calculated the average of the points given by the eight experts per reservoir and per region. That resulted in the following ranking order of reservoirs and regions.

Reservoir	Average	Total
Humans travelling from abroad to the Netherlands	21.25	170
Waste water from hospitals	16.88	135
Water from waste water treatment plants	14.25	114
Imported animal products	12.88	103
Imported livestock	12.63	101
Travelling pets	11.25	90
Humans hospitalized	10.88	87
Total	100.00	

Region	Average	Total
Asia	27.6	221
Africa	19.1	153
Southern Europe	15.5	124
Eastern Europe	11.4	91
Southern America	10.3	82
Western Europe	5.8	46
Northern America	5.6	45
Oceania	3.0	24
Northern Europe	1.75	14
Total	100.0	

Another way of analysing this part was making a ranking per expert, based on the points given (reservoir with most point was ranked 1, and so on), and then we calculated the average rank number. This resulted in:

Reservoir	Rank
Waste water from hospitals	1
Humans travelling from abroad to the Netherlands	2
Imported animal products	3
Travelling pets	4
Imported livestock	5
Waste water from waste water treatment plants	6
Humans hospitalized	7

Region	Rank
Asia	1
Africa	2
Southern Europe	3
Southern America	4
Eastern Europe	5
Northern America	6
Western Europe	7
Oceania	8
Northern Europe	9

We noticed some differences between the results with the different way of analysing. The most important difference is the rank of “waste water from waste water treatment plants”. This was ranked 3 based on average points and rank 6, based on rank number. This was caused by one expert who appointed many points to this reservoir.
For the regions the top 3 and the least important regions did not differ between analysing methods. There were only slight differences in the middle of the ranking list.

Discussion:
Not enough experts participated in this step of expert elicitation to draw significant conclusions. Answers for regions were very much alike; for reservoirs there were more differences between experts.
Conclusion “Conjoint analysis”:
The region of origin of a CPE reservoir was considered more important than the reservoir itself, with Asia and Africa as most important regions. The top 3 of most important reservoirs ánd regions was equal in part 1 (choice base conjoint analysis) and part 3 (giving points to reservoirs), which means that experts were consistent in their answers.
People immigrating from abroad to The Netherlands were considered the most important “type” of

human. The most important imported animal species were veal calves, followed by dogs and pigs.

3. Workshop to work out pathways in more detail

Date: December 3rd, 2018

Location: Utrecht, Faculty of Veterinary Medicine

Present (all Dutch experts):

- Nedzib Tafro, NVWA, importcontroles dieren en dierlijke producten op Schiphol (zendingen uit derde landen).
- Heike Schmidt, Centrum Zoönosen en Omgevingsmicrobiologie, RIVM en Universiteit Utrecht, AMR in water en mest
- Engeline van Duijkeren, clusterleider binnen het Centrum Zoönosen en Omgevingsmicrobiologie, RIVM, transmissie van resistentie tussen dier en mens, zowel food-borne als direct
- Arjan van Dijk, Nevedi, programmamanager veevoer; heeft voorheen bij Nepluvi gewerkt
- Alex Spieker, Avined, coördinatie van gezondheidszorg in diverse programma's, AI, monitoring van ziekten

Dik Mevius, WBVR en UU, projectteam

Arjan Stegeman, UU, projectteam

Natcha Dankittipong, UU, AIO in BEWARE project (spreekt (nog) geen Nederlands)

Jantien Backer, RIVM, projectteam

Manon Swanenburg, WBVR, projectteam

Clazien de Vos, WBVR, projectteam

Introduction by Arjan Stegeman:

Arjan Stegeman presented the aims and design of the BEWARE project. It consists of four workpackages: 1: Introduction risk of AMR (CPE) into Dutch livestock (pigs, poultry, veal calves)
2: Transmission of AMR within and between farms
3: Developing of an assay for sensitive and specific metagenomics detection of CPE
4: Developing of an early detection surveillance framework using a dynamic mathematical model
Next to BEWARE there is another project (WOT, which means it is paid by the government), carried out at WBVR, in which introduction risks of CPE are determined and suggestions for more efficient surveillance will be done. In this project also companion animals (cats, dogs, horses) are included.

Aim of the workshop:

To get more detailed knowledge about CPE reservoirs, and the pathways/routes from reservoirs to Dutch livestock. Another aim is to rank pathways for their importance.

Presentation workpackage 1:

Manon presents the plan for workpackage 1 of BEWARE: make an inventory of all reservoirs and routes that might contribute to the introduction of CPE in animals in The Netherlands. Reservoirs from abroad but also from within The Netherlands are taken into account. The aim for this work package is to rank the pathways, to identify the most important.

Ga je pdf van presentatie ook meesturen?

Introduction round:

Everybody shortly introduces him/herself. Participants attach a yellow sticky paper to the general model to indicate where their expertise is.

Project results until now:

Manon presents results of earlier expert elicitation rounds about reservoirs and introduction routes. There were 2 earlier expert elicitation rounds. This workshop is the 3rd round of expert elicitation. Results of the first and second round are described in this report (see page 2-6).

Some remarks were made in response to the results of the first expert elicitation:

- Arjan: (raw) animal products can only be processed in pet food; in livestock feed, fish meal might be used, but that is not raw (example: PAPS, these have undergone a processing step = risk reduction).
- Heike: waste water and hospital water cannot be distinguished from eachother; they are both processed via waste water treatment plant. The original reservoir of CPE are often humans.
- The participating experts say that they don't see water as a reservoir, but as a pathway. Humans are the reservoir, waste water is the route.
- Better definitions of reservoir and pathway (this was not further worked out during the workshop)

Remarks to the results of the second round (conjoint analysis):

- Engeline: was not able to fill in the conjoint analysis. She missed context and definitions.
- Engeline: the answer depends on how risk is defined. Is it for pigs, calves, etc?
- In the conjoint analysis imported animals/products were ranked as less important than in the first expert elicitation round. The workshop participants think this is logical: people are the most important risk for introduction into The Netherlands, and therefore most probably also for introduction into livestock. CPE has only seldomly been detected in livestock so far. However, imported animals might have the highest risk of having (direct) contact with livestock, but how big is this risk?
- Dik: the region North America is perhaps defined too broad. In the USA many CPE have been found. The Netherlands does not import many animals from the USA, but there is substantial import of horses.

There is a discussion about the variability (between experts) in the answers of the conjoint analysis. Ideally we would like to have more participants, but the question is if that is useful, or that the general trend will look the same.

Active participation of experts, drawing pathways:

The participating experts worked in groups (3-4 persons) to try to draw pathways from reservoir to Dutch livestock and pets. This was done in 3 rounds; per round 2 schemes were drawn (2 sectors). Group members changed each round, so that for each sector the "own" expert was in the group. import of veal calves was originally planned, but was not done, because the veal calf expert could not come. Manon will ask him to help with that on a later occasion.

Other pathways that will not be worked out during the workshop will be checked by experts who could not come, like the manure pathway (Paul Hoeksema). Arjan also suggests to contact Cumela (?) for manure.

Round 1: import pigs and import poultry
See photo for the results
Extra notes/remarks (not in the schemes):
- There is no testing in pigs for AMR (did they mean at the border or in general??)
- There are no health criteria/demands for AMR at import of animals from EU or 3rd countries.
- Most countries do not have surveillance for CPE. Therefore prevalences in animals are unknown.
- From 3rd countries only import of breeding material (does that mean sperm, ova, or also breeding animals??)
- Is genetic material a risk for AMR/CPE transmission? (antibiotics are added to sperm, gentacide (??)).
- Do pigs come via “collecting locations”? In the Netherlands we don’t have them anymore. What about other countries? Check with sector/NVWA.
- Imported pigs are going to the slaughterhouse or a farm in The Netherlands. From the slaughterhouse CPE/AMR can spread to humans via direct contact (slaughterhouse personnel) or consumption of animal products. There is also waste water that can go into the environment.
Side remark (other subject): Nedzib considers import of ornamental fish and fish products and shellfish a high risk. In water of ornamental fish many antibiotics were found (project with Olga Haenen). The water that is imported with the fish is discharged into the drain/sewer in The Netherlands. At Schiphol, CPE have been found in fish products. Dozens of consignments of fish products a day are imported.
Round 2: water and imported feed
Extra notes for water:
- Households and hospitals discharge their water at the same WWTP (waste water treatment plant)
- Water of WWTP is discharged on rivers (surface water); this can also be small rivers; water in sloten (little canals between grass land) is also partly originating from rivers.
- Overflow drain/sewer
- Households with separated waterflow: in about 2% the connection is constructed wrongly, and the waste water comes directly into the surface water.
- Also surface water from abroad via rivers.
- surface water
- Surface water is mainly drunk by animals that stay outside: horses, dairy cattle (partly), sheep. Poultry always gets tap water (strict rules for drinking water), and also pigs and veal calves. Tap water can originate from an own well, but in general this water is clean (filtration by sand).
- Drinking water from the tap almost contains no risk, after treatment for drinking water production.
- Exposure to CPE via surface water also for pets and humans (direct contact, taking in). Indirect exposure via humans to livestock. Pets can also contaminate the surface water.
- CPE are in surface water already; source is humans
- According to an ESBL study: waste water contributes to 60% of the risk (human risk??)
- Travellers

Reizigers veel groter risico voor CPE en ESBL (vormen zij een risico, of lopen zij een risico?). Groter risico dan

- Exposure from surface water is in general low; low concentrations, not much drinking from it, intake is only few CFU per intake. Meat is a much higher risk for humans.
- Dik: “evolutionary risks” (“evolutionaire risico’s”) --> in case of CPE surface water might play a role, because it is not spread widely. But for ESBL’s the contribution of surface water is very low, because other sources became more important.

Round 3: Travellers, import manure and import of pets (as an extra)

Notes for travellers:

- Two groups of people: general population and people who visit farms/work at farms professionally. In this last category we can distinguish between people who only come at one or a low number of farms (farmers, agricultural workers) and people who visit many farms (for example veterinarians).
- The general population has direct contact with pets and animals at “kinderboerderijen” and “zorgboerderijen” .
- The professional workers have direct contact with livestock
- The other route from travellers to livestock is from travellers via surface water to animals.
- risk depends on type of traveller: from which country, hospitalized or not, length of travel,
- It is assumed that the probability of becoming a carrier is higher if you have been a longer period abroad.
- Having “travellers diarrhoea”(with and without treatment) is a risk for being ESBL carrier (I think it was meant to say that it is a risk factor).
- Migrants that regularly travel to and from their home country are considered as a bigger risk.
- Travellers (migrants, many from eastern Europe) working in slaughterhouses might be a risk for contaminating the meat. This is a delicate point (ethnicity of slaughterhouse personnel). This cannot be externally communicated.
- When people are hospitalized, it is not checked if they have been abroad (it is asked if they have been in a hospital abroad or if they have been in contacts with pigs). Having been abroad is a high risk and should be part of the protocol.

<u>Completion</u>

The participants are asked to (again) list a top 3 of reservoirs/pathways that have the highest risk for introduction/exposure of CPE in Dutch animals. This top 3 is separately made for livestock and for pets, and is written on yellow sticky papers, which are attached to the pathways that were drawn during the workshop. The results were not analysed during the workshop.

After the workshop we analysed the results of these rankings.

For livestock the most often mentioned pathways were 1. Water, 2. Import poultry, 3. Travellers (these were also the 3 pathways with the lowest average ranking number).

For pets the most often mentioned pathways were: 1/2 (equal). Import pets/import pet food, 3. Travellers (import pets and pet food had the lowest average ranking number).

<u>Evaluating discussion</u>

Pathways have been sketched. When starting to work them out in a risk model it will probably turn out that more steps per block are needed. Getting real data for filling the model will be a problem in many cases.
Another point for discussion is Wat doe je met de impact van waar de CPE terechtkomt?
Drawing the pathways was a useful exercise, especially because of the presence of different expertises. The pathway of the veal calf sector has to be made. This sector is considered as a bigger risk for introduction of CPE than the poultry or pig sector.
Conclusion:
Water is a very important factor in the spread/transmission of CPE/AMR after introduction into The Netherlands. In many pathways it is part of the risk. Water in itself is not a reservoir. (The environment was often mentioned as a reservoir; we did not discuss what to do with it).

2. 7. 3 veal calves' CPE sample size inference

For countries that had no data on surveillance in calves, prevalence estimates were based on surveillance in bovine meat. In this approach we assumed that ratios of ESBL prevalence between veal calves and in bovine meat of individual MS were similar within the same EU regions and that the ratios of CPE prevalence between veal calves and bovine meat were similar to the ratios of ESBL prevalence between veal calves and bovine meat. Consequently, we inferred the number of veal calves sampled for CPE in MS by comparing **ESBL** prevalence in veal calves to ESBL in bovine meat from available MS:

$$N_{CPE\,sampleVC} = N_{CPE\,sampleM} * \frac{P_{ESBL_M}}{P_{ESBL_{vc}}}$$

Where $N_{CPV\,sampleVC}$was the expected number of veal calve samples collected to monitor CPE in individual Member States, $N_{CPE\,sampleM}$ was the number of bovine meat samples collected to monitor CPE in individual Member States, P_{ESBL_M} was the proportion ESBL positive in bovine meat detected in Member States, and $P_{ESBL_{vc}}$was the proportion ESBL positive in veal calves detected in all available Member States denoted [1].

In both CPE and ESBL surveillance, only 9 EU Member States and 2 EFTA countries have monitored ESBL and CPE in veal calves. Sample size and number of ESBL positive in bovine meat and veal calves from individual Member States (with available veal calve sample) was pooled together based on UN geoscheme regions (West, South, East, and North), P_{ESBL_M} and $P_{ESBL_{vc}}$ (Table 2.5). We used this pooled data based on the same regions to infer sample sizes for veal calves for countries that did not collect any samples from veal calve. For countries in East region, we used the pooled prevalence of ESBL in calves in the other regions because no veal calf samples were collected in any countries of the region (Table 2.6).

Table 2.5- *Proportion ESBL positive in bovine meat and in calves and their ratio for 4 UN regions in EU. Regions highlighted blue are regions with some available veal calves data. East EU data derived from pooled data from all regions*

Regions	P_{ESBL_M}	$P_{ESBL_{vc}}$	$P_{ESBL_M}/P_{ESBL_{vc}}$
West EU	0.023127753	0.47554698	0.049
South EU	0.078698846	0.49366086	0.159
North EU	0.020304569	0.18801997	0.108
East EU	0.038392857	0.4316652	0.089

Table 2.6- *CPE sample size in veal calves inferred from ESBL samples. From left: a) EU region b) EU Member States and EFTA countries c) bovine meat samples collected from each country d) veal calve samples collected from each country, Green: countries with no veal calves sampled and was inferred by multiplying samples collected in bovine meat with* $\frac{P_{ESBL_M}}{P_{ESBL_{VC}}}$.

		CPE Sample size	
Regions	Countries	bovine meat	<1 yr calf
West	Austria	297	303
West	Belgium	300	300
West	France	302	299
West	Germany	399	349
West	Luxemburg	26	1
West	Netherlands	486	302
West	Switzerland	299	304
East	Bulgaria	150	13
East	Czech republic	301	27
East	Hungary	184	16
East	Poland	300	27
East	Romania	146	13
East	Slovakia	150	13
South	Croatia	369	354
South	Cyprus	139	22
South	Greece	62	10
South	Italy	272	319
South	Malta	300	48
South	Portugal	220	289
South	Slovenia	151	24
South	Spain	300	300
North	Estonia	150	38
North	Finland	324	315
North	Ireland	300	32
North	Latvia	149	16
North	Lithuania	150	16
North	Sweden	286	31
North	United Kingdom	314	34
North	Iceland	95	10
North	Norway	343	303
North	Denmark	292	297

2. 7. 4 Community – Clinical prevalence

Probability of acquiring CPE from the community versus clinical during holiday was expected to be different since sources of CPE (patients) in the hospital setting is more saturated than CPE sources in the community (healthy adults, food contamination), and exposure time to CPE sources would be different. Given that we only have prevalence data from the clinical setting, we inferred community prevalence by a) using correlation between community and clinical setting in ESBL, b) using colonization period and travel times to narrow down exposure period of community CPE.

The probability of acquiring CPE from the clinical setting, $\boldsymbol{P_{CPE_m}}$, is $BETA(Positive_{pooled} + 1, Sample_{pooled} - Positive_{pooled} + 1)$ and the probability of acquiring CPE from the community was inferred from $\boldsymbol{P_{CPE_m}}$ multiplied by the correlation coefficient of ESBL in the community versus ESBL in the clinical setting, $\boldsymbol{CoefESBL_{com:cli}}$, (0.79) because we assumed the correlation between CPE from the community and clinical prevalence would follow the trend of ESBL.

The 77orrelateon coefficient of ESBL in the community versus ESBL in the clinical setting was calculated using 5 publications from EU Member States (Table 2.7). We applied clinical and community prevalence from the same year in the Pearson correlation test to calculate the correlation between community ESBL and clinical ESBL.

Table 2.7*- ESBL prevalence in community and clinical setting collected from the literature review.*

Regions	Countries	Years	Clinical prevalence	Community prevalence	References
EU_east	Czech republic	2010	2.1	0.4	[2]
EU_north	Ireland	2006	0.0	0.0	[3]
EU_north	Ireland	2007	0.0	0.0	[3]
EU_north	Ireland	2008	0.0	0.0	[3]
EU_north	Ireland	2009	0.0	0.0	[3]
EU_north	Ireland	2010	0.0	0.0	[3]
EU_north	Ireland	2011	0.0	0.0	[3]
EU_north	Ireland	2012	0.1	0.0	[3]
EU_north	Ireland	2013	0.1	0.0	[3]
EU_north	Ireland	2014	0.1	0.0	[3]
EU_west	Belgium	2006	0.2	0.1	[4]
EU_west	Belgium	2008	0.2	0.0	[5]
EU_west	Denmark	2009	0.1	0.0	[6]

Apart from the lesser probability of acquiring community CPE, we also calculated the exposure duration to community CPE. While exposure duration to nosocomial CPE would be total time spent in the hospital, the exposure duration to community CPE would be the total time spent on holiday per year. We calculated CPE incidence rate per day, which was the prevalence of CPE in hospital, $Prev_{CPE_m}$,

divided by average duration of colonization, $T_{days_{CPE}}$. We then multiplied this incidence rate by the average number of days of holiday abroad taken by Dutch citizens, T_{travel}.

$$P_{cCPE_m} = \frac{\boldsymbol{P_{CPE_m}} * CoefESBL_{com:cli}}{T_{days_{CPE}}} * T_{travel}$$

2. 7. 5 Estimated CPE in local and imported companion animals

We estimated the number of local companion animals (dog, cat) in the Netherlands, including housed animals and stray animals using data reported by the National Institute for Public Health and the Environment (RIVM) and Wageningen University & Research reports (Van Heijst et al., 2015; Radstake, 2016). We further estimated the imported companion animals from other EU regions, including stray animals and animals from commercial breeders, using reports from the Stray Animal Foundation and BUZhonden website(Radstake, 2016; Platform, 2022). To estimate the number of CPE-colonized companion animals in the farms with animal *i,* we first calculated the number of CPE-colonized companion animals in the Dutch companion animal population. Total number of local companion animals was multiplied by human CPE prevalence in the Netherlands, while the imported number of companion animals from EU regions was multiplied by human CPE prevalence of the same EU regions. Furthermore, we calculated the prevalence of CPE in companion animals of different sources (local & imports) by dividing the number of CPE-colonized companion animals by total companion animals. Subsequently, we estimated the number of farms with CPE-colonized companion animals (of different origin) by multiplying the individual CPE prevalence of different origin by the number of farms with companion animals.

Table 2.8- *Components for calculation of N_{colC_l} (preliminary). From left, a) Companion animals' countries of origin, b) CPE prevalence in humans, c) estimated number of colonized companion animals in Dutch companion animal population, d) estimated number of colonized companion animal in farms.*

Origin	No. dogs in NL	CPE_prev_human	No. colonized dogs	No. farms with colonized dog
NL	1,500,000	0.0011 (0.0004, 0.002)	1,628 (587, 2359)	12 (4, 18)
East	83,348	0.0004 (0.0004, 0.001)	35 (34, 114)	0 (0, 1)
West	20,202	0.0008 (0.0006, 0.001)	17 (14, 22)	0
South	7,616	0.0030 (0.002, 0.0034	23 (18, 27)	0
Origin	No. cats in NL	CPE_prev_human	No. colonized cats	No. farms with colonized cat
NL	2,299,566	0.0011 (0.0004, 0.002)	2,495 (900, 3617)	13 (7, 28)
East	120,221	0.0004 (0.0004, 0.001)	50 (49, 164)	0 (0, 1)
West	30,000	0.0008 (0.0006, 0.001)	25 (20, 32)	0
South	664	0.0030 (0.002, 0.0034)	2 (2, 2)	0

2. 7. 6 Model Input

Table 2.9- *Inputs to estimate the number of farms exposed to CPE.*

Parameters	Input parameter	Value (default)	Unit	Data source
N_{farm_i}	Number of broiler farms in the Netherlands	625	Farms	(Statline, 2020)
	Number of broiler breeder farms in the Netherlands	272	Farms	
	Number of pig-fattening farms in the Netherlands	2910	Farms	
	Number of pig-breeding (with piglets) farms in the Netherlands	1196	Farms	
	Number of farrow to finish pig farms in the Netherlands	640	Farms	
	Number of veal calves fattening farms in the Netherlands	1667	Farm	
N_{animal_i}	Number of broilers in the Netherlands	45230035	Animals	(Statline, 2020)
	Number of broiler parents in the Netherlands	8815525	Animals	
	Number of fattening pigs in the Netherlands	5211511	Animals	
	Number of breeding pigs in the Netherlands	1129564	Animals	
	Number of veal calves in the Netherlands	898107	Animals	
N_{vet_i}	Number of veterinarians working with broilers	109	Veterinarians	(Statline, 2020)
	Number of veterinarians working with parent broilers	109	Veterinarians	
	Number of veterinarians working with fattening pigs,	275	Veterinarians	
	Number of veterinarians working with breeding pigs and piglets	275	Veterinarians	
	Number of veterinarians working with farrow to finish farm	275	Veterinarians	
	Number of veterinarians working with veal calves	155	Veterinarians	
P_{T_m}	Proportion of Dutch citizens traveling to South Asia	0.003	Fraction	(Statline, 2020; Arcilla et al., 2017)
	Proportion of Dutch citizens traveling to Central and East Asia	0.005	Fraction	
	Proportion of Dutch citizens traveling to Western Asia	0.060	Fraction	
	Proportion of Dutch citizens traveling to Northern Africa	0.023	Fraction	
	Proportion of Dutch citizens traveling to Southeast Asia	0.014	Fraction	

Parameters	Input parameter	Value (default)	Unit	Data source
P_{T_m}	Proportion of Dutch citizens traveling to Central America and Caribbean	0.012	Fraction	(Statline, 2020; Arcilla et al., 2017)
	Proportion of Dutch citizens traveling to Central and Eastern Africa	0.003	Fraction	
	Proportion of Dutch citizens traveling to western Africa	0.004	Fraction	
	Proportion of Dutch citizens traveling to Southern America	0.004	Fraction	
	Proportion of Dutch citizens traveling to Southern Africa	0.003	Fraction	
	Proportion of Dutch citizens traveling to Western Europe	0.430	Fraction	
	Proportion of Dutch citizens traveling to Southern Europe	0.243	Fraction	
	Proportion of Dutch citizens traveling to Northern Europe	0.064	Fraction	
	Proportion of Dutch citizens traveling to Eastern Europe	0.022	Fraction	
P_{CPE_m}	Probability of acquiring CPE in a hospital in Southern Asia	BETA (4,587, 22,205)	Fraction	(WHO, 2014 ; Khan et al., 2020; Castanheira et al., 2011; Mohanty et al., 2011 ; Khajuria et al., 2014; Murali et al., 2015; Hsu et al., 2017; Nahid et al., 2017)
	Probability of acquiring CPE in a hospital in Central and Eastern Asia	BETA (11,879, 215,059)	Fraction	(WHO, 2014; Liu et al., 2018; Lie et al., 2012)
	Probability of acquiring CPE in a hospital in Western Asia	BETA (1,868, 19,110)	Fraction	(WHO, 2014; Ben-David et al., 2012; Schwaber et al., 2008; Johani et al., 2010)
	Probability of acquiring CPE in a hospital in Northern Africa	BETA (34, 610)	Fraction	(WHO, 2014)
	Probability of acquiring CPE in a hospital in Southeast Asia	BETA (18,066, 261,116)	Fraction	(WHO, 2014; Rimrang et al., 2012; Balm et al., 2013; Tran et al., 2015; CPE Thailand, 2018)
	Probability of acquiring CPE in a hospital in Central America and Caribbean	BETA (538, 17,162)	Fraction	(WHO, 2014)
	Probability of acquiring CPE in a hospital in Central and Eastern Africa	BETA (3, 1,056)	Fraction	(WHO, 2014)

Parameters	Input parameter	Value (default)	Unit	Data source
P_{CPE_m}	Probability of acquiring CPE in a hospital in Western Africa	BETA (10, 107)	Fraction	(WHO, 2014)
	Probability of acquiring CPE in a hospital in Southern America	BETA (729, 13,172)	Fraction	(WHO, 2014; Luci Correa et al., 2013)
	Probability of acquiring CPE in a hospital in Southern Africa	BETA (851, 1,554)	Fraction	(WHO, 2014; Singh-Moodley et al., 2016)
	Probability of acquiring CPE in a hospital in Western Europe	BETA (113, 66,129)	Fraction	(ECDC, 2017)
	Probability of acquiring CPE in a hospital in Southern Europe	BETA (2,066, 28,171)	Fraction	(ECDC, 2017)
	Probability of acquiring CPE in a hospital in Northern Europe	BETA (53, 58,021)	Fraction	(ECDC, 2017)
	Probability of acquiring CPE in a hospital in Eastern Europe	BETA (437, 13,888)	Fraction	(ECDC, 2017)
	Probability of acquiring CPE in a hospital in Northern America	BETA (998, 9,979)	Fraction	(WHO, 2014; Iregui et al., 2018; Patel et al., 2008))
	Probability of acquiring CPE in a hospital in Oceania	BETA (192, 2,925)	Fraction	(WHO, 2014)
c_{a_i}	Grams consumed per broiler per day	79	Average grams	(Animals, 2005)
	Grams consumed per broiler parent per day	39	Average grams	(Animals, 2005)
	Grams consumed per fattening pig per day	3,500	Average grams	(Rönnqvist et al., 2018)
	Grams consumed per sows per day	4,000	Average grams	(Rönnqvist et al., 2018)
	Grams consumed per rose veal calf per day	3,917	Average grams	(Bussel, 2020)
	Grams consumed per blank veal calf per day	1,429	Average grams	(Van Doremalen et al., 2014)

2. 7. 7 Queries to retrieve import livestock of interest from cbs.nl

URL: https://opendata.cbs.nl/statline/portal.html

➢ Click “Kies thema” at the top of the page

➢ Click the following options internationale handel> handel; goederen> goederensoorten, landen per jaar> natuur, voeding en tabak; jaar

➢ Click "Preview data" then Select the following animal species from the drop down “goederensoorten natuur, voeding en tabak”

0102291000: Cattle, live, with a weight of <= 80 kg (excl. pure-bred breeding cattle)

0102900500: Cattle/ domestic animals/live weighing <= 80 kg (excl. pure-bred breeding animals)

0103100000: Pure-bred breeding pigs

0103911000: Pigs/ domestic animals/ live pigs weighing <50 kg (excl. pure-bred breeding animals

0103921100: Sows /domestic animals /live ..."who have farrowed at least once, weighing> = 160 kg (excl. pure- bred breeding animals)

0105111900: Female breeding chicks of chickens/ poultry/weighing <= 185 g (excl. those of laying breeds)

0105119900: Roosters and chickens/ poultry/ weighing <= 185 g (excl. those of laying breeds and excl. female and breeding chicks)

➢ Specify imported animals by select drop down “Onderwerp” > Invoerhoeveelheid

Specify countries of import (European Member states in our analysis) by select drop down “Landen”

2. 7. 8 One-at-a-time sensitivity analysis on introduction

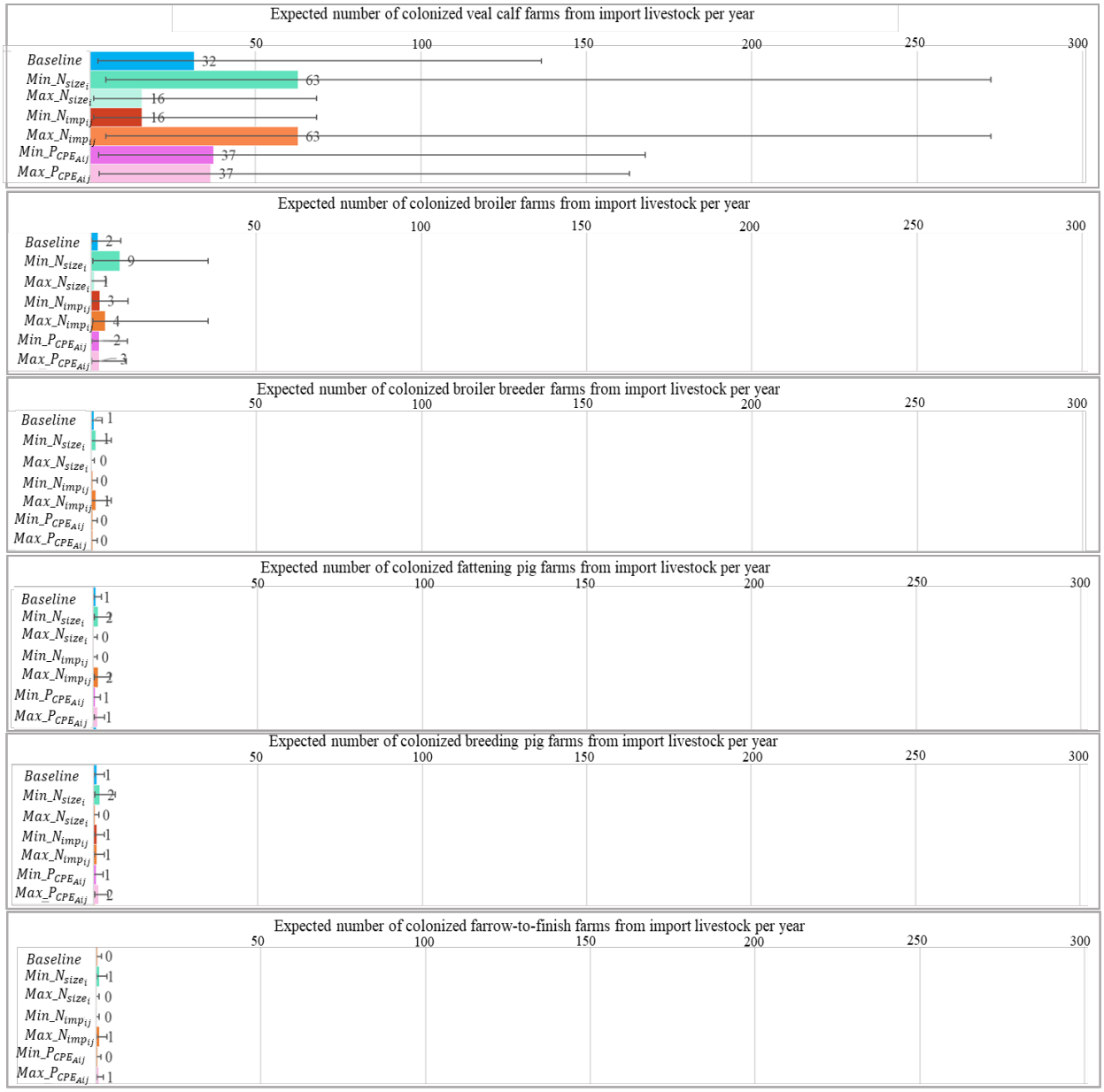

***Figure 2.7**- One-at-a-time sensitivity analysis from livestock import source. One input was discounted or raised two-fold in each round. The resulting number of introductions is shown here.*

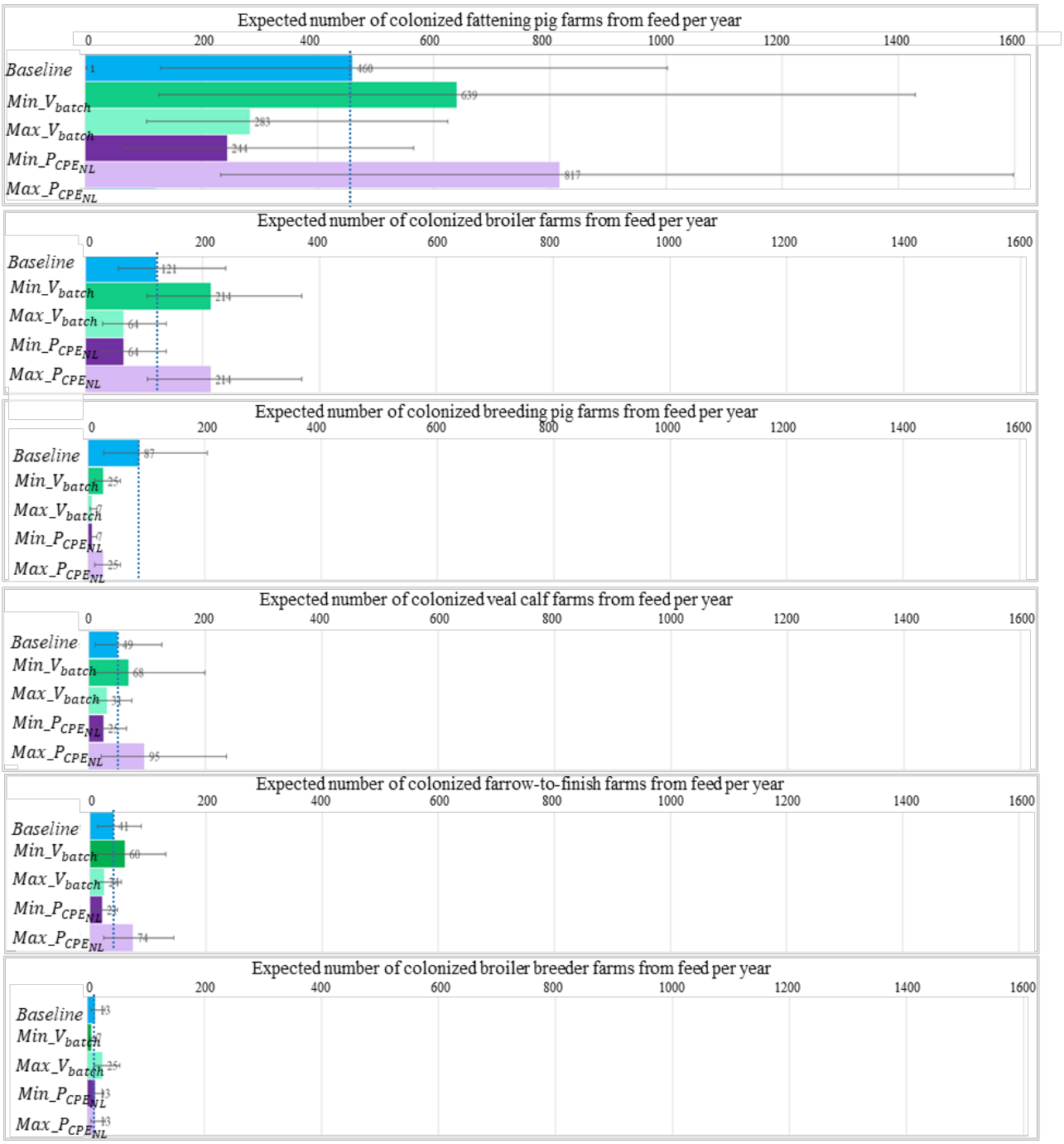

Figure 2.8- One-at-a-time additional parameters sensitivity analysis of feed source. One input was discounted or raised two-fold in each round. The resulting number of introductions is shown here.

2. 7. 9 Result of one-at-a-time between sources sensitivity analysis on introduction feed, imported livestock, companion animal, returning traveler, and hospitalized patients.

In every source, one input parameter was adjusted in each test and the resulting number of introductions were compared between sources.

***Table 2.10**- Total number of tests runs in which one parameter was discounted or increased two-fold.*

Sources	Total number of test runs	Number of tests in each farm type	Number of introductions in baseline model	Number of introductions in the least risk model	Number of introductions in the highest risk model
Feed	90	15	777	408	1,407
Import	42	7	44	22	87
Companion	102	17	0	0	3
Traveler	156	26	0	0	0
Hospital	156	26	0	0	0
Total	546	91	821	430	1,497

***Table 2.11-** Comparison of introduction between livestock feed and imported livestock. Livestock feed remain a higher risk source than import livestock except few tests in veal calf farms.*

	Total number of sensitivity tests	Number of tests where ranking remains the same	Probability of rank remains unchanged
Broiler	104	104	1
Broiler breeder	104	104	1
Fattening pig	104	104	1
Breeding pig	104	104	1
Farrow-to-finish	104	104	1
Veal calf	104	58	0.56
Total	624	578	0.93

Table 2.12- *Comparison of introduction between import and returning traveler sources. Imported livestock remain a higher risk source than returning traveler. Though, few test resulted in zero introduction from imported livestock to broiler breeder and farrow-to-finish farms which lower the high rank of imported livestock to low.*

import/ traveler						
	Total number of tests	Number of tests where ranking remains the same	Number of tests where ranking changes	Probability of rank remaining unchanged	Probability of rank changes	Probability of outcome from both sources equal/close to zero
broiler	182	182	0	1	0	0
broiler breeder	182	52	130	0.29	0.71	0.71
fattening pig	182	182	0	1	0	0
breeding pig	182	182	0	1	0	0
farrow-to-finish	182	104	78	0.57	0.43	0.43
veal calf	182	182	0	1	0	0
Total	1092	884	208	0.81	0.19	0.19

Table 2.13- *Comparison of introduction between import and companion animal sources. Imported livestock have significant probability to produce small introduction in broiler breeder and farrow-to-finish farms that is equal to introduction from companion animals. Proportion of time companion animal spends in barn (P_{barnC}) is the input parameter that started introduction from companion animal.*

import/ companion						
	Total number of tests	Number of tests where ranking remains the same	Number of tests where ranking changes	Probability of rank remaining unchanged	Probability of rank changes	Probability of outcome from both sources are equal (mostly close to zero)
broiler	119	119	0	1	0	0
broiler breeder	119	34	85	0.28	0	0.72
fattening pig	119	115	4	0.96	0	0.04
breeding pig	119	115	4	0.96	0	0.04
Farrow to finish	119	68	51	0.57	0	0.43
veal calf	119	119	0	1	0	0
Total	714	570	144	0.79	0	0.20

2. 7. 10 Introduction from imported livestock to veal calf farms

Table 2.14*- Top six countries with the highest number of introductions from imported livestock to veal calf farm.* $N_{intro_{s,i}}$ *is the median expected number of farms with introduction with upper and lower 95th percentile in brackets,* $N_{A_{ij}}$ *is number of imported batches of animals,* $P_{CPE_{A_{ij}}}$ *is the expected CPE prevalence estimated at the upper limit. Highlighted boxes are input with the top-five highest values.*

Member States	$N_{intro_{s,i}}$	$N_{A_{ij}}$	$P_{CPE_{A_{ij}}}$
Germany	10 (1, 43)	3293	0.001
Latvia	9 (1, 39)	151	0.116
Ireland	6 (0, 27)	211	0.033
Czech republic	4 (0, 28)	114	0.040
Lithuania	3 (0, 13)	54	0.037
Estonia	2 (0, 11)	93	0.065
Belgium	1 (0, 6)	370	0.002

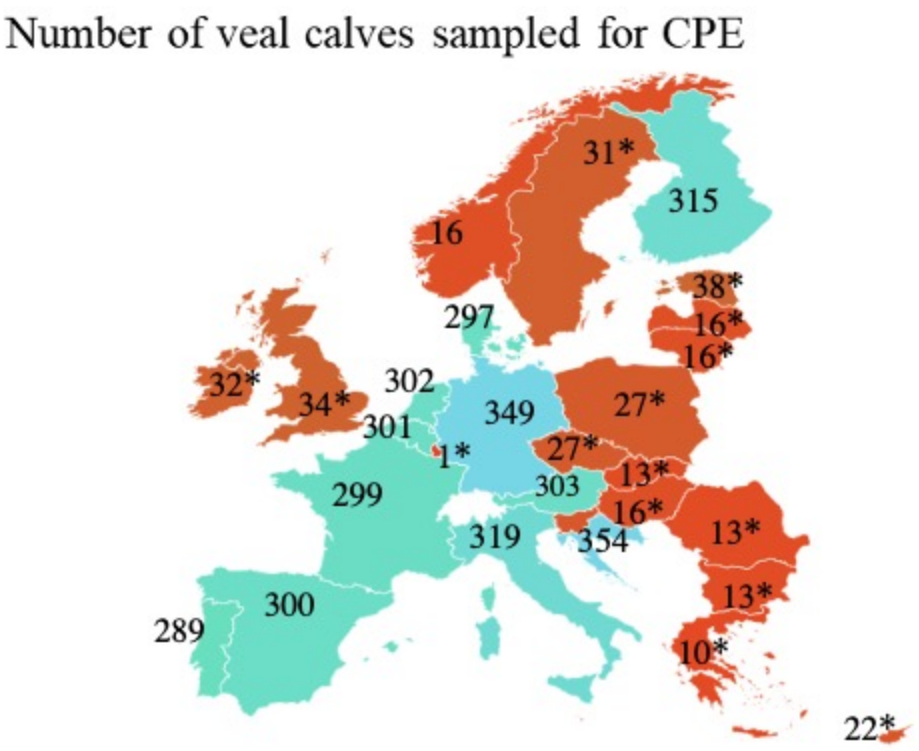

Figure 2.9- *Number of veal calves sampled in the import countries of origin reported by EARS-Net 2018. Number with * is the number of animals inferred from ESBL data in veal calf and bovine meat (Supplementary 2.7.3). All countries reported zero positive veal calf.*

Chapter 3

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

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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 5·102 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 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.

Keywords: Transmission experiment, S-I model, broiler chickens, Bayesian hierarchical model, Carbapenemase-producing Enterobacteriaceae, Extended-Betalactamase Enterobacteriaceae, Microbiome analysis

3. 1 Introduction

Carbapenemase-producing Enterobacteriaceae (CPE; also referred to as carbapenem-resistant Enterobacteriaceae) are potentially lifethreatening 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 (Kock ¨ 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 (Kock 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; Rob´e 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 blaCTX-M-1 and blaCMY-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.

3. 2 Material and method

3. 2. 1 Transmission experiment

The study protocol was approved by the local Animal Experiments Committee and all procedures were performed in full compliance with all legislation. All broilers were observed daily, and any abnormality and mortality were recorded.

3. 2. 1. 2 Inoculums

Three inoculums were prepared for this experiment, referred to as the CPE-strain, ESBL-strain, and catA1-strain throughout the paper (Table 3.1). 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 3.18 – 3.20). 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.

Table 3.1- *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)

3. 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 3.4 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 2,563 kcal of apparent metabolizable energy per kg and 20% of crude protein heated to 90 °C. From days 2 to 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 (Figure 3.1). 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 hours after inoculation on day 5, twice on day 6 (8 hours apart), and once per day on days 7 to 10, 12, and 14 (Figure 3.1) (Dame-Korevaar et al., 2020a). All broilers were euthanized on day 14 and their caecal content was collected for microbiome analysis.

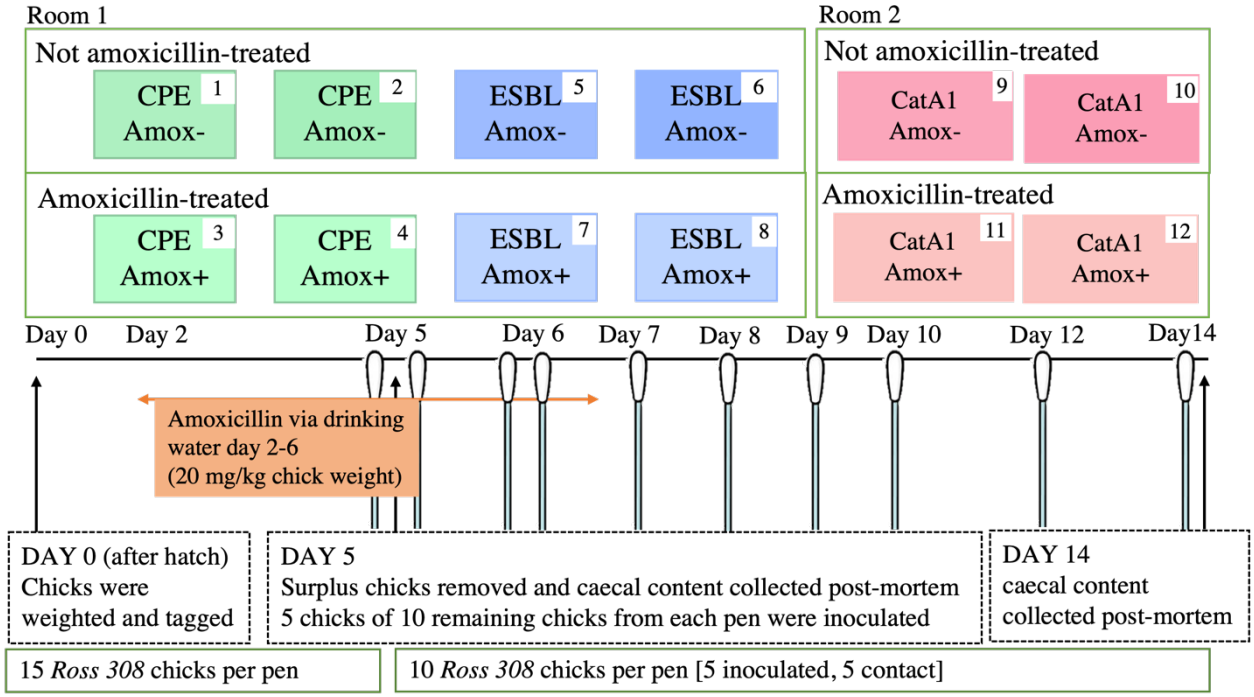

***Figure 3.1**- Setup of the pens (top) and timeline of the experimental design from the moment of hatch (day 0) to the end of the experiment on day 14, with the sampling time points indicated by the swabs (bottom). Abbreviations: Amox-: non-amoxicillin-treated; Amox+: amoxicillin-treated.*

3. 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 3.2).

3. 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 Figure 3.9 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 16S 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 minutes, 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 minutes. 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.

3. 2. 2. Data analysis

3. 2. 2. 1 SI- and SIS-models

The transmission of *E. coli* between broilers was modelled using a compartmental susceptible-infectious model (SI-model;Figure 3.2) 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 $\beta\, S_t\, E_t$, 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 3.3 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 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.

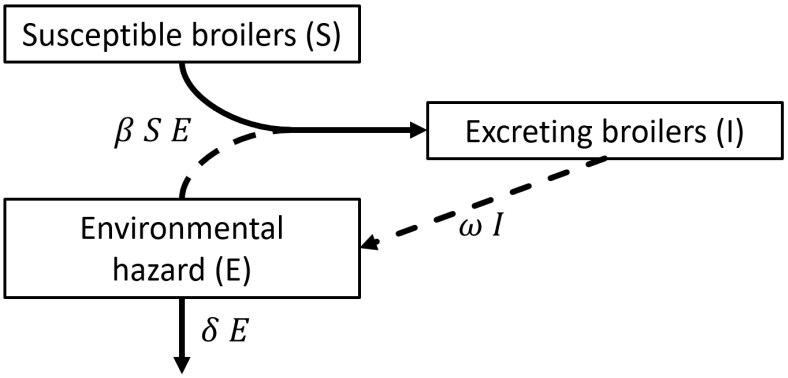

Figure 3.2: *compartmental SI-model of indirect transmission of E. coli between broilers. Excreting broilers (I; positive inoculated broilers and positive contact broilers) excrete bacteria into the environment at rate ω. Only negative contact broilers are counted as susceptible broilers (S) because 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.*

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 3.16) to find a suitable range of decay rates and ran the hierarchical model with several fixed decay rates ranging from 0.04 – 55 h^{-1} (Table 3.17). 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.

3. 2. 2. 2 Bayesian hierarchical inference

A Bayesian hierarchical model was used to infer the parameters of the SI-model (see section 3.7.3.2 '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 ($\bar{a}$) 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 ($\bar{a} + z_i$) 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 $\bar{a} + z_i$ 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 ($\bar{a}$) 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 (Figure 3.19) 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.

3. 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; Figure 3.10) 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 (Figure 3.11). 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.

3. 2. 2. 4 Used software

Transmission data were analysed with R version 4.1.2 (R Core Team, 2021) 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 Češnovar, 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. 3 Results

3. 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 3.2). 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. 3. 1 Transmission rates

3. 3. 1. 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 (Figure 3.3). 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.

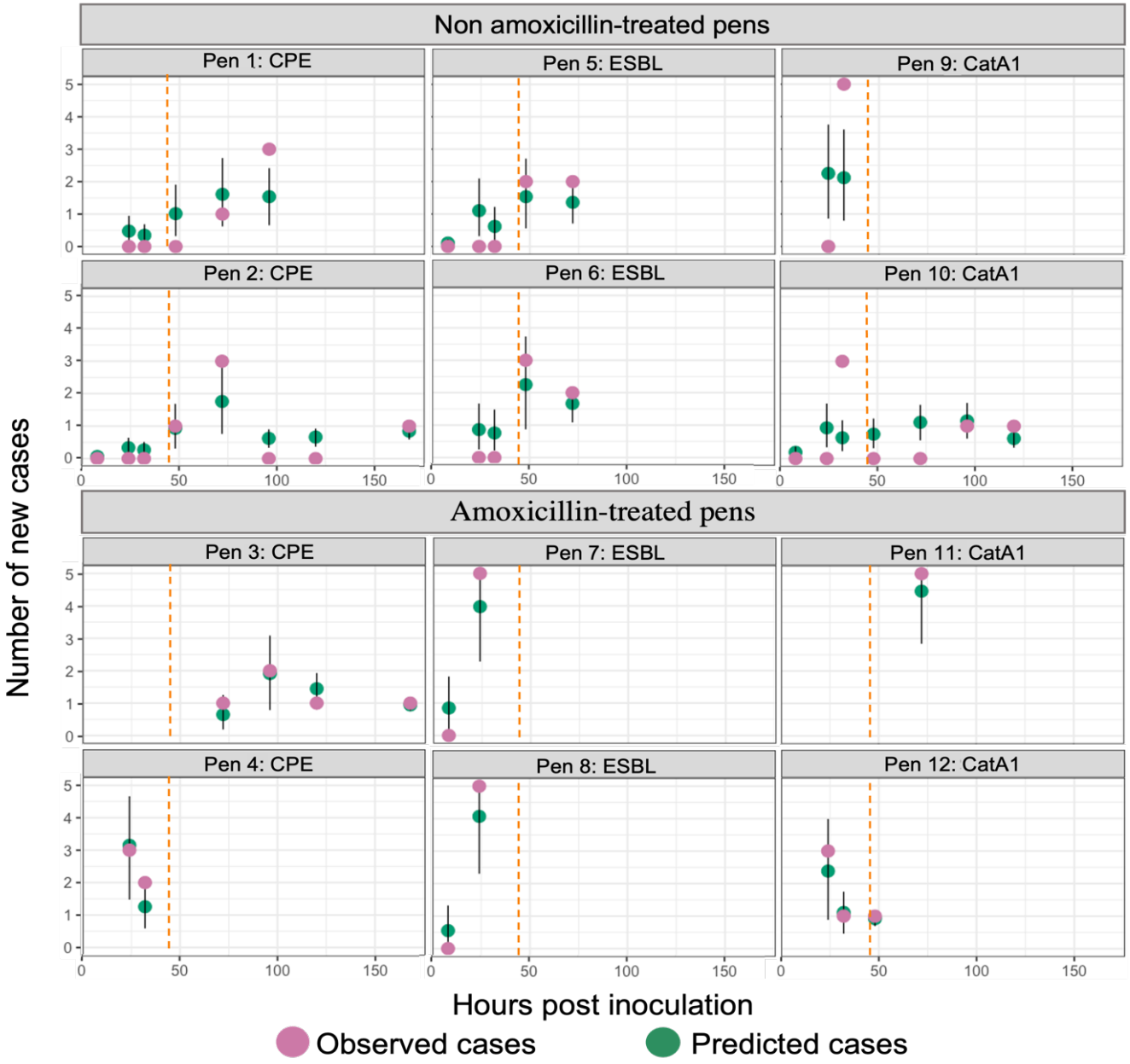

***Figure 3.3**- number of cases over time. Observed (violet) and predicted (green) number of new cases among the 5 susceptible broilers (i.e., susceptible contact broilers that became colonized) (vertical axis) in each of the 12 pens until the sampling time point in hours after inoculation (horizontal 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.*

3. 3. 1. 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 Figure 3.4 and Figure 3.5). The MAP suggests that CPE-strain has the lowest transmission rate of the 3 inoculums.

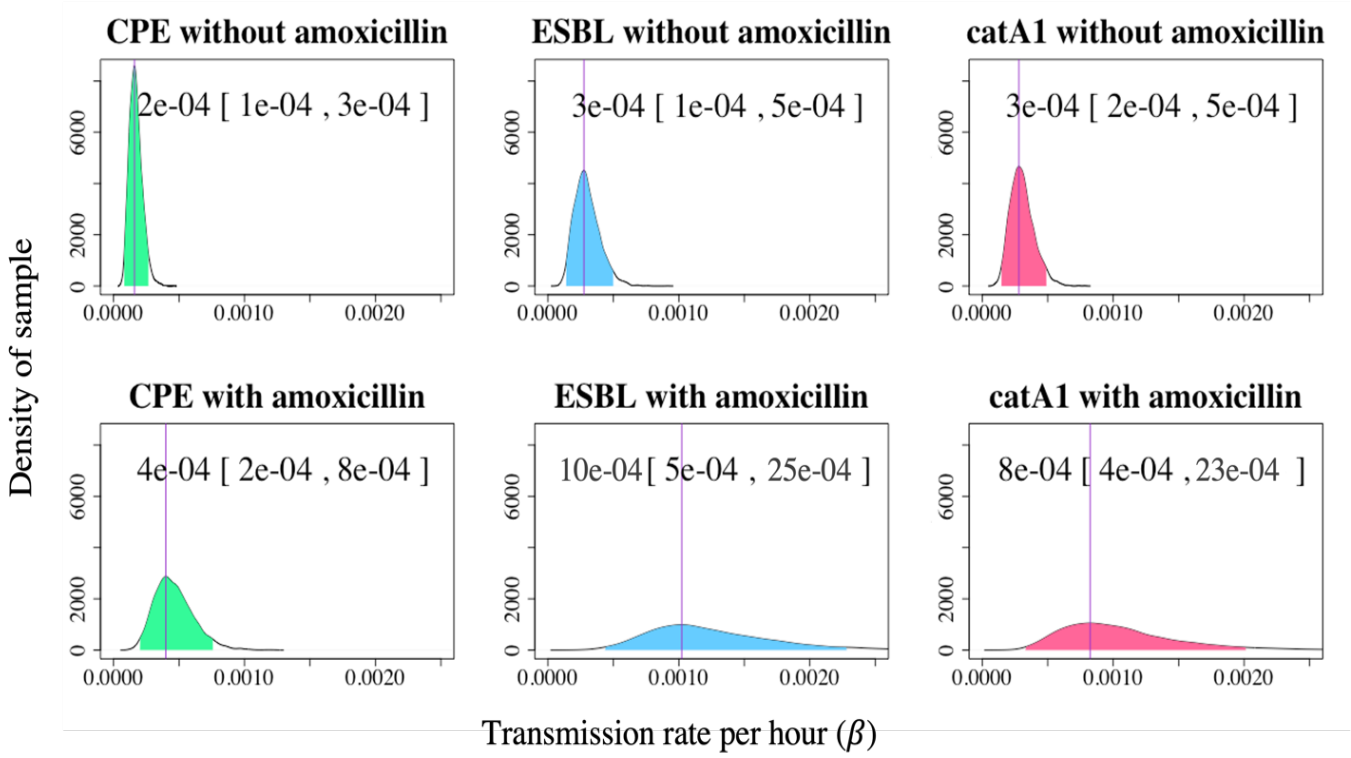

Figure 3.4- *Density (vertical axis) of the posterior distribution of the transmission rate per hour (horizontal axis) for the CPE-strain, ESBL-strain and catA1-strain. The top and bottom row show plots for the pens without and with amoxicillin treatment, respectively. Purple vertical lines indicate 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.*

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 (Figure 3.5). 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 (Figure 3.5). 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.

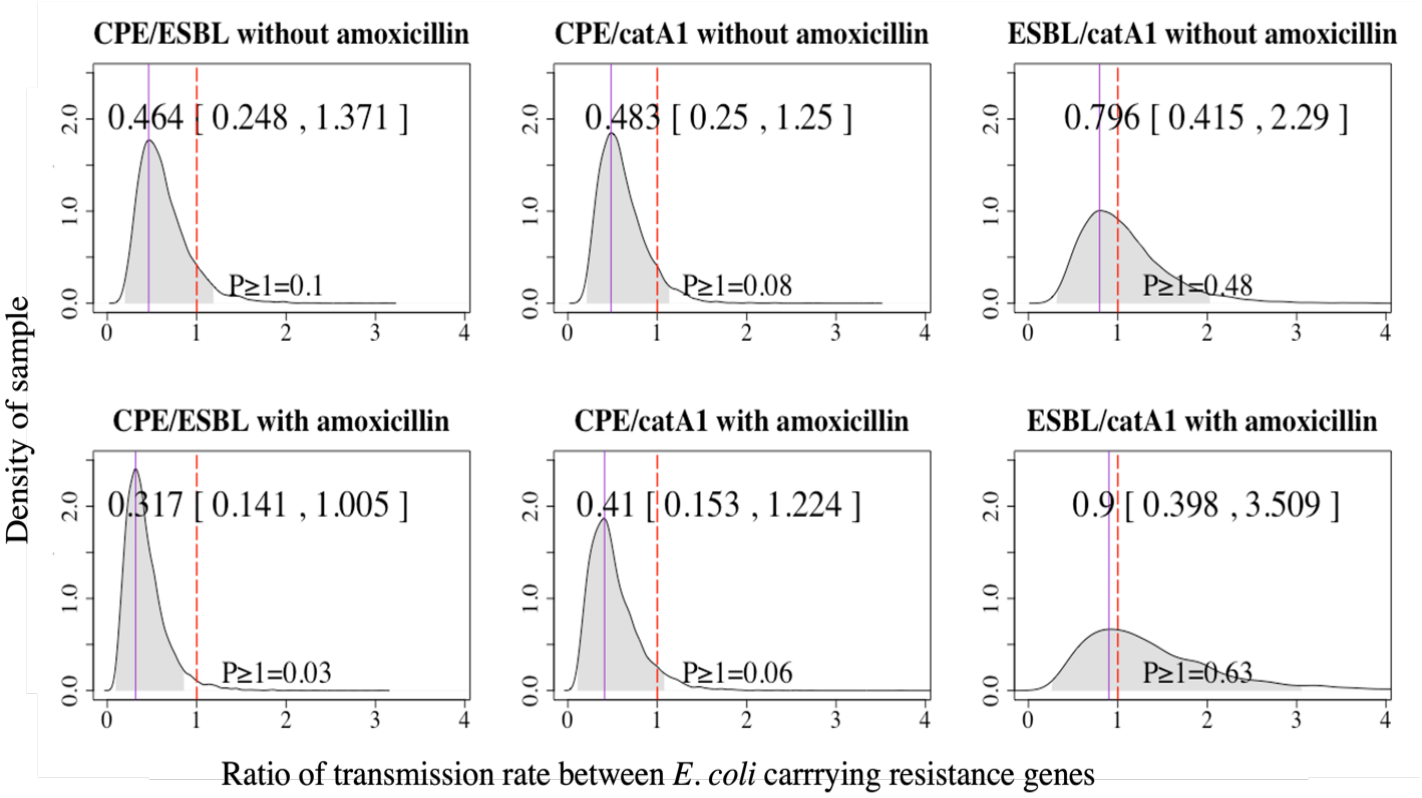

Figure 3.5- *Density (vertical axis) of the posterior distribution of the ratio of the transmission rates (horizontal axis) for different inoculums: CPE-strain to ESBL-strain, CPE-strain to catA1-strain, and ESBL-strain to catA1-strain. The top and bottom row show plots for the pens without and with 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.*

3. 3. 1. 3 Effect of amoxicillin

The transmission rates of all inoculums are smaller in the non-amoxicillin-treated groups than in the amoxicillin-treated groups (**Error! Reference source not found.**3.6). The difference between those groups is slightly larger for the ESBL-strain and catA1-strain than for the CPE-strain.

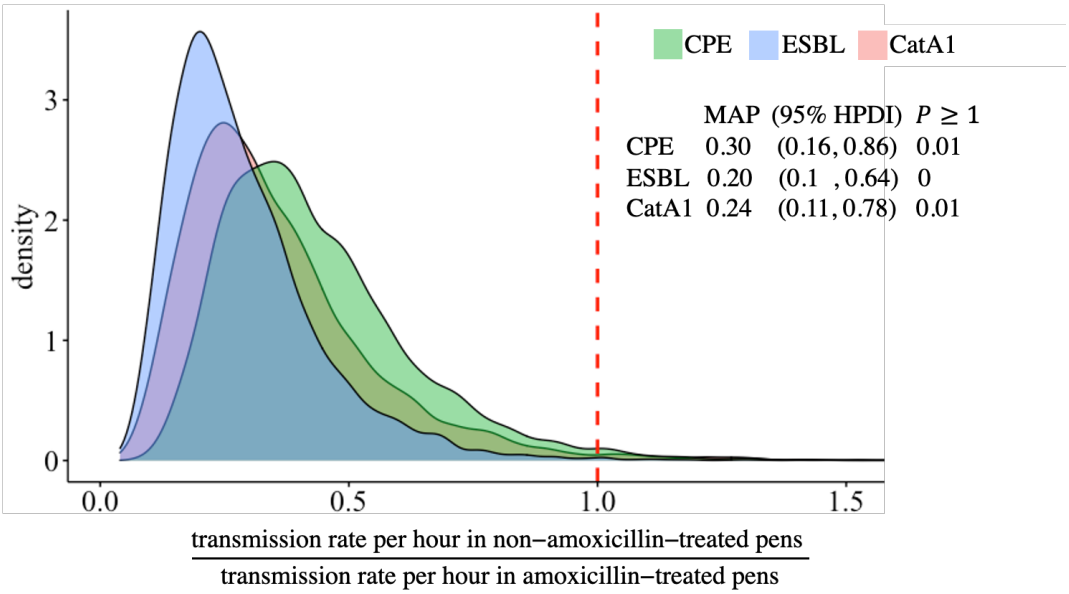

Figure 3.6*- Density (vertical axis) of the ratio of the transmission rates in non-amoxicillin-treated pens over amoxicillin-treated pens (horizontal axis) for the CPE-strain (green), ESBL-strain (blue) and catA1-strain (pink) in the SIS-model. The dotted red vertical line indicates a ratio of 1 (i.e., 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.*

3.3.2 Microbiome analysis

3. 3. 2. 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; Figure 3.7). 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 (Figure 3.16; Tables 3.7– 3.10).

Amoxicillin treatment affected the microbiome composition at class level, family level, and genus level (Figure 3.12; Figure 3.13; Figure 3.14). 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 (Figure 3.7), 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 (Figure 3.16; Table 3.7 – 3.10).

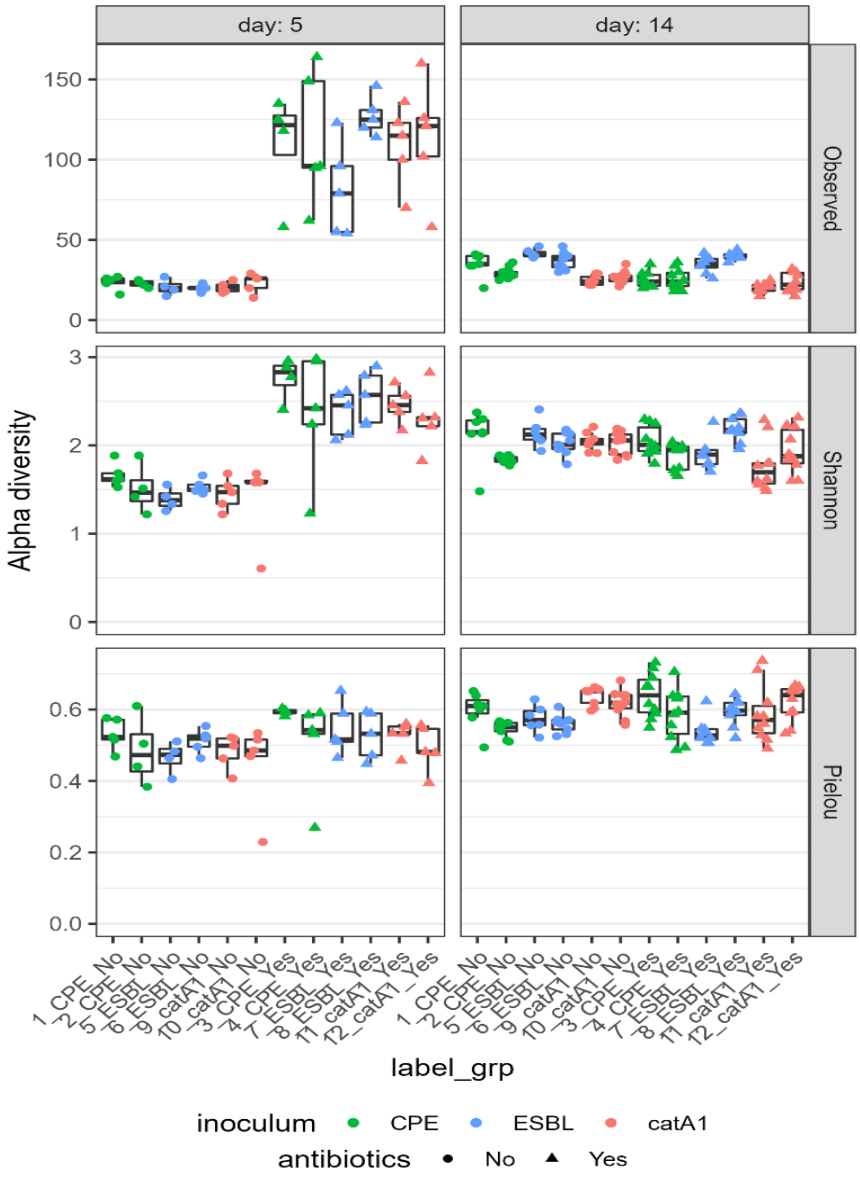

Figure 3.7*- Boxplots of alpha-diversity (vertical axis) by inoculum and antibiotic treatment (horizontal axis) at genus level. The box indicates the first and third quantiles and the whiskers extend to the smallest and largest values at most 1.5 times the interquartile range from the hinges. Colours 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).*

3. 3. 2. 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 3.11). Only groups without and with antibiotics were separated in the PCoA-plot (Figure 3.8). Repeating these analyses at the level of individual ASVs mostly gave the same results (see sections 3.7.2.5 ‘Beta-diversity at ASV level’ and 3.7.2.6 ‘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 3.13 – 3.15).

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 3.12). 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 (Figure 3.8) but separated on ASV level with Bray-Curtis dissimilarity (Figure 3.17).

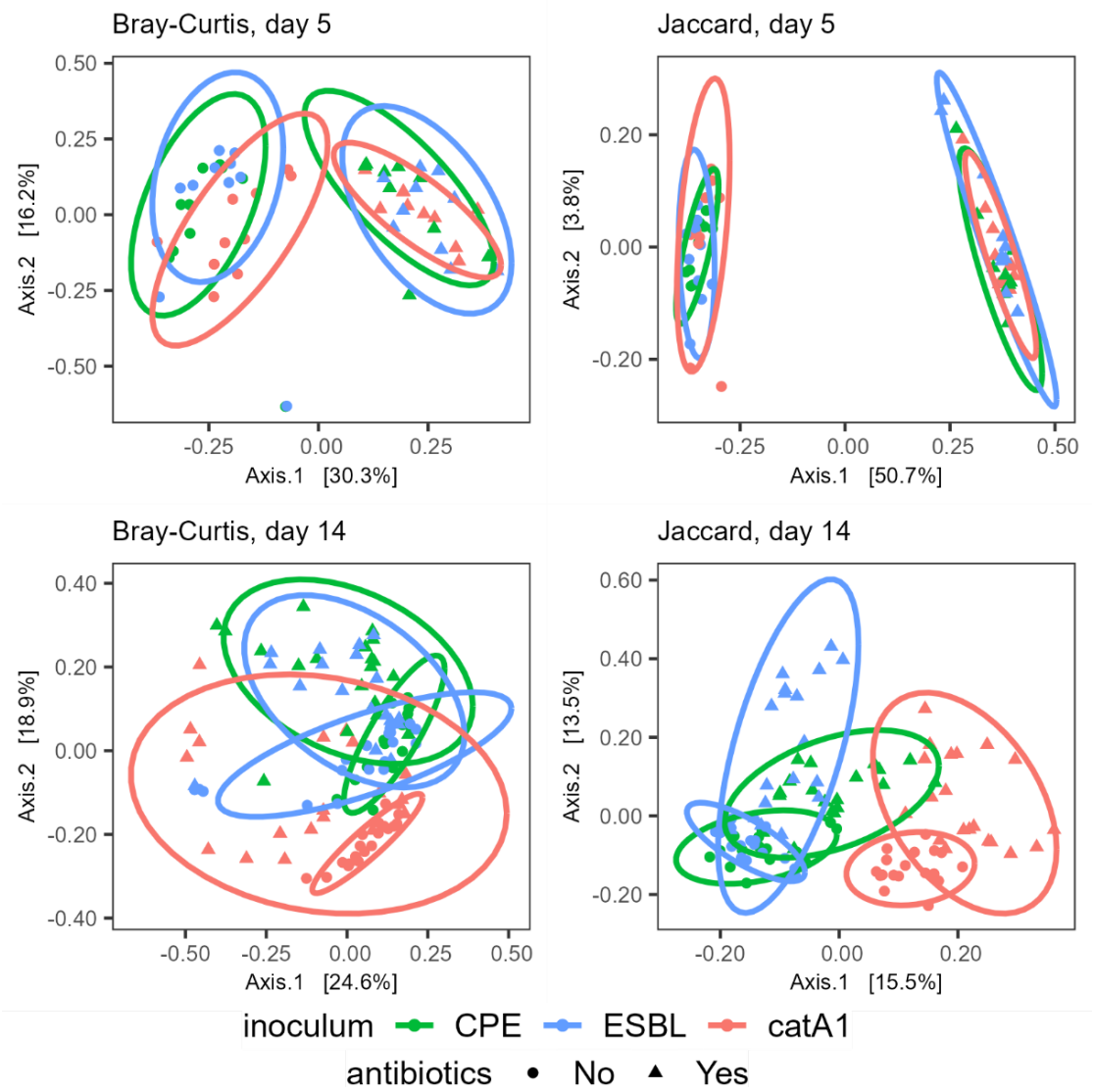

Figure 3.8- *Principal coordinate plots based on Bray-Curtis dissimilarity (left) and Jaccard distance (right) for day 5 (top) and day 14 (bottom) at genus level. Colours indicate different inoculums (CPE-strain: green; ESBL-strain: blue; catA1-strain: red) and symbols indicate the absence (circles) or presence (triangles) of antibiotic treatment. Ellipses represent 95% confidence regions assuming a multivariate t-distribution.*

3. 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).

3. 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 3.2.2.1 ‘SI- and SIS-models’ above and Tables 3.16 and 3.17). Using a higher decay rate would result in higher estimates for the transmission rates (Table 3.17), 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 Supplementary 3.7.3.6 ‘SIS-model result’) 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.

3. 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 (Figure 3.3), 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 (Figure 3.3).

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 3.18 – 3.20). 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 (Figure 3.5) when fitness costs are not expected to limit the growth and transmission

rates (see section ‘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 3.1), 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.

3. 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 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 (Figure 3.6). 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 (Figure 3.15), 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 capability for transmission were present in these bacterial strains, which are independent of the antibiotic resistance itself, as we already stated above.

3. 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 (Figure 3.13).

The lower alpha-diversity in non-amoxicillin-treated pens than in amoxicillin-treated pens on day 5 (Figure 3.7) 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 3.13 – 3.15). 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 (Figure 3.3). 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).

3. 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.

3. 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.

3. 6 Acknowledgement

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3. 7 Supplementary information

3. 7. 1 Data from the transmission experiment

3. 7. 1. 1 Raw transmission data

The pen, used inoculum, antibiotic treatment, and the test results of the cloacal swabs (i.e., positive or negative for the CPE-strain, ESBL-strain, or catA1-strain) at each sampling time point were recorded for all inoculated and contact broilers (Table 3.2).

Table 3.2- *The experimental treatment and status at each sampling time point for all inoculated and contact broilers. Light shading indicates a positive test result, dark shading indicates the absence of a test result because the broiler died. Abbreviations: AMU: antibiotic usage, ID: broiler ID.*

				Sampling time point (hours after inoculation)									
Pen	Inoculum	AMU	ID	0	8	24	32	48	72	96	120	168	216
1	CPE	-	0101	0	0	1	D	D	D	D	D	D	D
1	CPE	-	0114	0	0	1	0	1	1	1	1	1	1
1	CPE	-	0110	0	0	0	0	1	1	1	1	1	1
1	CPE	-	0108	0	0	0	0	0	1	0	1	1	1
1	CPE	-	0109	0	0	0	D	D	D	D	D	D	D
1		-	0113	0	0	0	0	0	1	0	1	1	1
1		-	0102	0	0	0	0	0	0	1	1	1	1
1		-	0103	0	0	0	0	0	0	1	0	1	1
1		-	0104	0	0	0	0	0	0	1	0	1	1
1		-	0107	0	0	0	0	0	D	D	D	D	D
2	CPE	-	0209	0	1	0	0	0	1	1	1	1	1
2	CPE	-	0202	0	0	0	1	1	1	1	1	1	1
2	CPE	-	0205	0	0	0	0	1	1	0	1	1	1
2	CPE	-	0211	0	0	0	0	0	1	1	0	1	1
2	CPE	-	0212	0	0	0	D	D	D	D	D	D	D

				Sampling time point (hours after inoculation)									
Pen	Inoculum	AMU	ID	0	8	24	32	48	72	96	120	168	216
2		-	0206	0	0	0	0	1	1	0	0	1	1
2		-	0204	0	0	0	0	0	1	0	1	1	1
2		-	0208	0	0	0	0	0	1	0	0	1	1
2		-	0215	0	0	0	0	0	1	1	1	1	1
2		-	0201	0	0	0	0	0	0	0	0	1	1
3	CPE	+	0304	0	0	0	0	0	1	1	1	1	1
3	CPE	+	0310	0	0	0	0	0	0	1	1	1	1
3	CPE	+	0312	0	0	0	0	0	0	1	1	1	1
3	CPE	+	0303	0	0	0	0	0	0	0	1	1	1
3	CPE	+	0313	0	0	0	0	0	0	0	0	1	1
3		+	0308	0	0	0	0	0	1	0	1	1	1
3		+	0306	0	0	0	0	0	0	1	1	1	1
3		+	0307	0	0	0	0	0	0	1	1	1	1
3		+	0315	0	0	0	0	0	0	0	1	1	1
3		+	0309	0	0	0	0	0	0	0	0	1	1
4	CPE	+	0402	0	0	1	1	1	1	1	1	1	1
4	CPE	+	0404	0	0	1	0	1	1	1	1	1	1
4	CPE	+	0405	0	0	1	1	1	1	1	1	1	1
4	CPE	+	0406	0	0	1	1	1	1	1	1	1	1
4	CPE	+	0415	0	0	1	1	1	1	1	1	1	1
4		+	0401	0	0	1	1	1	1	1	1	1	1
4		+	0409	0	0	1	1	1	1	1	1	1	1
4		+	0413	0	0	1	1	1	1	1	1	1	1
4		+	0407	0	0	0	1	1	1	1	1	1	1
4		+	0412	0	0	0	1	1	1	1	1	1	1
5	ESBL	-	0514	0	1	1	1	1	1	1	1	1	1
5	ESBL	-	0510	0	0	1	0	1	1	1	1	1	1
5	ESBL	-	0511	0	0	1	1	1	1	1	1	1	1
5	ESBL	-	0513	0	0	0	0	1	1	1	1	1	1
5	ESBL	-	0505	0	0	0	D	D	D	D	D	D	D
5		-	0501	0	0	0	0	1	1	1	1	1	1
5		-	0512	0	0	0	0	1	1	1	1	1	1
5		-	0504	0	0	0	0	0	1	1	1	1	1
5		-	0507	0	0	0	0	0	1	1	1	1	1
5		-	0503	0	0	0	D	D	D	D	D	D	D
6	ESBL	-	0606	0	0	1	1	1	1	1	1	1	1
6	ESBL	-	0612	0	0	1	1	1	1	1	1	1	1
6	ESBL	-	0602	0	0	0	1	1	1	1	1	1	1

				Sampling time point (hours after inoculation)									
Pen	Inoculum	AMU	ID	0	8	24	32	48	72	96	120	168	216
6	ESBL	-	0614	0	0	0	0	1	1	1	1	1	1
6	ESBL	-	0610	0	0	0	0	0	1	1	1	1	1
6		-	0605	0	0	0	0	1	0	1	1	1	1
6		-	0611	0	0	0	0	1	1	1	1	1	1
6		-	0615	0	0	0	0	1	1	1	1	1	1
6		-	0604	0	0	0	0	0	1	1	1	1	1
6		-	0613	0	0	0	0	0	1	1	1	1	1
7	ESBL	+	0701	0	1	1	1	1	1	1	1	1	1
7	ESBL	+	0702	0	1	1	1	1	1	1	1	1	1
7	ESBL	+	0705	0	0	1	1	1	1	1	1	1	1
7	ESBL	+	0708	0	0	1	1	1	1	1	1	1	1
7	ESBL	+	0713	0	0	1	1	1	1	1	1	1	1
7		+	0703	0	0	1	1	1	1	1	1	1	1
7		+	0709	0	0	1	1	1	1	1	1	1	1
7		+	0710	0	0	1	1	1	1	1	1	1	1
7		+	0712	0	0	1	1	1	1	1	1	1	1
7		+	0704	0	0	1	1	1	1	1	D	D	D
8	ESBL	+	0807	0	1	1	1	1	1	1	1	1	1
8	ESBL	+	0804	0	0	1	1	1	1	1	1	1	1
8	ESBL	+	0810	0	0	1	1	1	1	1	1	1	1
8	ESBL	+	0812	0	0	1	1	1	1	1	1	1	1
8	ESBL	+	0811	0	0	1	D	D	D	D	D	D	D
8		+	0801	0	0	1	1	1	1	1	1	1	1
8		+	0802	0	0	1	1	1	1	1	1	1	1
8		+	0805	0	0	1	1	1	1	1	1	1	1
8		+	0809	0	0	1	1	1	1	1	1	1	1
8		+	0815	0	0	1	1	1	1	1	1	1	1
9	catA1	-	0906	0	0	1	1	0	1	1	1	1	1
9	catA1	-	0912	0	0	1	1	0	1	1	1	1	1
9	catA1	-	0915	0	0	1	0	1	1	1	1	1	1
9	catA1	-	0908	0	0	0	1	0	1	1	1	1	1
9	catA1	-	0913	0	0	0	1	1	1	1	1	1	1
9		-	0901	0	0	0	1	1	1	1	1	1	1
9		-	0902	0	0	0	1	1	1	1	1	1	1
9		-	0910	0	0	0	1	0	1	1	1	1	1
9		-	0914	0	0	0	1	1	1	1	1	1	1
9		-	0904	0	0	0	1	0	D	D	D	D	D
10	catA1	-	1002	0	1	0	1	0	0	0	1	1	1

				Sampling time point (hours after inoculation)									
Pen	Inoculum	AMU	ID	0	8	24	32	48	72	96	120	168	216
10	catA1	-	1004	0	1	0	0	0	0	0	1	1	1
10	catA1	-	1010	0	1	0	0	0	0	0	1	0	1
10	catA1	-	1013	0	1	0	0	0	0	0	1	1	1
10	catA1	-	1003	0	0	0	1	0	0	0	1	1	1
10		-	1006	0	0	0	1	0	0	0	1	1	1
10		-	1007	0	0	0	1	0	0	0	1	1	1
10		-	1009	0	0	0	1	0	0	1	1	1	1
10		-	1005	0	0	0	0	0	0	1	1	1	1
10		-	1015	0	0	0	0	0	0	0	1	1	1
11	catA1	+	1103	0	0	0	0	0	1	1	1	1	1
11	catA1	+	1104	0	0	0	0	0	1	1	1	1	1
11	catA1	+	1105	0	0	0	0	0	1	1	1	1	1
11	catA1	+	1110	0	0	0	0	0	1	1	1	1	1
11	catA1	+	1112	0	0	0	0	0	1	1	1	1	1
11		+	1102	0	0	0	0	0	1	1	1	1	1
11		+	1106	0	0	0	0	0	1	1	1	1	1
11		+	1107	0	0	0	0	0	1	1	1	1	1
11		+	1108	0	0	0	0	0	1	1	1	1	1
11		+	1115	0	0	0	0	0	1	1	1	1	1
12	catA1	+	1204	0	0	1	1	1	1	1	1	1	1
12	catA1	+	1207	0	0	1	1	1	1	1	1	1	1
12	catA1	+	1209	0	0	1	1	1	1	1	1	1	1
12	catA1	+	1214	0	0	0	1	1	1	1	1	1	1
12	catA1	+	1213	0	0	0	0	1	1	1	1	1	1
12		+	1201	0	0	1	1	1	1	1	1	1	1
12		+	1203	0	0	1	1	1	1	1	1	1	1
12		+	1210	0	0	1	1	1	1	1	1	1	1
12		+	1202	0	0	0	1	1	1	1	1	1	1
12		+	1212	0	0	0	0	1	1	1	1	1	1

3. 6. 1. 2 Protocols to adjust raw transmission data

Since all broilers were excreting (status “1” in Table 3.2) at the end of the experiment, we assumed transmission of *E. coli* bacteria between broilers follows SI-dynamics in which broilers will remain excreting (status “1” in Table 3.2) until the end of the experiment once their cloacal swabs test positive for the resistant bacteria. However, several broilers with a positive sample became negative at the following sampling time point, and then positive again at a later sampling time point. We adjusted the status of

these negative samples according to 1 of the 3 protocols described below and fitted a Bayesian hierarchical model to each of these adjusted data sets. Model diagnostics indicated the models fitted all 3 data sets well. We selected the third protocol to create our final input data because considering reoccurrences of negative samples to be false negatives best reflects the nature of transmission and sequencing, with false-negative samples arising because of limitations in sampling and detecting small numbers of resistant bacteria.

Protocol 1: Only a single reoccurrence of a negative status is treated as false negative

Rules to change the data in each row from the first occurrence of a positive swab (i.e., status “1”):

- 1: When “1” is followed by a single “0”, this “0” is assumed to be a false negative and is changed to “1”.
- 2: When “1” is followed by more than one “0”, all preceding “1” are assumed to be false positive and are changed to “0”.
- 3: When “1” is followed by “0” followed by “D” (i.e., the broiler died), the “0” is assumed to be a false negative and is changed to “1”.

Protocol 2: Prolonged reoccurrence of a negative status is treated as false negative if it follows a single occurrence of positive status

Rules to change the data in each row from the first occurrence of a positive swab (i.e., status “1”):

- 1: When “1” is followed by a single “0”, this “0” is assumed to be a false negative and is changed to “1”.
- 2: When a single “1” is followed by more than one “0”, this “1” is assumed to be a false positive and is changed to “0”.
- 3: When more than one “1” is followed by any number of “0”, the “0” are assumed to be false negative and are changed to “1”
- 4: When “1” is followed by “0” followed by “D” (i.e., the broiler died), the “0” is assumed to be a false negative and is changed to “1”.

Protocol 3: Each reoccurrence of a negative status is treated as a false negative

Rules to change the data in each row from the first occurrence of a positive swab (i.e., status “1”):

- 1: When “1” is followed by any number of “0”, all “0” are assumed to be false negative and are changed to “1”.

Table 3.3- *Examples of the status of 3 broilers at different time points (hours after inoculation) giving the raw data and adjustments according to each of the 3 protocols. The status at the last two time points (168 and 216 hours) was 1 for all 3 broilers and has been omitted to reduce the width of the table. Shading indicates changes compared to the raw data. Abbreviations: ID: broiler ID, P: protocol*

ID	0113								1002								1004							
time	0	8	24	32	48	72	96	120	0	8	24	32	48	72	96	120	0	8	24	32	48	72	96	120
raw	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1
P1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
P2	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1
P3	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1

3. 7. 1. 3 Summary statistics

Table 3.4- *Overview of the distribution of the sexes in the different experimental groups. Abbreviations: F: female, M: male, No: pens without antibiotics, T: total, Yes: pens with antibiotics.*

		Day 5				Day 14				Total			
Group	Pens	F	M	T	%F	F	M	T	%F	F	M	T	%F
CPE_No	1, 2	6	4	10	60	9	7	16	56	15	11	26	58
ESBL_No	5, 6	8	2	10	80	8	10	18	44	16	12	28	57
catA1_No	9, 10	6	4	10	60	8	11	19	42	14	15	29	48
CPE_Yes	3, 4	4	5	9	44	10	10	20	50	14	15	29	48
ESBL_Yes	7, 8	6	4	10	60	11.5	6.5	18	64	17.5	10.5	28	63
catA1_Yes	11, 12	2	7	9	22	10	10	20	50	12	17	29	41
CPE	1 - 4	10	9	19	53	19	17	36	53	29	26	55	53
ESBL	5 - 8	14	6	20	70	19.5	16.5	36	54	33.5	22.5	56	60
catA1	9 - 12	8	11	19	42	18	21	39	46	26	32	58	45
No	1, 2, 5, 6, 9, 10	20	10	30	67	25	28	53	47	45	38	83	54
Yes	3, 4, 7, 8, 11, 12	12	16	28	43	31.5	26.5	58	54	43.5	42.5	86	51
Total	1 - 12	32	26	58	55	56.5	54.5	111	51	88.5	80.5	169	52

Table 3.5- *Overview of the mean time for broilers to become positive (in hours after inoculation) in the different experimental groups. Abbreviations: No: pens without antibiotics, Yes: pens with antibiotics.*

Group	Pens	Role	Number of dead broilers	Mean time (h after inoculation)
CPE_No	1, 2	I	3	38
ESBL_No	5, 6	I	1	33
catA1_No	9, 10	I	0	20
CPE_No	1, 2	S	1	84
ESBL_No	5, 6	S	1	55
catA1_No	9, 10	S	1	47
CPE_Yes	3, 4	I	0	67
ESBL_Yes	7, 8	I	1	19
catA1_Yes	11, 12	I	0	51
CPE_Yes	3, 4	S	0	69
ESBL_Yes	7, 8	S	1	24
catA1_Yes	11, 12	S	0	51
CPE	1 - 4	I	3	52
ESBL	5 - 8	I	2	26
catA1	9 - 12	I	0	36
CPE	1 - 4	S	1	76
ESBL	5 - 8	S	2	40
catA1	9 - 12	S	1	49
No	1, 2, 5, 6, 9, 10	I	4	30
Yes	3, 4, 7, 8, 11, 12	I	1	46
No	1, 2, 5, 6, 9, 10	S	3	62
Yes	3, 4, 7, 8, 11, 12	S	1	48
Total	1 – 12	both	9	47

3. 7. 2 Data from the microbiome analysis

3. 7. 2. 1 Quality controls for microbiome sequencing

3. 7. 2. 1. 1 Composition of the spiked negative controls

The composition of the negative controls spiked with a low concentration of microbial community DNA standard (ZymoBIOMICS; Zymo Research Corporation, Irvine, CA) was similar for all controls, was close to the theoretical composition indicated by the manufacturer, and contained less than 0.15% foreign DNA (Figure 3.2). This shows the sequencing runs went well, the use of multiple 96-well plates did not lead to large deviations in obtained composition, and little contamination occurred.

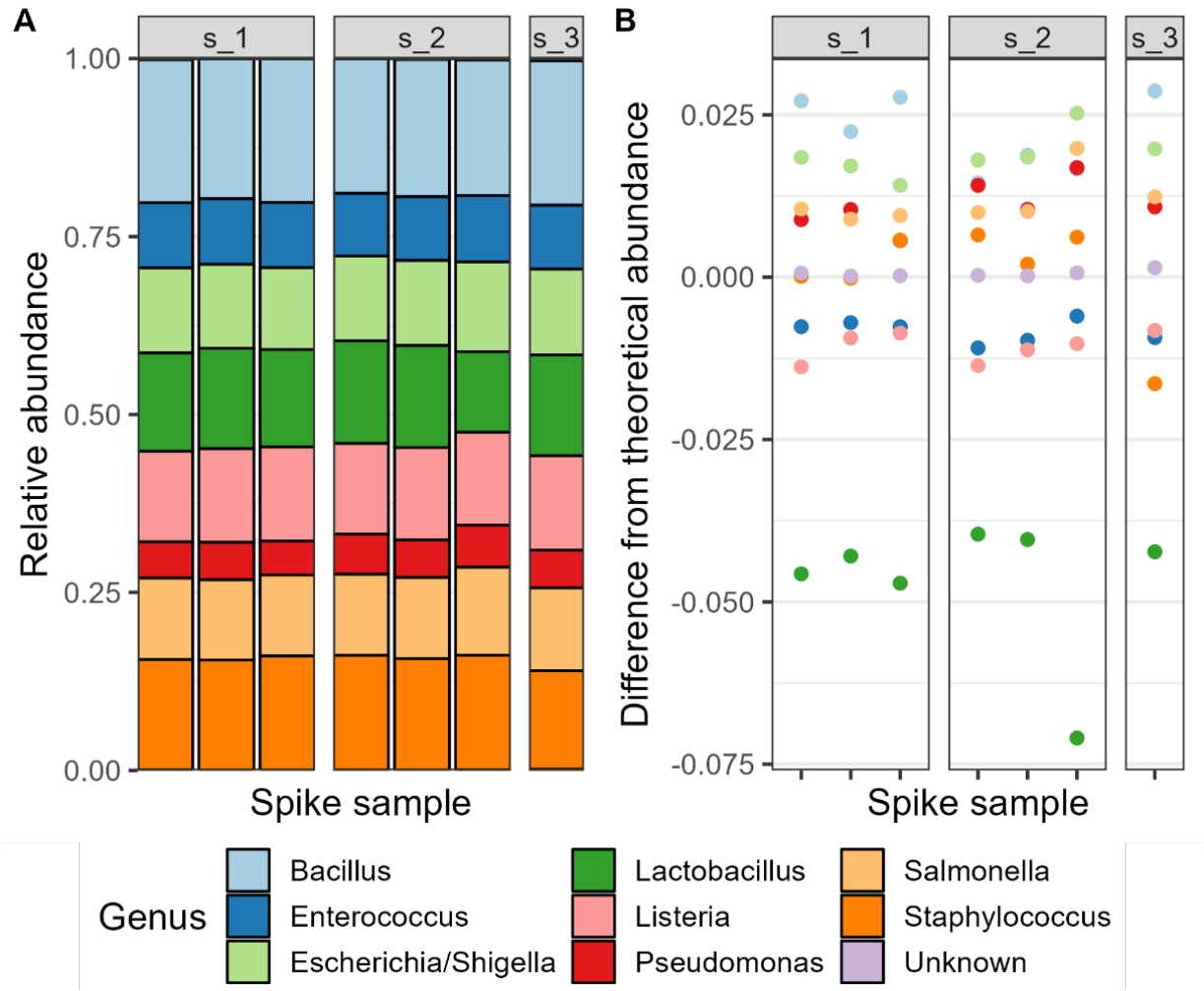

Figure 3.9- *A composition of the spike samples at genus level as determined by sequencing. B: The difference between the observed composition and the theoretical composition indicated by the manufacturer at genus level. s_1, s_2, and s_3 indicate the different well plates the spike samples were on.*

3. 7. 2. 1. 2 Number of reads

Forty-seven per cent of the sequences could not be assigned on genus level after removing the spike samples and non-bacterial sequences, with better coverage on higher taxonomic levels (Table 3.12). We left unassigned sequences as-is, i.e., we did not use the subsequent higher taxonomic ranks to fill in NAs.

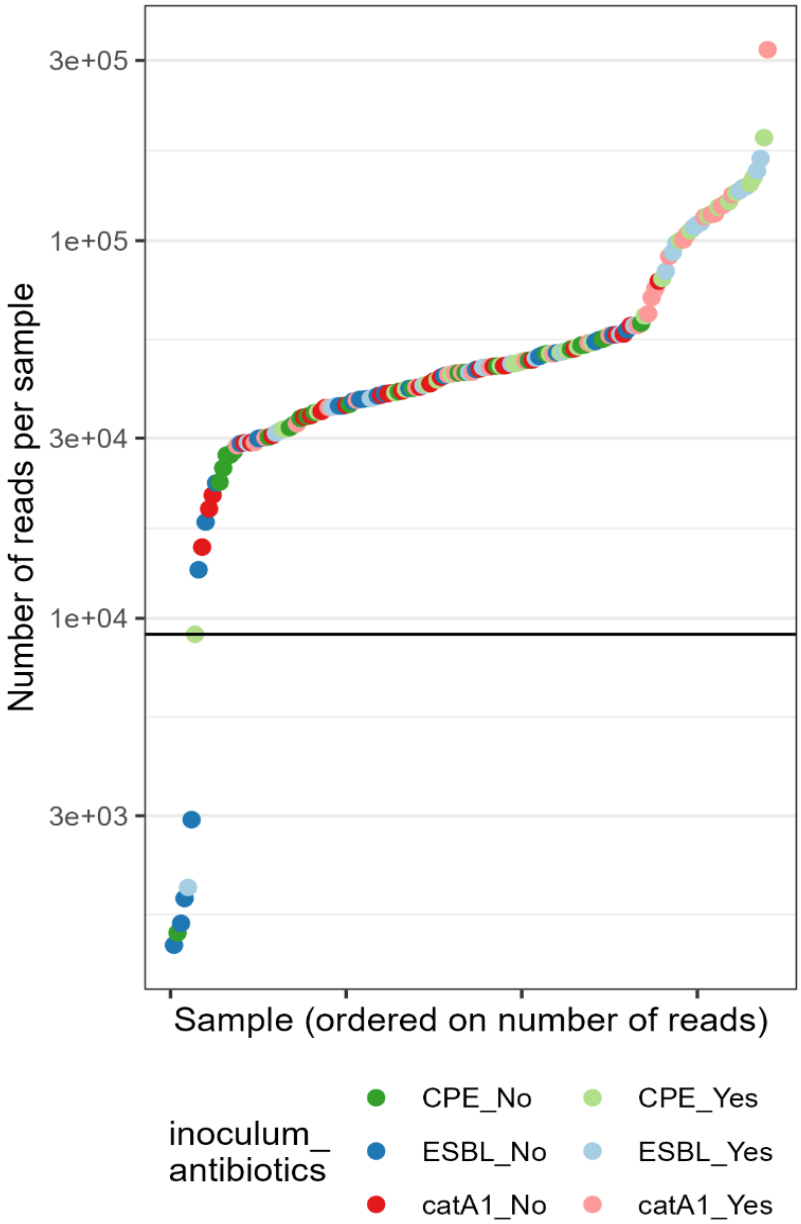

Figure 3.10- *The number of reads in the samples. The black horizontal line indicates the rarefying depth of 9071 reads (7th least number of reads). Colours indicate the absence ('No') or presence ('Yes') of antibiotic treatment and the different inoculums (CPE-strain, ESBL-strain, catA1-strain).*

Table 3.6*- Numbers and percentages of sequences not assigned to a taxon for each taxonomic rank, after removal of the non-bacterial sequences and the spiked negative controls.*

Rank	n NA	% NA
Phylum	53	0.7
Class	152	1.9
Order	608	7.7
Family	1406	17.7
Genus	3715	46.8

3. 7. 2. 1. 3 Rarefaction curves

Rarefaction curves on amplicon sequence variant (ASV) level for samples without antibiotics on day 5, and for most samples on day 14 did not level off (bottom row in Figure 3.11), indicating not all ASVs present in the sample have been sequenced. This is less prominent at the genus level (top row in Figure 3.11).

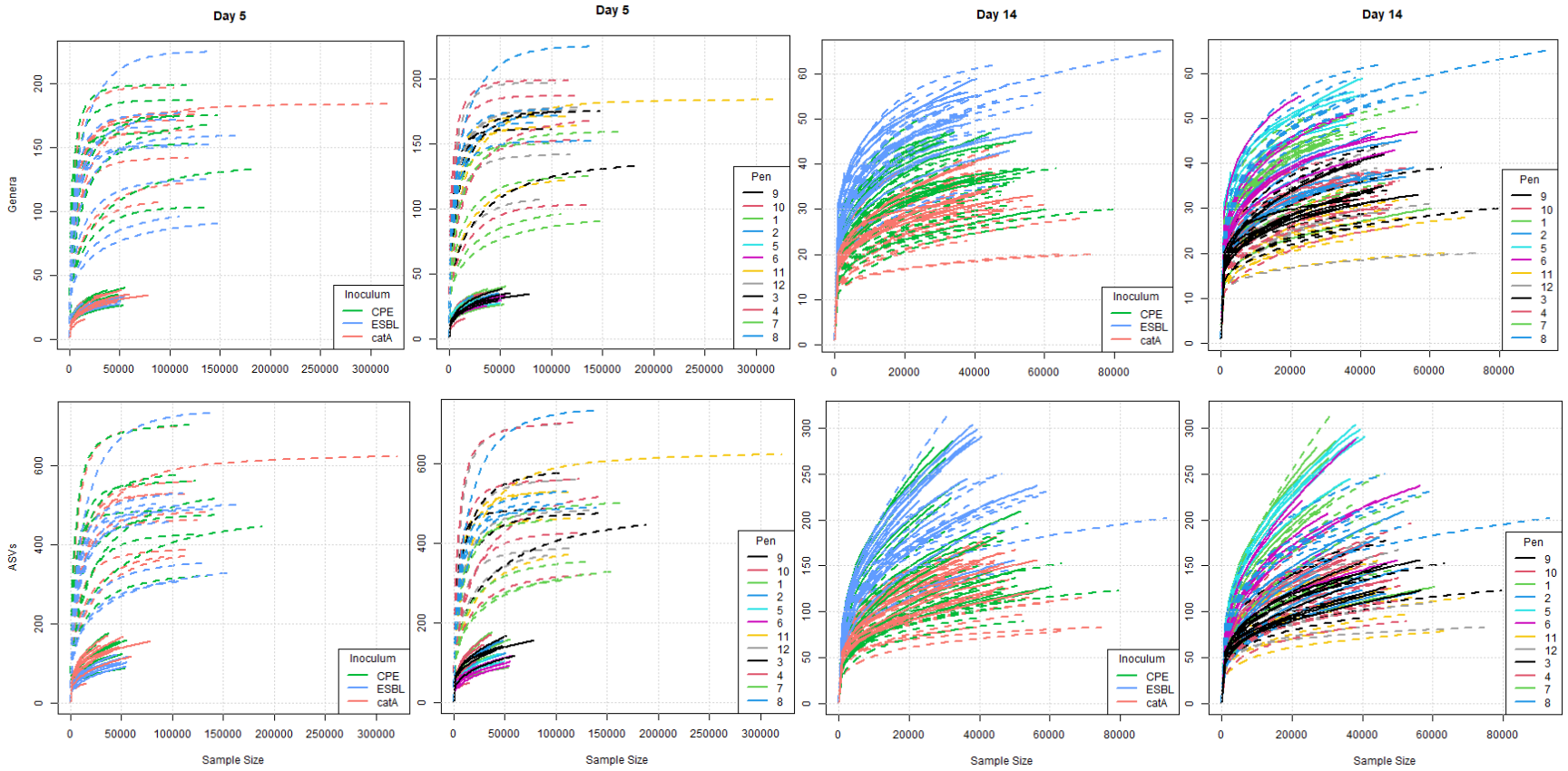

Figure 3.11- *Rarefaction curves for samples on day 5 (first 2 columns) and day 14 (last 2 columns) at genus level (top row) and ASV level (bottom row). Solid lines indicate samples without antibiotic treatment and dashed lines indicate samples with antibiotic treatment. Note the axes differ between days and between the genus level and ASV level*

3. 7. 2. 2 Microbiome composition

The microbiome of all broilers was dominated by the classes Gammaproteobacteria, Clostridia, and Bacilli (Figure 3.12) On day 5 the distinction between groups with and without antibiotics is clear at class, family and genus level (Figures 3.12, 3.13, 3.14) On day 14, the composition in pens 11 and 12 (catA1-strain with antibiotics) resembles the composition in pens 9 and 10 (catA1-strain without antibiotics) more closely than the composition in other pens with antibiotics at family level (Figure 3.13), which could reflect a room effect as pens 9 to 12 were in the same room. This is not the case at class level (Figure 3.12).

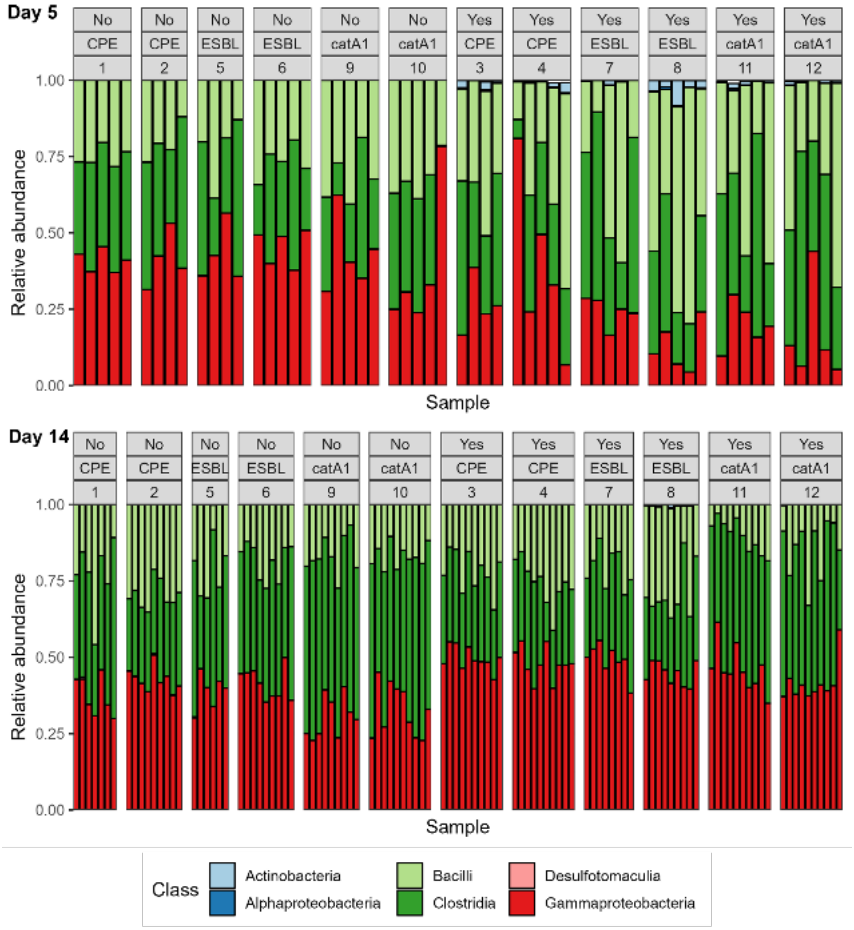

***Figure 3.12**- Relative abundance (vertical axis) of the 6 most-abundant classes considering the total abundance over all samples. Facet labels indicate the absence ('No') or presence ('Yes') of antibiotic treatment, the different inoculums (CPE-strain, ESBL-strain, catA1-strain), and the pen number.*

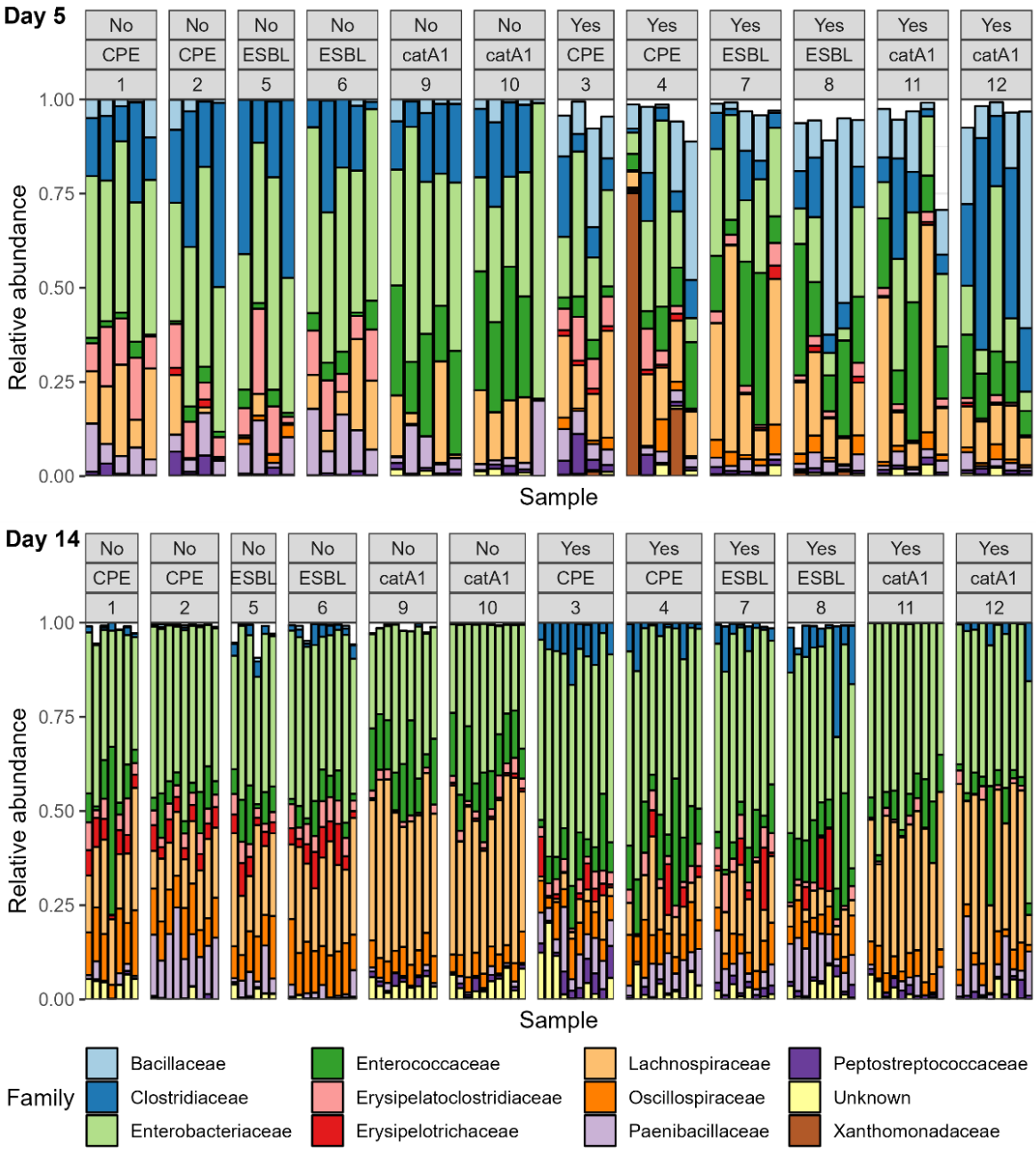

Figure 3.13- *Relative abundance (vertical axis) of the 12 most-abundant families considering the total abundance over all samples. Facet labels indicate the absence ('No') or presence ('Yes') of antibiotic treatment, the different inoculums (CPE-strain, ESBL-strain, catA1-strain), and the pen number.*

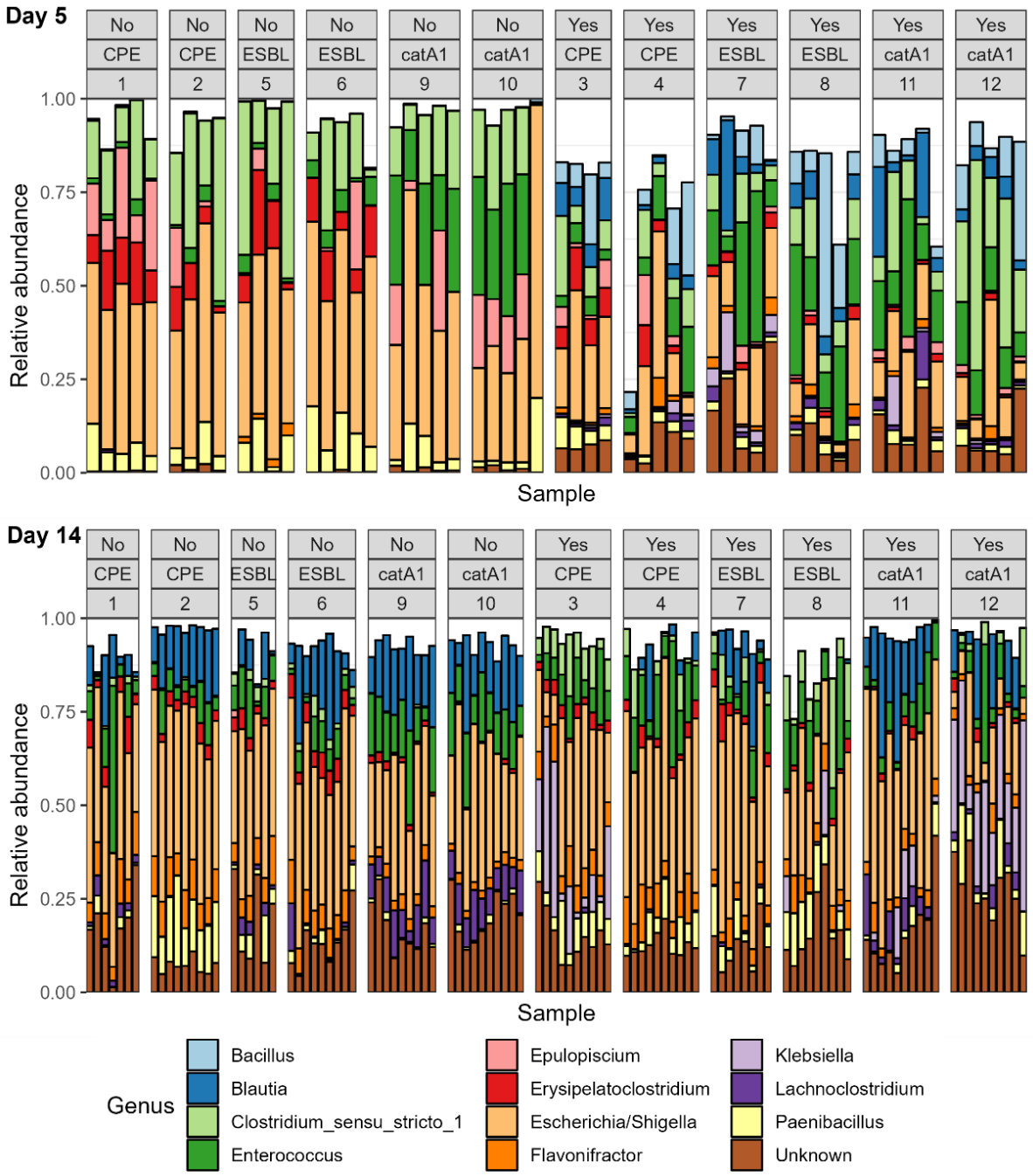

***Figure 3.14**- Relative abundance (vertical axis) of the 12 most-abundant genera considering the total abundance over all samples. Facet labels indicate the absence ('No') or presence ('Yes') of antibiotic treatment, the different inoculums (CPE-strain, ESBL-strain, catA1-strain), and the pen number.*

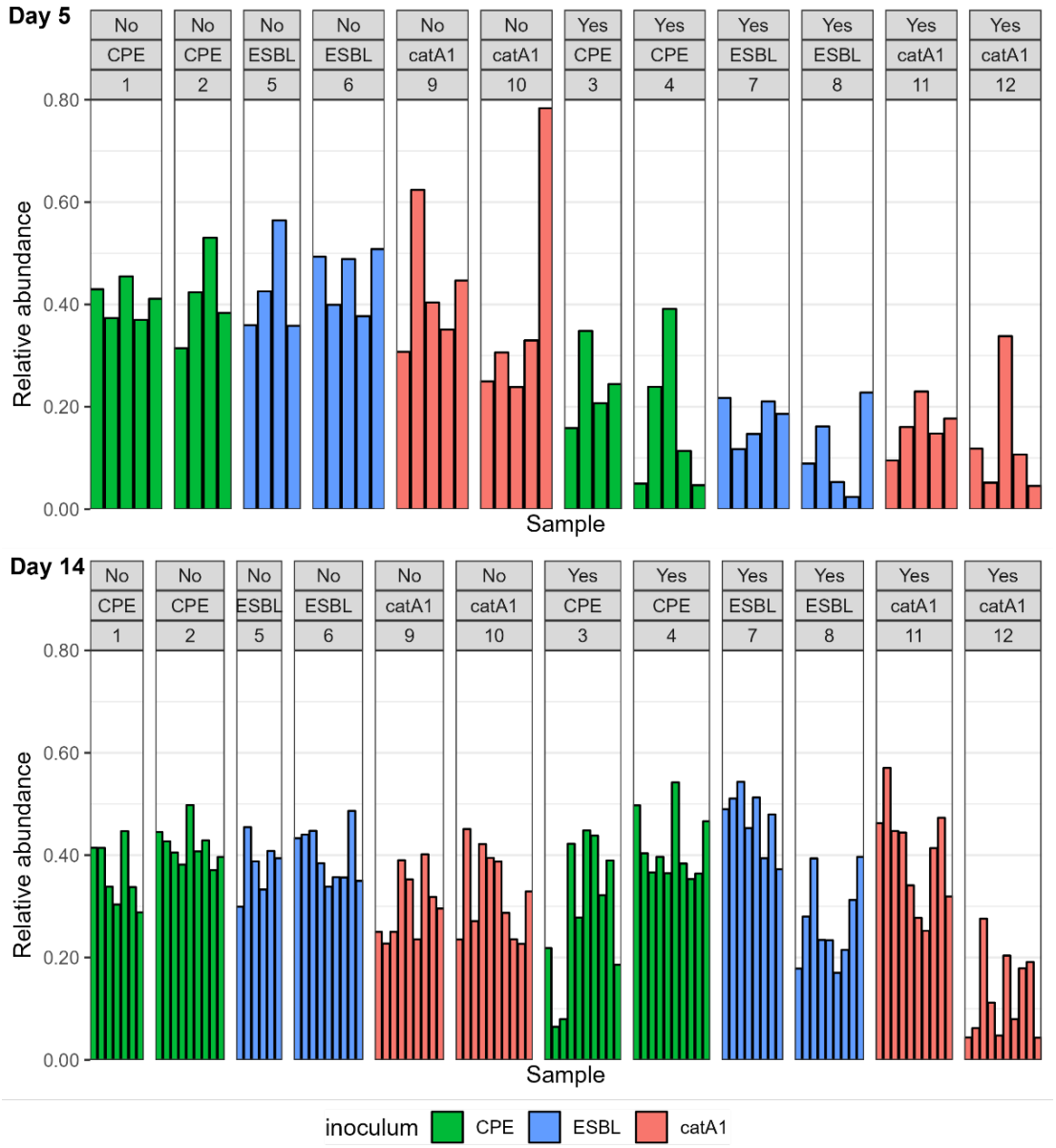

Figure 3.15- *Relative abundance (vertical axis) of the Escherichia/Shigella genus. Facet labels indicate the absence ('No') or presence ('Yes') of antibiotic treatment, the different inoculums (CPE-strain, ESBL-strain, catA1-strain), and the pen number.*

3. 7. 2. 3 Alpha-diversity on ASV level

As expected, because caecal samples on day 5 were taken before inoculation, observed richness, Shannon's diversity, and Pielou's evenness at ASV level on day 5 were not different between groups inoculated with the different inoculums (i.e., CPE-strain, ESBL-strain, catA1-strain; Figure 3.16 and Table 3.9). Observed richness on day 14 was slightly higher in broilers inoculated with the ESBL-strain than in broilers inoculated with the CPE-strain or catA1-strain, both in the non-amoxicillin-treated group and in the amoxicillin-treated group (Figure 3.16 and Table 3.10). Shannon's diversity on day 14 was not different between the inoculums. Pielou's evenness on day 14 was higher in the catA1-strain than in the CPE-strain or ESBL-strain in groups without and with antibiotics.

Observed richness and Shannon's diversity at ASV 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 (Figure 3.16 and Table 3.9) indicating fewer different ASV were present in the non-amoxicillin-treated groups but the distribution of their abundances was similar to the distribution of their abundance in the amoxicillin-treated groups. Shannon's diversity on day 14 was higher in non-amoxicillin-treated groups than in amoxicillin-treated groups, but no differences in observed richness or Pielou's evenness were found between those groups (Figure 3.16 and Table 3.10).

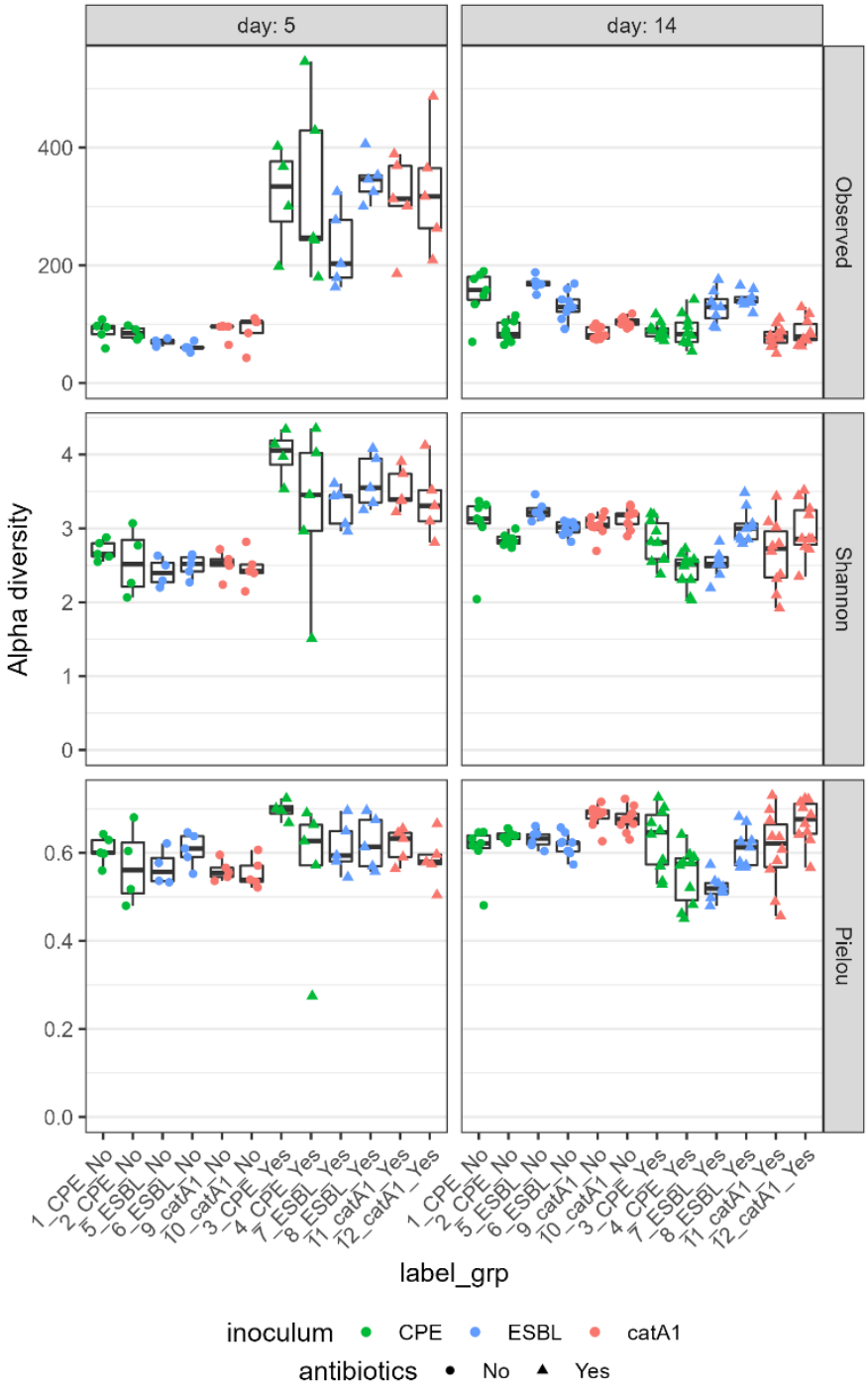

***Figure 3.16**- Boxplots of alpha-diversity (y-axis) by inoculum and antibiotic treatment (horizontal axis) at ASV level. The box indicates the first and third quantiles and the whiskers extend to the smallest and largest values at most 1.5 times the interquartile range from the hinges. Colours indicate 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).*

3. 7. 2. 4 Alpha-diversity: tables

Table 3.7- *Differences in alpha-diversity between groups at genus level on day 5 based on Kruskal-Wallis rank sum test and post hoc Dunn's test. P-values have been adjusted with Benjamini-Hochberg correction. 'No' and 'Yes' refers to pens without and with antibiotics, respectively.*

	Observed		Shannon		Pielou	
comparisons	Z	P.adj	Z	P.adj	Z	P.adj
CPE_No – ESBL_No	0.782	0.6516	0.795	0.6397	0.795	0.5816
CPE_No – catA1_No	0.401	0.8605	0.523	0.8195	1.077	0.4694
ESBL_No – catA1_No	-0.401	0.9388	-0.293	0.8246	0.261	0.8510
CPE_Yes – ESBL_Yes	0.272	0.8418	0.181	0.8566	1.186	0.4418
CPE_Yes – catA1_Yes	0.003	0.9977	0.469	0.7987	1.789	0.2208
ESBL_Yes – catA1_Yes	-0.276	0.9026	0.296	0.8849	0.620	0.6178
CPE_No – CPE_Yes	-3.347	0.0015	-3.181	0.0031	-1.832	0.3349
ESBL_No – ESBL_Yes	-3.964	0.0004	-3.899	0.0007	-1.509	0.3280
catA1_No – catA1_Yes	-3.937	0.0003	-3.408	0.0016	-1.199	0.4940

Table 3.8- *Differences in alpha-diversity between groups at genus level on day 14 based on Kruskal-Wallis rank sum test and post hoc Dunn's test. P-values have been adjusted with Benjamini-Hochberg correction. 'No' and 'Yes' refers to pens without and with antibiotics, respectively.*

	Observed		Shannon		Pielou	
comparisons	Z	P.adj	Z	P.adj	Z	P.adj
CPE_No – ESBL_No	-2.192	0.0425	-1.602	0.4094	0.145	1.0000
CPE_No – catA1_No	2.295	0.0362	-1.190	0.3903	-3.319	0.0045
ESBL_No – catA1_No	4.536	0.0000	0.498	0.7729	-3.411	0.0048
CPE_Yes – ESBL_Yes	-4.522	0.0000	-1.286	0.4250	2.322	0.0759
CPE_Yes – catA1_Yes	1.446	0.1709	1.269	0.3835	0.510	0.7630
ESBL_Yes – catA1_Yes	5.908	0.0000	2.502	0.0925	-1.834	0.1430
CPE_No – CPE_Yes	2.566	0.0193	-0.091	0.9273	-2.172	0.0745
ESBL_No – ESBL_Yes	0.442	0.7051	0.341	0.8459	-0.042	0.9664
catA1_No – catA1_Yes	1.683	0.1155	2.417	0.0783	1.743	0.1355

Table 3.9- *Differences in alpha-diversity between groups at ASV level on day 5 based on Kruskal-Wallis rank sum test and post hoc Dunn's test. P-values have been adjusted with Benjamini-Hochberg correction. 'No' and 'Yes' refers to pens without and with antibiotics, respectively.*

	Observed		Shannon		Pielou	
comparisons	Z	P.adj	Z	P.adj	Z	P.adj
CPE_No – ESBL_No	1.214	0.3063	0.88	0.5679	0.213	0.8313
CPE_No – catA1_No	-0.224	0.8818	0.801	0.5767	1.584	0.2832
ESBL_No – catA1_No	-1.470	0.2125	-0.102	0.9188	1.365	0.3229
CPE_Yes – ESBL_Yes	0.310	0.8733	0.255	0.9216	1.072	0.4726
CPE_Yes – catA1_Yes	-0.077	0.9384	0.360	0.8987	1.557	0.2558
ESBL_Yes – catA1_Yes	-0.397	0.8638	0.108	0.9795	0.498	0.7727
CPE_No – CPE_Yes	-3.409	0.0016	-2.982	0.0061	-1.789	0.2207
ESBL_No – ESBL_Yes	-4.433	0.0000	-3.708	0.0010	-0.982	0.4892
catA1_No – catA1_Yes	-3.443	0.0017	-3.597	0.0008	-1.913	0.2091

Table 3.10 - *Differences in alpha-diversity between groups at ASV level on day 14 based on Kruskal-Wallis rank sum test and post hoc Dunn's test. P-values have been adjusted with Benjamini-Hochberg correction. 'No' and 'Yes' refers to pens without and with antibiotics, respectively.*

	Observed		Shannon		Pielou	
comparisons	Z	P.adj	Z	P.adj	Z	P.adj
CPE_No – ESBL_No	-2.626	0.0162	-1.522	0.1920	0.371	0.7109
CPE_No – catA1_No	1.121	0.3281	-1.605	0.1808	-3.024	0.0075
ESBL_No – catA1_No	3.833	0.0004	0.007	0.9945	-3.356	0.0030
CPE_Yes – ESBL_Yes	-4.292	0.0001	-1.226	0.2751	1.136	0.3198
CPE_Yes – catA1_Yes	0.711	0.5112	-1.793	0.1367	-2.344	0.0477
ESBL_Yes – catA1_Yes	4.973	0.0000	-0.493	0.6664	-3.383	0.0036
CPE_No – CPE_Yes	1.967	0.0738	2.577	0.0249	1.177	0.3262
ESBL_No – ESBL_Yes	0.530	0.5962	2.842	0.0168	1.797	0.1207
catA1_No – catA1_Yes	1.574	0.1575	2.628	0.0258	2.122	0.0635

3. 7. 2. 5 Beta-diversity at ASV level

The inoculum explained 9% and 6% of the variation in Bray-Curtis dissimilarity and Jaccard distance at ASV level on day 5 (i.e., before inoculation), antibiotic treatment explained 22% of the variation for both measures, and their interaction explained 5% of the variation for both measures (Table 3.11). The different inoculums were not separate in the principal coordinate plot (Figure 3.17), apart from the catA1-strain without

antibiotics being separate from the CPE-strain and ESBL-strain for Jaccard distance. In contrast, the groups without and with antibiotics were clearly separate. No differences in dispersion were found.

The inoculum explained 21% and 15% of the variation in Bray-Curtis dissimilarity and Jaccard distance at ASV level on day 14, antibiotic treatment explained 17% and 7% of the variation, and their interaction explained 6% and 5% of the variation (Table 3.12). The catA1-strains were clearly separate from the CPE-strains and ESBL-strains in the principal coordinate plots, whereas the CPE-strains and ESBL-strains overlapped much with each other (Figure 3.17). The groups without and with antibiotics clearly separated with Bray-Curtis dissimilarity but not with Jaccard distance, indicating they differ mostly in the most-abundant ASVs. For both beta-diversity measures, the dispersion in the catA1-strain without antibiotics was different from dispersion in all other groups, and dispersion in the CPE-strain without antibiotics was smaller than dispersion in the ESBL-strain with antibiotics and smaller than dispersion in the catA1-strain with antibiotics, such that the differences could be a difference in location, a difference in dispersion, or both.

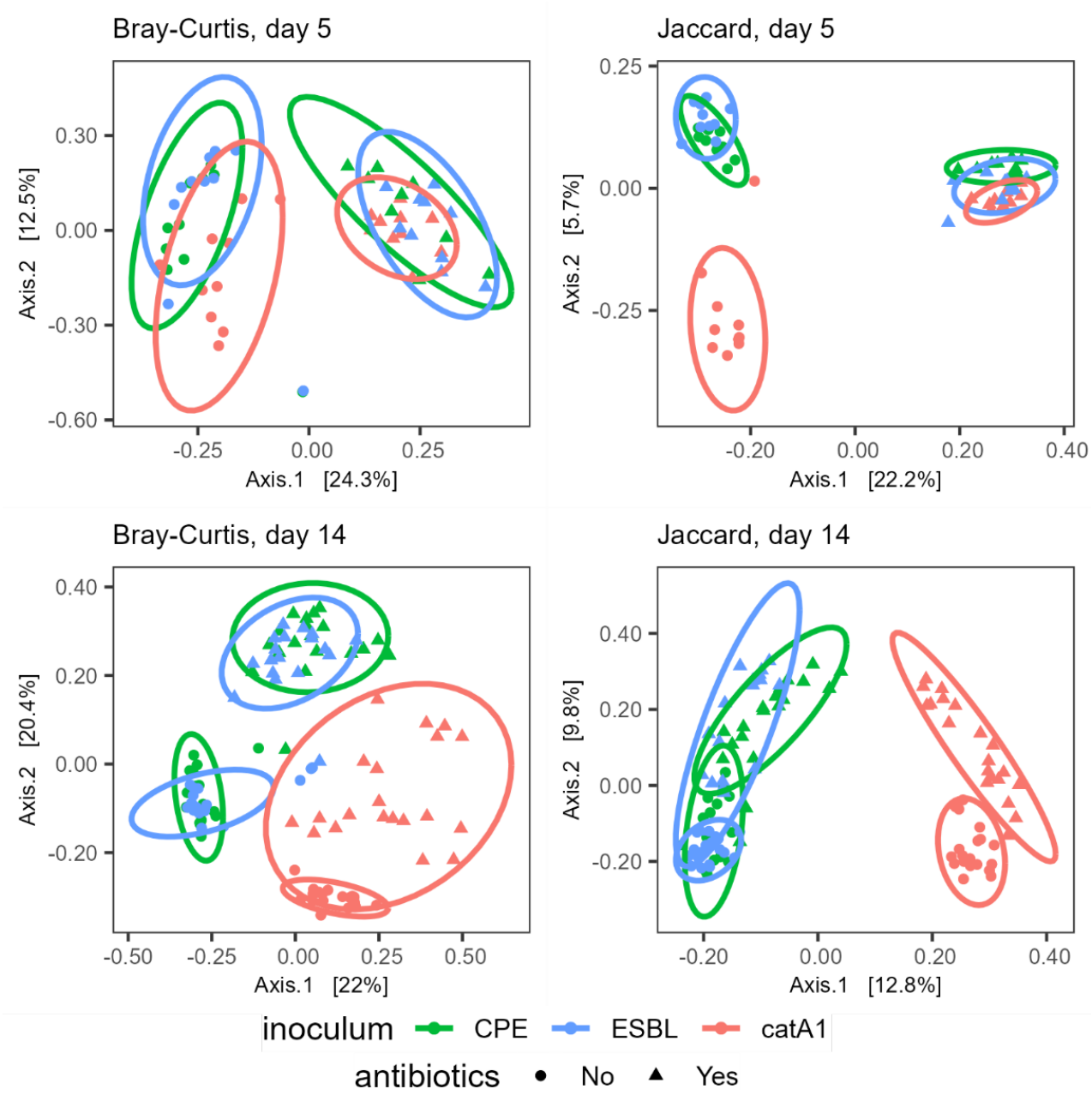

***Figure 3.17**- Principal coordinate analysis of Bray-Curtis dissimilarity (left) and Jaccard distance (right) for day 5 (top) and day 14 (bottom) at ASV level. Colours indicate different inoculums (CPE-strain: green; ESBL-strain: blue; catA1-strain: red) and symbols indicate the absence (circles) or presence (triangles) of antibiotic treatment. Ellipses represent 95% confidence regions assuming a multivariate t-distribution.*

3. 7. 2. 6 Beta-diversity: tables

***Table 3.11**- Permutational multivariate analysis of variance for the effects of E. coli, antibiotics, and their interaction on beta-diversity measured with Bray-Curtis dissimilarity and Jaccard distance at genus level and ASV level on day 5.*

	Bray-Curtis dissimilarity						Jaccard distance					
	Genus level			ASV level			Genus level			ASV level		
	Df	R^2	Pr(>F)	Df	R^2	Pr(>F)	Df	R^2	Pr(>F)	Df	R^2	Pr(>F)
Inoculum	2	0.060	0.003	2	0.092	0.001	2	0.026	0.140	2	0.060	0.001
Antibiotic treatment	1	0.271	0.001	1	0.222	0.001	1	0.501	0.001	1	0.219	0.001
Interaction	2	0.046	0.025	2	0.052	0.004	2	0.027	0.135	2	0.050	0.007
Residual	53	0.623		53	0.634		53	0.446		53	0.672	
Total	58	1.000		58	1.000		58	1.000		58	1.000	

***Table 3.12**- Permutational multivariate analysis of variance for the effects of E. coli, antibiotics, and their interaction on beta-diversity measured with Bray-Curtis dissimilarity and Jaccard distance at genus level and ASV level on day 14.*

	Bray-Curtis dissimilarity						Jaccard distance					
	Genus level			ASV level			Genus level			ASV level		
	Df	R^2	Pr(>F)	Df	R^2	Pr(>F)	Df	R^2	Pr(>F)	Df	R^2	Pr(>F)
Inoculum	2	0.163	0.001	2	0.209	0.001	2	0.169	0.001	2	0.146	0.001
Antibiotic treatment	1	0.093	0.001	1	0.170	0.001	1	0.085	0.001	1	0.072	0.001
Interaction	2	0.045	0.002	2	0.061	0.001	2	0.056	0.001	2	0.046	0.001
Residual	105	0.700		105	0.559		105	0.690		105	0.737	
Total	110	1.000		110	1.000		110	1.000		110	1.000	

3. 7. 2. 7 Similarity percentage analyses: tables

Table 3.13- *Genera that contributed more than 1 per cent to the Bray-Curtis dissimilarity on day 5 (i.e., before inoculation) between groups without and with antibiotic treatment in pens inoculated with CPE. 'No' and 'Yes' refers to pens without and with antibiotics, respectively. Abbreviations: p: permutation p-value.*

Genus	Average contribution	Standard deviation	Average abundance No	Average abundance Yes	p
Escherichia/Shigella	0.104	0.086	15239	27758	0.981
Stenotrophomonas	0.082	0.184	0	13163	0.001
Bacillus	0.072	0.060	84	11411	0.001
Enterococcus	0.050	0.037	831	8920	0.001
Clostridium sensu stricto 1	0.047	0.046	8217	11052	1.000
Blautia	0.036	0.026	8	6018	0.001
Unknown	0.036	0.020	17	6064	0.001
Erysipelatoclostridium	0.033	0.027	3489	7332	0.933
Epulopiscium	0.030	0.028	4027	4490	1.000
Aeribacillus	0.026	0.028	1148	5211	0.022
Paenibacillus	0.020	0.018	2340	5006	0.984
Terrisporobacter	0.013	0.014	649	2441	0.507
Flavonifractor	0.013	0.017	6	2153	0.001
Enterobacter	0.013	0.025	1	2247	0.001

Table 3.14- *Genera that contributed more than 1 per cent to the Bray-Curtis dissimilarity on day 5 (i.e., before inoculation) between groups without and with antibiotic treatment in pens inoculated with ESBL. 'No' and 'Yes' refers to pens without and with antibiotics, respectively. Abbreviations: p: permutation p-value.*

Genus	Average contribution	Standard deviation	Average abundance No	Average abundance Yes	p
Enterococcus	0.142	0.093	1534	24727	0.001
Bacillus	0.081	0.105	70	12864	0.001
Unknown	0.079	0.070	7	12944	0.001
Escherichia/Shigella	0.071	0.048	17265	18512	1.000
Blautia	0.056	0.058	6	8685	0.001
Clostridium sensu stricto 1	0.047	0.033	8585	9634	1.000
Aeribacillus	0.035	0.068	115	6019	0.001
Klebsiella	0.024	0.034	5	3999	0.001
Erysipelatoclostridium	0.018	0.014	4100	2683	1.000

Genus	Average contribution	Standard deviation	Average abundance No	Average abundance Yes	p
Paenibacillus	0.017	0.012	4226	2864	1.000
Epulopiscium	0.014	0.021	1683	1314	0.999
Flavonifractor	0.014	0.011	317	2515	0.002
Anaerostipes	0.013	0.014	1927	1249	1.000
Lachnoclostridium	0.012	0.009	1	1902	0.001

Table 3.15- *Genera that contributed more than 1 per cent to the Bray-Curtis dissimilarity on day 5 (i.e., before inoculation) between groups without and with antibiotic treatment in pens inoculated with catA1. 'No' and 'Yes' refers to pens without and with antibiotics, respectively. Abbreviations: p: permutation p-value.*

Genus	Average contribution	Standard deviation	Average abundance No	Average abundance Yes	p
Clostridium sensu stricto 1	0.122	0.118	6410	24651	0.001
Escherichia/Shigella	0.068	0.056	16952	18135	1.000
Bacillus	0.064	0.067	58	11960	0.001
Enterococcus	0.062	0.071	9330	20546	0.588
Blautia	0.059	0.066	12	13291	0.001
Unknown	0.046	0.045	12	9629	0.001
Unknown	0.023	0.045	9	3900	0.001
Epulopiscium	0.022	0.020	4102	2349	1.000
Thermoactinomyces	0.021	0.058	0	3016	0.001
Aeribacillus	0.021	0.014	824	4300	0.003
Lachnoclostridium	0.018	0.028	4	3408	0.001
Klebsiella	0.014	0.029	3	2068	0.001
Paenibacillus	0.013	0.011	2255	2436	1.000

3. 7. 3 Background to the models

3. 7. 3. 1 Susceptible-infectious model

Environmental bacteria are the source of transmission in the susceptible-infectious model. The overall compartmental model is the following: susceptible animals (S_t) are infected by environmental bacteria (B_t) at a rate given by transmission rate parameter β:

$$\frac{dS_t}{dt} = -\beta S_t B_t \quad (1)$$

$$\frac{dI_t}{dt} = \beta S_t B_t \quad (2)$$

The environmental bacteria are produced by excreting animals (I_t) excreting viable bacteria into the environment at a constant rate of ω units per hour, and decay at rate δ per hour. As a result, the change in the amount of environmental bacteria at time t is given by:

$$\frac{dB_t}{dt} = \omega I_t - \delta B_t \quad (3)$$

The number of bacteria in the environment at time *t* produced by an individual broiler *k* is defined by the time since the start of excretion of broiler *k*, τ_k. The amounts of excreted bacteria are summed within each pen, such that the total amount of environmental bacteria at each time point *t* in a pen with *n* individuals is given by:

$$B_t = \sum_{k=1}^{n} \frac{\omega}{\delta}\left(1 - e^{-\delta\tau_k}\right) \quad (4)$$

The excretion rate (ω) is unknown and unidentifiable in the inference method and therefore we set the excretion rate (ω) to $\frac{\delta^2}{\delta + e^{-\delta} - 1}$ so that the total hazard produced by one excreting broiler during one time unit equals one (Chang and de Jong, 2023). By scaling the excretion parameter, we do not model the number of bacteria in the environment, but the hazard of colonization. This hazard of colonization by environmental bacteria produced by all excreting individuals until that time will now be denoted by E_t, and is found by substitution of $\omega = \frac{\delta^2}{\delta + e^{-\delta} - 1}$ in equation (4):

$$E_t = \sum_{k=1}^{n} \frac{\delta}{\delta + e^{-\delta} - 1}\left(1 - e^{-\delta \cdot \tau_k}\right) \quad (5)$$

β can be interpreted as the rate of transmission per time unit given the hazard $E_t = 1$, which is the hazard presented by one chicken during one time interval. Substitution equation (5) and $\omega = \frac{\delta^2}{\delta + e^{-\delta} - 1}$ in equations (1-3) gives:

$$\frac{dS_t}{dt} = -\beta S_t E_t \quad (6)$$

$$\frac{dI_t}{dt} = \beta S_t E_t \quad (7)$$

$$\frac{dE_t}{dt} = \frac{\delta^2}{\delta + e^{-\delta} - 1} I_t - \delta E_t \quad (8)$$

3. 7. 3. 2 Bayesian hierarchical inference

We defined the likelihood function for the transmission rate parameter of each pen, with the number of new cases ($C_{i,t}$) binomially distributed with the probability of colonization (p_i) given the environmental hazard, where *i* is the numerical index for each pen.

$$C_i \sim Binomial(S_i, p_i) \quad (9)$$

The transmission rate parameter of each pen (β_i) was inferred with non-centred parameterization where the average transmission rate parameter of all pens ($\bar{a}$) is calculated and combined with the variation in the transmission rate parameter between pens (z_i).

$$\beta_i = \bar{a} + z_i \quad (10)$$

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 $\bar{a} + z_i$ of all pens in that specific cluster.

This leads to the number of cases following a likelihood function (Eq. 11) combining the probability of transmission during the time interval between sampling points, p_i ($t \rightarrow t + \Delta t$), environmental hazard (E_t), the decay rate (δ) and input data. In the likelihood function $E_t\left(1 - e^{-\delta \Delta t}\right)$ characterizes a cumulative exposure during the sample interval by the amount of viable environmental bacteria at the beginning of the sampling interval and $I_t \frac{\delta^2}{\delta + e^{-\delta} - 1}\left(\frac{e^{-\delta \Delta t} - 1}{\delta} + \Delta t\right)$ is the cumulative exposure of individuals (I_t) by environmental bacteria during the sampling interval. Both expressions are obtained by integrating the system defined by equations (6) – (8) over a time interval Δt starting at time t with initial values E_t and I_t. I_t are all inoculated and contact broilers

excreting resistant bacteria during a time interval and all animals were assumed to have the same level of infectiousness.

$$\mathrm{E}(C_t + \Delta t) = S_t \times p(t \to t + \Delta t) = S_t \left(1 - e^{-\frac{\beta}{\delta}\left(E_t\left(1-e^{-\delta\Delta t}\right)+I_t\frac{\delta^2}{\delta+e^{-\delta}-1}\left(\frac{e^{-\delta\Delta t}-1}{\delta}+\Delta t\right)\right)} \right) \quad (11)$$

3. 7. 3. 3 Decay rate

Broilers were excreting until the end of the experiment (Table 3.2), making it impossible to estimate the decay rate of *E. coli* by sampling from a prior probability distribution and simultaneously estimate the transmission rate with the Bayesian model in our study because a given number of cases can be explained equally well by a higher transmission rate or by a lower decay rate. We reviewed the literature on decay rates (Table 3.16) to find a suitable range of decay rates and ran the hierarchical model with several fixed decay rates ranging from 0.04 – 55 h^{-1} (Table 3.17). 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 (Burrows and Rankin, 1970; Kovács and Tamási, 1977; Rogers et al., 2011), and *E. coli* excreted from broilers was found to have a decay rate of zero for 14 days (van Bunnik et al., 2014). A decay rate of zero could not be used with the model, therefore we selected the lowest fixed decay rate (δ) of 0.04 h^{-1} in the final model.

Table 3.16- *Decay of Escherichia coli outside a live host. Abbreviations: fc: field capacity.*

ID	Temperature (°C)	Humidity	pH	Environment	Decay rate (per day)	Reference
1	Autumn			Lab	0.102	(Burrows and Rankin, 1970)
2	Autumn			Lab	0.287	(Burrows and Rankin, 1970)
3	4		7	Lab	0.686	(Kovács and Tamási, 1977)
4	January			Lab	0.109	(Rankin and Taylor, 1969)
5	26		7.4	Soil	0.896	(Klein and Casida, 1967)
6	10		7.4	Soil	0.195	(Klein and Casida, 1967)
7				Soil	0.115	(Mallmann and Litsky, 1951)
8			7	Soil	0.371	(Van Donsel et al., 1967)
9				Soil	0.143	(Mallmann and Litsky, 1951)
10		1/3 bar	6.16	Soil	0.473	(Tate, 1978)
11		saturated	6.64	Soil	0.839	(Tate, 1978)
12		100% fc	6.16	Soil	0.796	(Tate, 1978)
13		flooded		Soil	0.382	(Tate, 1978)
14	0			Inoculated water	0.192	(Mitchell, 1968)
15	10	60% fc		Swine manure-amended soil	0.22	(Rogers et al., 2011)
16	10	80% fc		Swine manure-amended soil	0.19	(Rogers et al., 2011)
17	25	60% fc		Swine manure-amended soil	0.40	(Rogers et al., 2011)
18	25	80% fc		Swine manure-amended soil	0.28	(Rogers et al., 2011)
19	10	60% fc		Beef manure-amended soil	0.17	(Rogers et al., 2011)
20	10	80% fc		Beef manure-amended soil	0.15	(Rogers et al., 2011)
21	25	60% fc		Beef manure-amended soil	0.33	(Rogers et al., 2011)
22	25	80% fc		Beef manure-amended soil	0.37	(Rogers et al., 2011)
23	Optimal condition to rear broilers			Broiler pen floor	0.0	(van Bunnik et al., 2014)

Table 3.17- *Transmission rate parameters per hour for different decay rates of Escherichia coli outside a live host. Values are the point estimate at the highest posterior density and ranges are the 95% highest posterior density intervals of the posterior distribution.*

Decay rate (h^{-1})	CPE without amoxicillin	ESBL without amoxicillin	catA1 without amoxicillin	CPE with amoxicillin	ESBL with amoxicillin	catA1 with amoxicillin
0.04	0.0001 [0.0001, 0.0003]	0.0003 [0.0001, 0.0005]	0.0003 [0.0002, 0.0005]	0.0004 [0.0002, 0.0008]	0.0010 [0.0005, 0.0024]	0.0008 [0.0004, 0.0024]
0.05	0.0002 [0.0001, 0.0003]	0.0003 [0.0002, 0.0006]	0.0003 [0.0002, 0.0006]	0.0005 [0.0002, 0.0009]	0.0012 [0.0006, 0.0026]	0.0009 [0.0005, 0.0026]
0.14	0.0004 [0.0003, 0.0008]	0.0006 [0.0004, 0.0013]	0.0007 [0.0004, 0.0013]	0.0010 [0.0005, 0.0019]	0.0021 [0.0011, 0.0048]	0.0018 [0.0010, 0.0052]
0.17	0.0006 [0.0003, 0.0011]	0.0008 [0.0005, 0.0015]	0.0008 [0.0005, 0.0015]	0.0012 [0.0007, 0.0023]	0.0024 [0.0013, 0.0057]	0.0021 [0.0012, 0.0061]
0.22	0.0008 [0.0004, 0.0015]	0.0011 [0.0006, 0.0021]	0.0012 [0.0006, 0.0021]	0.0017 [0.0009, 0.0031]	0.0034 [0.0017, 0.0073]	0.0028 [0.0016, 0.0086]
0.27	0.0010 [0.0006, 0.0018]	0.0014 [0.0008, 0.0026]	0.0014 [0.0008, 0.0025]	0.0021 [0.0011, 0.0040]	0.0041 [0.0021, 0.0088]	0.0036 [0.0020, 0.0104]
0.37	0.0014 [0.0008, 0.0026]	0.0018 [0.0011, 0.0037]	0.0018 [0.0011, 0.0036]	0.0028 [0.0016, 0.0054]	0.0057 [0.0029, 0.0120]	0.0051 [0.0027, 0.0141]
0.45	0.0018 [0.0010, 0.0032]	0.0023 [0.0013, 0.0045]	0.0024 [0.0014, 0.0044]	0.0034 [0.0019, 0.0068]	0.0063 [0.0036, 0.0147]	0.0063 [0.0034, 0.0172]
0.61	0.0025 [0.0013, 0.0045]	0.0033 [0.0018, 0.0063]	0.0033 [0.0019, 0.0061]	0.0048 [0.0027, 0.0093]	0.0086 [0.0049, 0.0200]	0.0087 [0.0047, 0.0246]
0.74	0.0030 [0.0017, 0.0056]	0.0041 [0.0023, 0.0077]	0.0042 [0.0024, 0.0075]	0.0061 [0.0033, 0.0117]	0.0113 [0.0062, 0.0251]	0.0109 [0.0056, 0.0308]
1.00	0.0043 [0.0024, 0.0076]	0.0056 [0.0032, 0.0108]	0.0055 [0.0033, 0.0104]	0.0079 [0.0046, 0.0159]	0.0157 [0.0084, 0.0344]	0.0146 [0.0080, 0.0422]
1.35	0.0056 [0.0032, 0.0105]	0.0076 [0.0043, 0.0147]	0.0071 [0.0044, 0.0143]	0.0114 [0.0062, 0.0214]	0.0209 [0.0112, 0.0480]	0.0193 [0.0111, 0.0567]
1.65	0.0071 [0.0039, 0.0131]	0.0093 [0.0052, 0.0183]	0.0097 [0.0054, 0.0175]	0.0145 [0.0076, 0.0269]	0.0252 [0.0141, 0.0576]	0.0243 [0.0130, 0.0679]
2.23	0.0090 [0.0054, 0.0178]	0.0124 [0.0071, 0.0247]	0.0131 [0.0074, 0.0240]	0.0181 [0.0104, 0.0362]	0.0353 [0.0190, 0.0771]	0.0325 [0.0182, 0.0944]
2.72	0.0118 [0.0066, 0.0216]	0.0162 [0.0088, 0.0305]	0.0163 [0.0090, 0.0293]	0.0229 [0.0131, 0.0447]	0.0436 [0.0234, 0.0960]	0.0411 [0.0220, 0.1140]
3.67	0.0156 [0.0089, 0.0292]	0.0223 [0.0120, 0.0410]	0.0228 [0.0122, 0.0398]	0.0310 [0.0173, 0.0602]	0.0620 [0.0327, 0.1316]	0.0572 [0.0298, 0.1572]
4.48	0.0190 [0.0108, 0.0356]	0.0267 [0.0143, 0.0500]	0.0250 [0.0153, 0.0486]	0.0371 [0.0210, 0.0736]	0.0697 [0.0384, 0.1606]	0.0698 [0.0357, 0.1978]
6.05	0.0262 [0.0146, 0.0487]	0.0360 [0.0200, 0.0683]	0.0348 [0.0201, 0.0664]	0.0527 [0.0288, 0.1000]	0.0978 [0.0523, 0.2203]	0.0885 [0.0500, 0.2644]
7.39	0.0317 [0.0181, 0.0590]	0.0446 [0.0237, 0.0838]	0.0444 [0.0253, 0.0804]	0.0616 [0.0352, 0.1213]	0.1245 [0.0641, 0.2661]	0.1168 [0.0626, 0.3162]
20.09	0.0862 [0.0491, 0.1595]	0.1250 [0.0649, 0.2245]	0.1183 [0.0691, 0.2153]	0.1739 [0.0938, 0.3321]	0.3245 [0.1757, 0.7294]	0.3069 [0.1697, 0.8615]
54.60	0.2278 [0.1350, 0.4424]	0.3181 [0.1797, 0.6136]	0.3219 [0.1857, 0.5967]	0.4699 [0.2545, 0.8954]	0.8932 [0.4778, 1.9827]	0.9289 [0.4530, 2.3571]

3. 7. 3. 4 Posterior distribution of the model parameters

The posterior distribution is a multiplicative product of the prior distribution and the likelihood of producing the observed data. Figure 3.18 shows the overlapping prior and posterior distribution for the average transmission rate parameter over all pens ($\bar{a}$; left) and the between-pen variation of the transmission rate parameter (z_i; right).

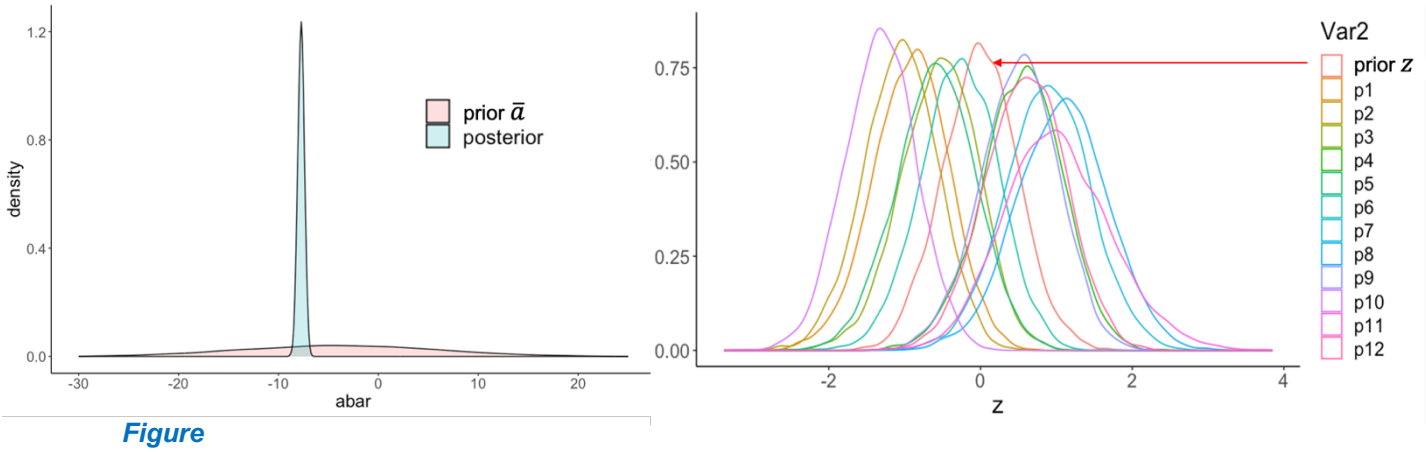

Figure 3.18*- Prior and posterior distributions for the average transmission rate parameter of all pens ($\bar{a}$; left) and the variation in the transmission rate parameters between pens (z_i; bottom) that are needed to obtain the transmission rate parameters (β).*

3. 7. 3. 5 Model diagnostics

Four thousand samples were drawn from each chain in which the first 2000 samples were warm-up samples. The model resulted in effective sample sizes (*ESS*) of 1200 in the average transmission rate over all pens ($\bar{a}$) and 2200 in the between-pen variation of the transmission rate ($z_i\sigma$). This effective sample size is above the number of samples, indicating efficient sampling of the posterior distribution. Gelman-Ruben convergences ($\hat{R}$) of 1 indicated that the Markov Chains converged. Similarly, patterns of the chains showed they rapidly explore a wide distribution and have a similar central tendency with a similar location with high probability, showing their good mixing, stability and convergence (McElreath, 2020; Stan Development Team) (Figure 3.19).

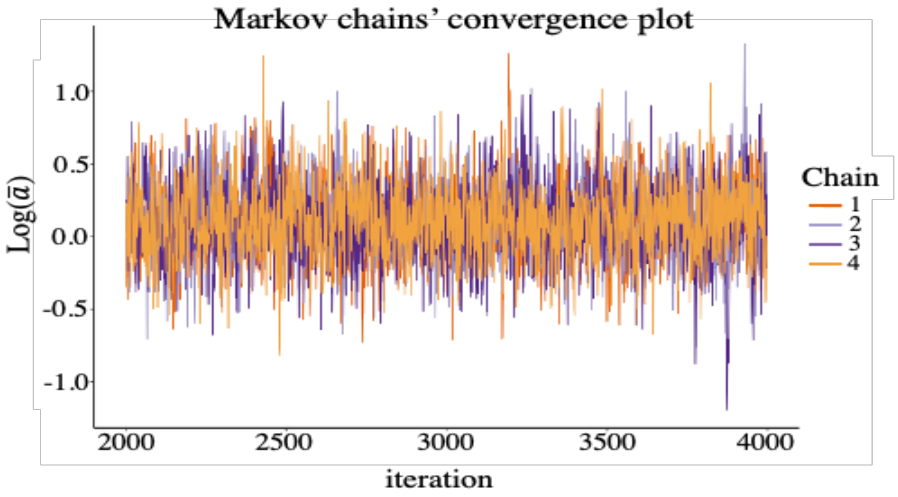

Figure 3.19- *Samples from each Markov chain are plotted sequentially in a trace plot. The horizontal axis indicates the serial number of draws (the first 2000 draws are the warm-up and have been removed) and the y-axis indicates the parameter value.*

3. 7. 3. 6 SIS-model result

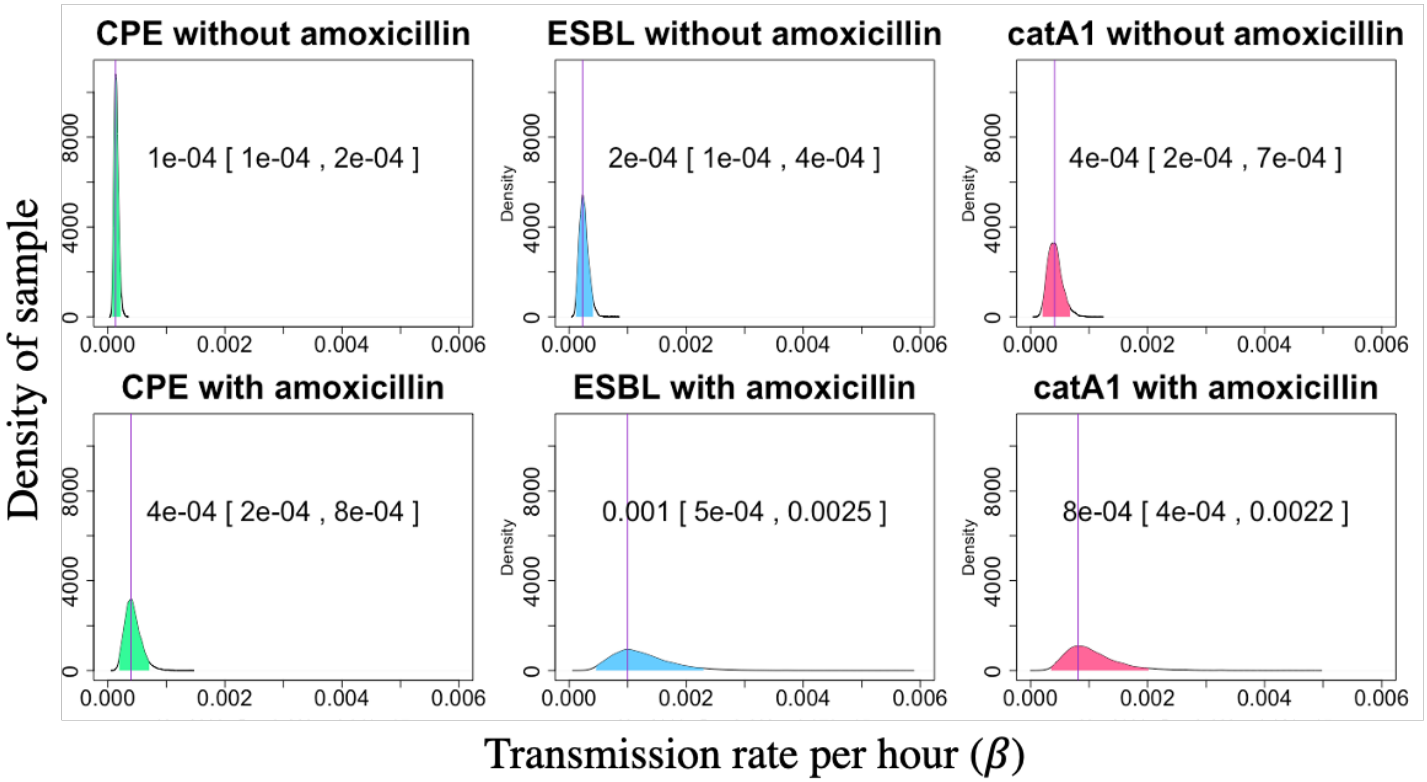

Figure 3.20 - *Density (vertical axis) of the posterior distribution of the transmission rate per hour (horizontal axis) for the CPE-strain, ESBL-strain and catA1-strain in the SIS-model. The top and bottom row show plots for the pens without and with amoxicillin treatment, respectively. Purple vertical 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.*

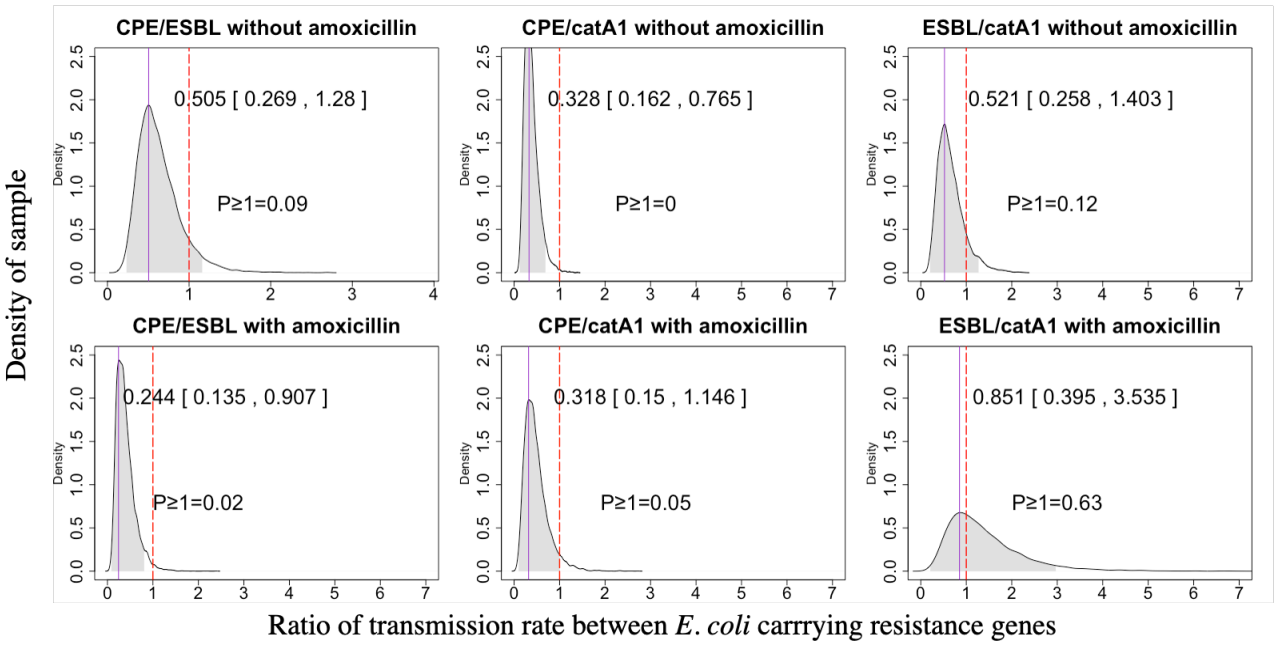

Figure 3.21- *Density (vertical axis) of the posterior distribution of the ratio of the transmission rates (horizontal axis) for different inoculums in the SIS-model: CPE-strain to ESBL-strain, CPE-strain to catA1-strain, and ESBL-strain to catA1-strain. The top and bottom row show plots for the pens 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.*

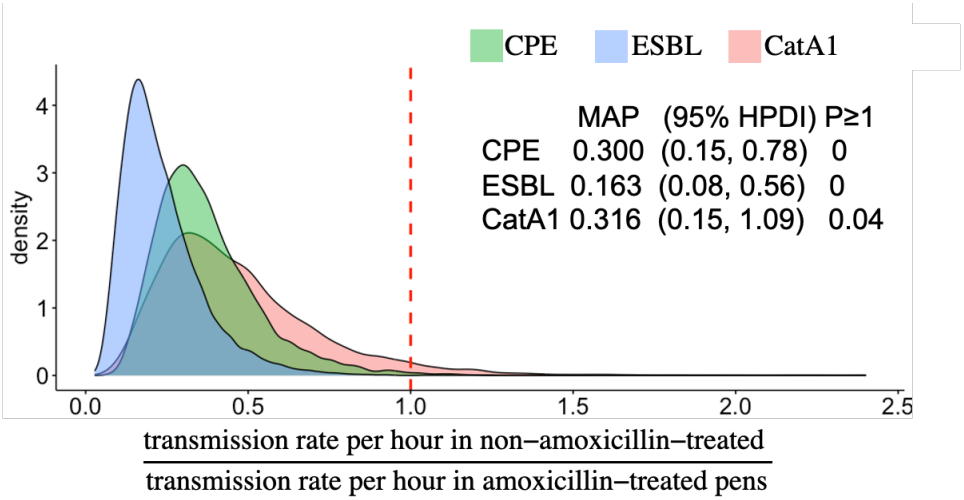

Figure 3.22- *SIS-model: Density (vertical axis) of the ratio of the transmission rates in non-amoxicillin-treated pens over amoxicillin-treated pens (horizontal axis) for the CPE-strain (green), ESBL-strain (blue) and catA1-strain (pink). The dotted red vertical line indicates a ratio of 1 (i.e., the 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.*

3. 7. 4 Plasmids and resistance genes

ResFinder 4.1 (Florensa et al., 2022) and PlasmidFinder 2.0 (Carattoli et al., 2014) were used to characterize the resistance genes and plasmids in the different inoculums. The CPE strain used in the animal experiment contained resistance genes from 6 families (*Table*) and 3 plasmids with incompatibility types IncHI2, IncHI2A, and IncQ1. The ESBL strain contained resistance genes from 4 families (*Table*) and 6 plasmids with incompatibility types ColpVC, IncFIB, IncFII, IncHI2, IncHI2A, and IncQ1. The catA1 strain contained resistance genes from 4 families (*Table*) and 4 plasmids with incompatibility types IncFIB, IncFII, IncI1-I, and IncI2.

***Table 3.18**- The resistance genes present in the CPE strain.*

Antimicrobial class	Genetic background	Antimicrobial resistance
amphenicol	floR	chloramphenicol
beta-lactam	blaOXA-162	ampicillin, cefepime, ertapenem, imipenem, meropenem, piperacillin, tazobactam
beta-lactam	blaTEM-1B	ampicillin
folate pathway antagonist	sul2	sulfamethoxazole
polymyxin	mcr-1.1	colistin
quinolone	gyrA (p.S83L)	ciprofloxacin, nalidixic acid
tetracycline	tet(A)	tetracycline

***Table 3.19**- Resistance genes present in the ESBL strain.*

Antimicrobial class	Genetic background	Antimicrobial resistance
beta-lactam	blaCTX-M-2	ampicillin, cefepime, cefotaxime, ceftazidime
beta-lactam	blaTEM-1B	ampicillin
folate pathway antagonist	dfrA1	trimethoprim
folate pathway antagonist	sul1, sul2	sulfamethoxazole
quinolone	gyrA (p.S83L)	ciprofloxacin, nalidixic acid
tetracycline	tet(A), tet(B)	tetracycline

***Table 3.20**- Resistance genes present in the catA1 strain.*

Antimicrobial class	Genetic background	Antimicrobial resistance
amphenicol	catA1	chloramphenicol
beta-lactam	blaTEM-1B	ampicillin
folate pathway antagonist	dfrA1	trimethoprim
folate pathway antagonist	sul2	sulfamethoxazole
tetracycline	tet(A)	tetracycline

Chapter 4

Transmission rates of veterinary and clinically important antibiotic resistant *Escherichia coli*: a meta-analysis

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Abstract

The transmission rate per hour between hosts is a key parameter for simulating transmission dynamics of antibiotic-resistant bacteria, and might differ for antibiotic resistance genes, animal species, and antibiotic usage. We conducted a Bayesian meta-analysis of resistant *Escherichia coli (E. coli)* transmission in broilers and piglets to obtain insight in factors determining the transmission rate, infectious period, and reproduction ratio. We included $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{OXA-162}}$, *catA1, mcr-1,* and fluoroquinolone resistant *E. coli*. The Maximum a Posteriori (MAP) transmission rate in broilers without antibiotic treatment ranged from $0.4 \cdot 10^{-3}$ to $2.5 \cdot 10^{-3}$ depending on type of broiler (SPF vs conventional) and inoculation strains. For piglets, the MAP in groups without antibiotic treatment were between $0.7 \cdot 10^{-3}$ and $0.8 \cdot 10^{-3}$, increasing to $0.9 \cdot 10^{-3}$ in the group with antibiotic treatment. In groups without antibiotic treatment, the transmission rate of resistant *E. coli* in broilers was almost twice the transmission rate in piglets. Amoxicillin increased the transmission rate of *E. coli* carrying $bla_{\text{CTX-M-2}}$ by three-fold. The MAP infectious period of resistant *E. coli* in piglets with and without antibiotics is between 971 and 1,065 hours (40 – 43 days). The MAP infectious period of resistant *E. coli* in broiler without antibiotics is between 475 and 2,306 hours (20 – 96 days). The MAP infectious period of resistant *E. coli* in broiler with antibiotics is between 2,702 and 3,462 hours (113 – 144 days) which means a lifelong colonization. The MAP basic reproduction ratio in piglets of infection with resistant *E. coli* when using antibiotics is 27.70, which is higher than MAP in piglets without antibiotics between 15.65 and 18.19. The MAP basic reproduction ratio in broilers ranges between 3.46 and 92.38. We consider three possible explanations for our finding that in the absence of antibiotics the transmission rate is higher among broilers than among piglets: i) due to the gut microbiome of animals, ii) fitness costs of bacteria, and iii) differences in experimental set-up between the studies. Regarding infectious period and reproduction ratio, the effect of the resistance gene, antibiotic treatment, and animal species are inconclusive due to limited data.

Keywords; Meta analysis, Antibiotic resistant bacteria, Bayesian hierarchical, Transmission rate, Infectious period

4. 1 Introduction

Transmission dynamics of antibiotic-resistant bacteria between livestock hosts are widely unknown despite the damaging impact of therapeutic failure due to antibiotic resistance in all animal species including humans. Cases of resistant bacteria against important last resort antibiotics such as carbapenem-resistant *E. coli* (CPE) have been occurring worldwide in livestock since 2010 (Köck et al., 2018). However up to now, CPE has not spread as extensively among livestock as extended spectrum beta-lactamase *E. coli* (ESBL) (Dahms et al., 2015).

Simulation modelling is a helpful tool to assess antibiotic resistant bacteria transmission dynamics and to evaluate intervention programs. While transmission simulations have traditionally been instrumental in comprehending the spread of infectious diseases within populations (Keeling and Rohani, 2008), their utility extends to the domain of antibiotic-resistant bacteria dynamics. Numerous studies have utilized simulation method to detangle the intricacies of resistant bacteria dissemination and persistence within livestock populations, thereby providing essential insights for bolstering surveillance efforts (Lanzas et al., 2011; Sorenson et al., 2017; Schulz et al., 2018). With modeling, we can simulate transmission dynamics that would otherwise be difficult to study in real-world situations due to economical and ethical constraints. Still, these simulations require a wide range of parameters, including the transmission rate. The accurate determination of the transmission rate (β) hold great importantance, as it significantly determines the model's outcomes and subsequent predictions (Kirkeby et al., 2017). Furthermore, transmission rate (β) is essential for the calculation of another vital parameter -the basic reproduction ratio (R_0). The basic reproduction ratio (R_0) is a vital parameter in epidemiology due to its role in predicting the number of new infections originating from an infectious animal during its period of infectivity. Following the introduction of antibiotic-resistant bacteria, R_0 is instrumental in gauging whether the bacteria will succeed in invading the susceptible population (Keeling and Rohani, 2008). A successful invasion becomes feasible when R_0 exceeds the threshold of 1. R_0 is calculated from the infectious period and the transmission rate, where the infectious period is the length of time that individual animal had been infectious until it returned to uncolonized state.

Transmission rates and infectious periods are most precisely estimated from transmission experiments in which animals are inoculated and the infection is allowed to spread to susceptible contact animals. To calculate the transmission rate, the infection status of individual animals is tracked over time. However, transmission experiments are restricted in size, treatment groups, housing and management conditions and limited sampling times due to costs (labor intensive), and ethical reasons (Hu et al., 2017). These restrictions often result in censored data for the infectious period because the moment that animal return to uncolonized state is beyond the end of experiment (Turkson et al., 2021). Consequently, there are no transmission experiments for resistant *E. coli* that have observed the full infectious period or that test multiple relevant factors such as antibiotic treatment, resistance gene, and animal species. To quantify the impact of antibiotic treatment, resistance gene, and animal species on the transmission rate of resistant *E. coli*, we conducted Bayesian meta-analysis of available transmission experiments.

Through the combination of multiple studies and incorporation of prior knowledge into the analysis, Bayesian meta-analysis can enhance the precision of the estimations of transmission rates and infectious period obtained from longitudinal experimental studies. The Bayesian hierarchical method, although well-established in various fields, is relatively uncommon in the veterinary domain (Gelman and Hill, 2006). However, its adoption here proves invaluable. The probabilistic prediction produced by this method is informative of both the data and the model, providing a more accurate representation of the uncertainty surrounding the estimations (McElreath, 2020). Meta-analysis increases sampling power by joining small scale studies with partial pooling, while penalizing against overfitting by using regularizing priors (McElreath, 2020). Bayesian inference is flexible and intuitive due to its adjustable prior and likelihood components (McElreath, 2020). Also, Bayesian inference produces a prediction in the form of a posterior distribution which is more informative of the model and data than a confidence interval (Gelman et al., 2021; Hiura et al., 2021; Vilares and Kording, 2011). The posterior distribution reflects the variability of the data, likelihood model and prior information while the confidence interval assumes that the entire range of the confidence interval of a uniform distribution has equal opportunity to be the true value.

Here, Bayesian meta-analysis was employed to infer transmission rates and infectious periods of *E. coli* with different resistance genes in both piglets and broilers from transmission experiments. Environmental transmission was assumed, because bacteria such as *E. coli* are transferred between animal hosts through the faecal-oral route and can survive in the farm environment as long as 30 days (Lister and Barrow, 2008; van Bunnik et al., 2014; van Elsas et al., 2011). We aimed to identify factors determining the transmission rate and whether these resistant bacteria will successfully invade livestock populations after their introduction.

4. 2 Materials and methods

4. 2. 1 Systematic literature review and data extraction

This Bayesian meta-analysis was conducted following the PRISMA-P: Preferred Reporting Items for Systematic review and Meta-Analysis Protocols 2015 checklist. The extensive protocol is included in Supplementary 4.7.1.

First author (ND) performed the literature search in 2022. Pubmed and Google Scholar were the online database in which the search is performed. The search strategy encompassed a combination of three distinct categories of search terms: those related to meat-producing livestock, antimicrobial-resistant bacteria, and longitudinal data. The initial search results were carefully screened to remove duplicate records. Subsequently, a set of specific selection criteria were applied to identify relevant studies, which included: 1) inclusion of longitudinal data 2) presence of distinct contact and challenge animals, with the challenge animals being inoculated with non-pathogenic resistant bacteria 3) restriction to studies involving non-pathogenic resistant bacteria and meat-producing animal as the host species. Throughout this process, we implemented a hierarchical screening approach. We began by thoroughly reviewing the titles of the identified records to identify relevant studies. Next, we proceeded to screen the abstracts of the remaining records, further narrowing down the selection. Finally, the smallest subset of records underwent a comprehensive review, with the entire manuscripts being scanned with the selection criteria.

4. 2. 1. 1 Outcome

The excreting status (positive or negative for resistance markers) of individual animals was extracted at each sampling time point. For each individual animal, we extracted the pen information, the inoculation strain, any antibiotic treatments (yes/no), and the inoculation status (inoculated animal versus contact animal). Contact animals were classified as susceptible animals and could become cases, subsequently becoming infectious animals, whereas the inoculated animals could only become infectious but were not counted as cases.

The number of hours that an animal (contact and inoculated) excreted *E. coli* carrying resistance was extracted as an input for the infectious period (*D*). Resistance is defined as either resistance gene or phenotypic resistance. We assumed that all individuals would stop excreting the *E. coli* carrying resistance at the end of their infectious period (return to uncolonized state). Hence, to extract the infectious period, we counted hours from the first sampling time point that an animal is excreting (positive for resistance marker) until the first sampling time point that an animal stop excreting (negative for resistance marker). Only animals that exhibit at least two consecutive negative samples were considered to have undergone loss of colonization and potentially became colonized again. Animals that showed a single negative sample following a positive result, and return to an uncolonized state, were adjusted by reclassifying that negative sample as positive. Additionally, we run the analysis in the dataset that did not have reclassification of single negative sample. The result of the analysis is included in Supplementary 4.7.5. Animals that return to uncolonized state and became infectious again could have more than one infectious period.

If the time that an animal's return to an uncolonized state is censored, indicating that the animal continues to excrete *E. coli* carrying resistance genes until the end of the experiment, we calculate the infectious period by measuring the time from the initial sampling time point, when the animal starts excreting (positive for the resistance marker) until the last observed sampling time point. In this context, we assume that the period of time during which an animal returns to an uncolonized state extends beyond the actual end of the experiment. This assumption about the time for an animal to return to an uncolonized state follows the gamma distribution, accounting for variations in return dynamics among the subjects.

4. 2. 2 Data synthesis

Before we apply the Bayesian hierarchical model, we adopted the Meta-analysis of Individual Participant Data technique to extract individual animal outcomes, such as excretion status across time points. These data were subsequently organized into pen clusters, facilitating analysis. Subsequently, the Bayesian hierarchical model was applied, incorporating the complete individual dataset, and treating pen clusters as random effects.

4. 2. 2. 1 Transmission model

We used a susceptible-infectious-susceptible (SIS) transmission model with environmental transmission (Gerhards et al., 2022). Within the same pen, susceptible animals (S_t) may become colonized (I_t) through infectious material deposited in the environment and can subsequently return to uncolonized state and become susceptible again. Infectious material deposited in the environment determines the instantaneous environmental hazard (E_t). The excreted bacteria and thus the hazard will decay with a constant rate (δ) per hour and the hazard due to viable bacteria results in colonization with rate (β) per hour of susceptible animals (Dankittipong et al., 2023). Because we do not know the exact number of bacteria excreted by a broiler chicken or pig, we scaled this excretion into one unit of excreted bacteria by one animal per hour (Gerhards et al., 2022) using scaling factor $\omega = \frac{\delta^2}{\delta + e^{-\delta} - 1}$. The R_0 in this model is for an average infectious period (D) : $R_0 = \frac{\beta\omega}{\delta} D = \beta \frac{\delta}{\delta + e^{-\delta} - 1} D$. (Gerhards et al., 2022).

4. 2. 2. 2 Bayesian hierarchical inference for transmission rate per hour

We applied Bayesian inference for the parameters of the transmission model for each pen i. The transmission rate (hour^{-1}) parameter of each pen (β_i) was calculated in two steps. The number of new cases during a time interval is taken to be binomially distributed with logit link function comprised of the number of trials equalling the number of susceptible animals in pen (S_i) and the probability of transmission (p_i). The probability of transmission during an interval is calculated given a pen-specific transmission rate per hour (β_i). In the log likelihood function, the log transmission rate

per hour is modeled with the mean log population transmission rate per hour ($\log(\bar{\beta})$) and the variation of transmission rate between pens (z_i). The exponent of the log transmission rate per hour is then multiplied by the instantaneous hazard of colonization (E_t). This hazard is obtained by scaling the excretion to the total amount of bacteria excreted by an animal per unit of time, (Gerhards et al., 2022). The decay rate per hour of *E. coli* carrying resistance genes in environment could not be estimated from our data. Therefore, we reviewed literature for estimated decay rates of *E. coli* in environment and applied these to the model (Table 4.4). We used weighted Akaike information criterion (WAIC) and the number of divergences to select the decay rate thus based on the model's goodness-of-fit to the observed data while considering its complexity. WAIC is particularly useful for comparing models with different parameters, while divergences can help diagnose issues with the Markov chain Monte Carlo (MCMC) algorithm's convergence and posterior estimates.

A weakly informative prior for the log mean transmission rate per hour ($\log(\bar{\beta})$) follows a normal distribution with mean of -10 and standard deviation of 10. The prior for variation of transmission rates per hour between pens (z_i) follows a normal distribution with mean of 0 and standard deviation of 1 (Table 4.5). We used a fixed decay rate per hour (δ) of 0.13 hour^{-1}.

The posterior distribution of the transmission rate per hour in each pen (β_i) was extracted and, for comparisons, grouped and averaged by the resistance gene in the inoculation, host species and antibiotic treatment. The posterior distributions are either presented in figures or by the Maximum A Posteriori (MAP) and 97% Highest Probability Density Interval (97% HDPI).

To compare two transmission rates per hour for different factors such as animal species, we determined the entire posterior distribution of ratios between the transmission rates per hour of two factors by dividing the rates per hour in each sample of the posterior distribution. A ratio of one means the transmission rates per hour are equal for the two factors. Furthermore, we determined whether the transmission for one factor was lower than the other by calculating the probability that the ratio is lesser than one (P<1) by summing iterations that resulted in ratio lessor than one and dividing the sum with the total number of iterations.

4. 2. 2. 3 Bayesian parametric survival analysis for infectious period

Bayesian parametric survival analysis was used to quantify the infectious period (*D*) in each pen (i). The infectious period (*D*) refers to the duration (in hours) during which an animal is colonized before returning to an uncolonized state and becoming susceptible to the disease. We assumed that the observed infectious periods of all animals in each pen (D_{obs_i}) follows a gamma distribution with the pen-specific shape (a_i) and a rate parameter which is same at each animal (b), where the shape parameter is normally distributed with the mean population shape ($\bar{a}$) and variation of the shape for each pen (σ_i). For censored values of the infectious period, we characterized the distribution of the infectious period to be a cumulative gamma distribution of shape (a_i) and rate (b_i).

Prior information of the infectious period was obtained from studies of *E. coli* O157 in one-day-old specific-pathogen free (SPF) layer chickens and extended-spectrum cephalosporin-resistant *E. coli* in commercial piglets and fattening pigs (Moor et al., 2021; Ragione et al., 2005). In broiler, a regularizing weakly informative prior for the mean population shape ($\bar{a}$) follows a normal distribution with mean of 0 and standard deviation of 1. The variation of the shape of gamma from each pen (σ_i) follows an exponential distribution with the rate of 1. The rate parameter (b_i) follows a standard normal distribution with mean of 0 and standard deviation of 1. In piglets, a regularizing weakly informative prior for the mean population shape ($\bar{a}$) follows a normal distribution with mean of 0 and standard deviation of 2 and the same prior for other parameters. The entire posterior distributions of shape in each pen (a_i) and rate (b_i) were extracted. The estimated mean infectious period of each pen (D_i) was calculated by dividing shape parameter in each pen (a_i) with rate (b_i).

4. 2. 2. 4 Bayesian hierarchical inference for reproduction ratio

The posterior distribution for the basic reproduction ratio of each pen (R_{0_i}) is derived from each sample of the posterior distribution of the transmission rate per hour in each pen (β_i) combined by each sample from the posterior distribution of the infectious period of each pen (D_i).

$$R_{0_i} = (\beta_i \otimes D_i) \ \frac{\delta}{\delta + e^{-\delta} - 1}$$

To illustrate, we take 20,000 samples from the posterior distribution of transmission rate per hour in each pen. We then perform a multiplication process where each sample from this distribution is paired with the corresponding sample from the posterior distribution of the mean infectious period of each pen (D_i). This element-by-element multiplication ensures that the posterior distribution of R_{0_i} encompass all potential combinations of transmission rates per hour and infectious periods (hours), thereby accurately accounting for their relationship. Consequently, we obtain the posterior distribution of the basic reproduction ratio of each pen (R_{0_i}) resulting in a total of 4×10^8 derived from $20{,}000^2$ samples. In the total, we obtained 6.4×10^{10} estimated for the basic reproduction ratio across 40 pens.

Similary to transmission rate per hour, reproductive ratio (R_{0_i}) and mean infectious period of each pen (D_i) were grouped based on the resistance gene in the inoculation, the host species and the antibiotic treatment. We extracted and presented the average transmission rate per hour from multiple pens with the same variables and presented as Maximum A Posteriori (MAP) and 97% Highest Probability Density Interval (97% HDPI).

All analysis were done in R version 4.1.2 (R development Core Team, 2022) and Bayesian inference was done in RStan 2.21.5 (Stan Development Team, n.d.) with 14 tree depth, 0.99 acceptance rate and 4 chains, each chain with 10,000 iterations. 5,000 iterations from each chain were excluded as warm-up samples resulting in a total of 20,000 iterations from 4 chains. The codes will be provided as supplementary 4.7.4.

4. 3 Result

4. 3. 1 Literature search result

The initial search across the Pubmed and Google Scholar databases yielded a total of 2,055 papers. Following a review, 3 duplicate papers were identified and subsequently excluded from the dataset. Among the remaining entries, 21 publications were found to be pertinent to the topic of resistant bacteria in livestock. After a screening process, 5 publications met the criteria for inclusion in the final analysis

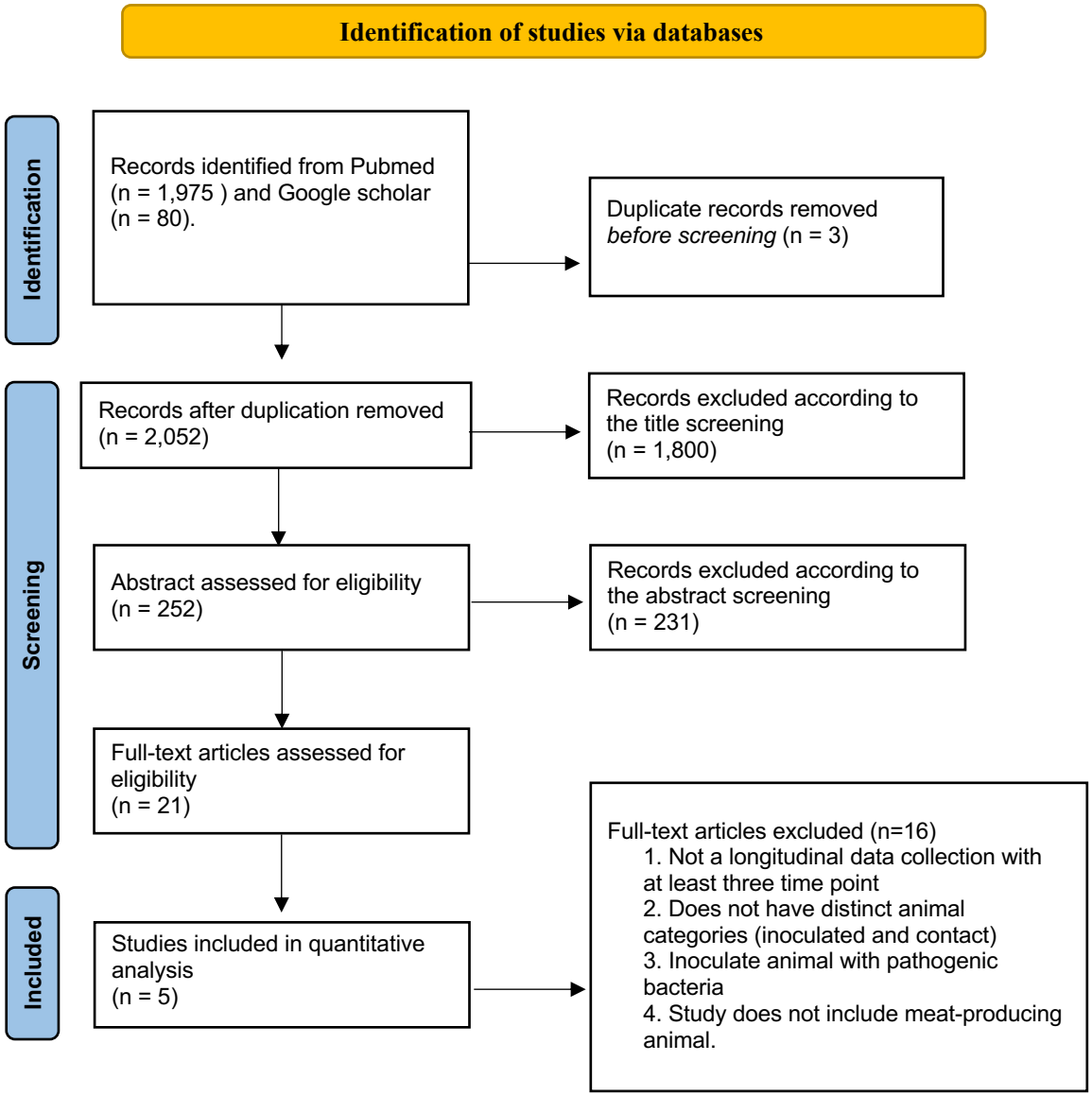

Figure 4.1- PRISMA protocol for systematic literature review to collect longitudinal data of resistance genes transmission between meat-producing animals.

4. 3. 2 Risk of bias

Cochrane's risk of bias assessment was applied to all included studies (Higgins et al., 2011). Overall, the studies share a similar experimental design, phenotypic resistance analysis, and individual resistance reporting. Consequently, the risk of biases in all studies is low, although there are minor concerns related to the absence of blinding the experimenters to the treatment and the lack of a pre-specified experiment plan in the records. We consider these concerns negligible since the outcomes, including

resistance and susceptible status of individual animals at each sampling time point, are objectively determined by the EU protocol (ECDC, 2023)(Figure 4.1).

4. 3. 3 Transmission experiment data

We extracted three longitudinal experimental studies in broilers, with in total 170 one-day old conventional broilers and 36 five-day old SPF broilers. Cloacal samples were enriched overnight and then inoculated onto selective MacConkey plates supplemented with antibiotics of interest. In broilers, the concentration of antibiotics and antibiotics of choice were consistent across all studies, comprising 1 mg/L of cefotamine, 0.5 mg/L of ertapenem, or 64 mg/L chloramphenicol. Dame-Korevaar et al. (2018) investigated the transmission rate of *E. coli* carrying $bla_{\text{CTX-M-1}}$ resistance gene with $0.5{\cdot}10^{1}$ and $0.5{\cdot}10^{2}$ cfu/animal inoculation doses in one-day old conventional broilers (Table 4.1). Ceccarelli et al. (2017) inoculated five-day old SPF broilers with *E. coli* carrying $bla_{\text{CTX-M-1}}$ genes with doses of $0.5{\cdot}10^{6}$ and $0.5{\cdot}10^{8}$ cfu/animal. Dankittipong et al. (2023) evaluated the transmission of *E. coli* carrying $bla_{\text{OXA-162}}$, *E. coli* carrying $bla_{\text{CTX-M-2}}$, and *E. coli* carrying *catA1*, all of which were inoculated at $0.5{\cdot}10^{3}$ cfu/animal in five-day old conventional broilers. In this study, half of the animals received amoxicillin treatment (20 mg/kg of broiler) for five days starting three days before inoculation (Dankittipong et al., 2023). Thus, half of the animal were inoculated during antibiotic treatment In all three studies, the inoculated and susceptible chicks acquired *E. coli* carrying resistance genes, except for one pen of conventional broilers that were inoculated with $0.5{\cdot}10^{1}$ cfu/animal of *E. coli* carrying $bla_{\text{CTX-M-1}}$ in Dame-Korevaar et al (2018). In this pen none of the inoculated animals started shedding and thus the transmission rates cannot be estimated. From five studies, we extracted a total of 204 infectious periods from 191 broilers, 13 of which returned to uncolonized state and were recolonizied.

For piglets, we extracted three longitudinal experimental studies with in total 101 SPF piglets of seven to eight weeks old. Rectals samples from piglets were enriched overnight and cultured on Chromagar plates with relevant antibiotic supplements. Antibiotic concentrations varied slightly between studies. For the *mcr-1* resistance study by Mourand et al. (2018, 2019), plates were supplemented with 250 mg/L rifampicin, while the fluoroquinolone resistant study by Andraud et al. (2011) employed 0.5 mg/L ciprofloxacin. Two experiments were conducted by Mourand et al. (2018,

2019) to test transmission rate of *E. coli* carrying *mcr-1* resistance genes with $2.5 \cdot 10^5$ and $2.5 \cdot 10^8$ cfu/animal inoculation doses. In the study of Mourand et al. (2019), colistin was administered at a dosage of 12,500 IU/kg (which is 4 mg/kg) live weight for three days. This administration occurred through two separate protocols within two distinct groups of piglets. In the first group, colistin treatment was initiated seven days before the planned inoculation. In contrast, the second group received colistin administration just one hour before the planned inoculation on day 7. Subsequently on day 7, piglets of eight weeks old from both groups were inoculated with $2.5 \cdot 10^8$ cfu/animal inoculation doses. The two pens, previously treated with colistin seven days before inoculation, were excluded from the analysis because these results could not be compared between piglets and broilers (i.e., inoculation during antibiotic treatment). In the study of Mourand et al. (2018), *E. coli* carrying *mcr-1* resistance genes with $2.5 \cdot 10^5$ and $2.5 \cdot 10^8$ cfu/animal inoculation doses were inoculated to seven-week old piglets. Two pens inoculated with $2.5 \cdot 10^5$ cfu/animal did not result in any shedding in the inoculated animals and thus were excluded from the analysis. Point-mutated fluoroquinolone resistant *E. coli* transmission between seven weeks old piglets was studied by Andraud et al. (2011) with an inoculation dose of 10^{10} cfu/animal. All piglets in seven pens became colonized with *E. coli* carrying fluoroquinolone resistance. A total count of 81 piglets were obtained from three separate studies. Out of these, 18 piglets experienced a return to uncolonized state and recolonization. As a result, a cumulative total of 99 instances of infectious periods were considered for the estimation of the infectious period. Overall, 27 pens of broilers and 13 pens of piglets were included in the inference of transmission rate per hour.

4. 3. 4 Transmission rate of resistant bacteria within same host species

Overall transmission rates per hour of *E. coli* carrying resistance in piglets ranged from $0.4 \cdot 10^{-3}$ h^{-1} to $2.5 \cdot 10^{-3}$ h^{-1}, according to the lowest to highest value of 97% highest posterior density interval (97% HPDI). Among piglets, the highest Maximum a Posteriori (MAP) transmission rate ($0.9 \cdot 10^{-3}$ h^{-1}) is from *E. coli* carrying *mcr-1* in piglets treated with colistin. In the piglet group without antibiotic treatment, the MAP transmission rate of *E. coli* carrying fluoroquinolone resistance and *mcr-1* are $0.7 \cdot 10^{-3}$ h^{-1} and $0.8 \cdot 10^{-3}$ h^{-1} respectively.

In broilers, the transmission rates of *E. coli* carrying resistance genes ranged from $0.1 \cdot 10^{-3}$ h^{-1} to $9.4 \cdot 10^{-3}$ h^{-1}(97% HPDI). The highest MAP transmission rate among *E. coli* carrying resistance genes in broilers without antibiotic treatment was observed for *E. coli* carrying $bla_{CTX\text{-}M\text{-}1}$ ($2.5 \cdot 10^{-3}$ h^{-1}). In the broiler group with antibiotic treatment, *E. coli* carrying $bla_{CTX\text{-}M\text{-}2}$ had the highest MAP transmission rate ($2.6 \cdot 10^{-3}$ h^{-1}).

Furthermore, the studies with *E. coli* carrying $bla_{CTX\text{-}M\text{-}1}$ involved multiple inoculation doses ranging from $0.5 \cdot 10^{1}$ cfu/animal to $0.5 \cdot 10^{8}$ cfu/animal. The low inoculation dose of $0.5 \cdot 10^{1}$ cfu/animal resulted in a lower transmission rate per hour compared to the transmission rate per hour of other inoculation dosages. This lower dosage even prevented transmission in one pen. Despite the use of this lower inoculation dosage of *E. coli* carrying $bla_{CTX\text{-}M\text{-}1}$, the transmission rate per hour surpasses the rates observed for the other resistance genes at higher dosages under no antibiotic treatment (Table 4.1)

Table 4.1- *Transmission rate per hour from different host species, status, mode of transmission, resistance, and antibiotic treatment. MAP denotes Maximum a priori and 97% HPDI denotes 97% higest posterior density distribution.*

Species	Status	Resistance	Antibiotic	Number of animals	Inoculated dose (cfu/animal)	MAP (h^{-1})	97% HPDI	Reference
Broilers	Specific pathogen free animals	*bla*$_{CTX-M-1}$	No	20	$0.5{\cdot}10^6$	$2.5 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}, 9.4 \cdot 10^{-3}$	Ceccarelli et al., 2017
		bla$_{CTX-M-1}$	No	16	$0.5 \cdot 10^8$	$1.6 \cdot 10^{-3}$	$0.9 \cdot 10^{-3}, 3.3 \cdot 10^{-3}$	Ceccarelli et al., 2017
	Conventional	*bla*$_{CTX-M-1}$	No	10	$0.5 \cdot 10^1$	$0.4 \cdot 10^{-3}$	$0.1 \cdot 10^{-3}, 2.4 \cdot 10^{-3}$	Dame-Korevaar et al., 2020
		bla$_{CTX-M-1}$	No	30	$0.5 \cdot 10^2$	$2.2 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}, 4.6 \cdot 10^{-3}$	Dame-Korevaar et al., 2020
		bla$_{CTX-M-2}$	No	20	$0.5 \cdot 10^3$	$0.7 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}, 1.1 \cdot 10^{-3}$	Dankittipong et al., 2023
		bla$_{OXA-162}$	No	20	$0.5 \cdot 10^3$	$0.4 \cdot 10^{-3}$	$0.3 \cdot 10^{-3}, 0.8 \cdot 10^{-3}$	Dankittipong et al., 2023
		catA1	No	20	$0.5 \cdot 10^3$	$1.0 \cdot 10^{-3}$	$0.6 \cdot 10^{-3}, 1.6 \cdot 10^{-3}$	Dankittipong et al., 2023
		bla$_{CTX-M-2}$	Amoxicillin	20	$0.5 \cdot 10^3$	$2.6 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}, 4.3 \cdot 10^{-3}$	Dankittipong et al., 2023
		bla$_{OXA-162}$	Amoxicillin	20	$0.5 \cdot 10^3$	$0.6 \cdot 10^{-3}$	$0.3 \cdot 10^{-3}, 1.3 \cdot 10^{-3}$	Dankittipong et al., 2023
		catA1	Amoxicillin	20	$0.5 \cdot 10^3$	$0.8 \cdot 10^{-3}$	$0.3 \cdot 10^{-3}, 3.3 \cdot 10^{-3}$	Dankittipong et al., 2023
Piglets	Specific pathogen free animals	*mcr-1*	No	10	$2.5 - 9 \cdot 10^8$	$0.8 \cdot 10^{-3}$	$0.5 \cdot 10^{-3}, 1.4 \cdot 10^{-3}$	Mourand et al., 2019
		Fluoroquinolone	No	51	$1.0 \cdot 10^{10}$	$0.7 \cdot 10^{-3}$	$0.5 \cdot 10^{-3}, 1.1 \cdot 10^{-3}$	Andraud et al., 2011
		mcr-1	Colistin	20	$2.5 - 9 \cdot 10^8$	$0.9 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}, 2.5 \cdot 10^{-3}$	Mourand et al., 2019

4. 3. 5 Comparing the transmission rate between groups with and without antibiotic treatment

Amoxicillin accelerated the transmission of *E. coli* carrying $bla_{\text{CTX-M-2}}$ resistance genes but had no effect on the transmission rate per hour of the other resistance genes. Figure 4.2 shows the 3.34 fold higher transmission rate per hour for *E. coli* carrying $bla_{\text{CTX-M-2}}$ in the group treated with amoxicillin than the untreated group. Amoxicillin and colistin seemed to slightly increase the transmission rate per hour of *E. coli* carrying $bla_{\text{OXA-162}}$ and *E. coli* carrying *mcr-1*. However, the ratio range of 0.42 to 3.55 (97% HPDI) cannot decisively establish the influence of antibiotics on the transmission of *E. coli* carrying $bla_{\text{OXA-162}}$ and *E. coli* carrying *mcr-1*. This range encompasses values from 0.42 (suggesting no significant effect from colistin and amoxicillin) up to 3.55 (indicating a potential tripling of the transmission rate per hour under colistin and amoxicillin treatment). For *E. coli* carrying *catA1* genes, our observations indicate a generally reduced transmission rate per hour when subjected to amoxicillin treatment. However, this finding was even less conclusive due to the fact that nearly half (0.46) of the posterior distribution indicates lower transmission rates per hour (Figure 4.2).

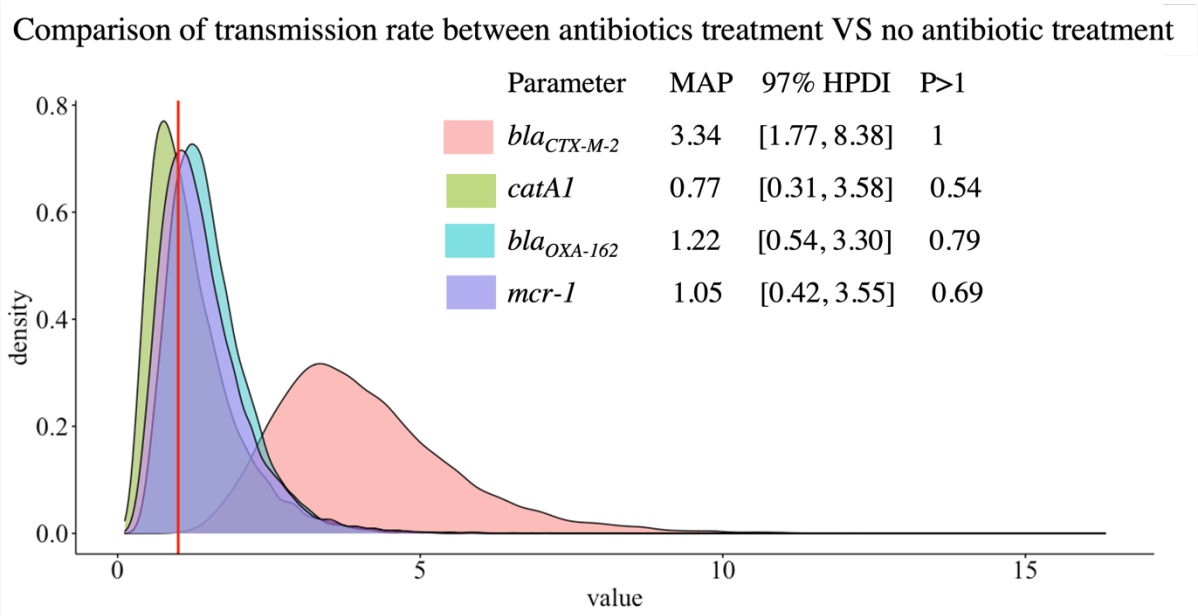

***Figure 4.2**- Posterior distributions of the ratio of transmission between antibiotic treatments (no antibiotic treatment vs. with antibiotic treatment) for different resistance genes in broilers and piglets.*

4. 3. 6 Comparing the transmission rate in broilers versus piglets

Without antibiotics, the transmission rate per hour between broilers was higher (probability of 0.99) than between piglets, and this was on average two-fold higher. In contrast, under antibiotic treatment the same transmission rate per hour was found for piglets and broilers (Figure 4.3).

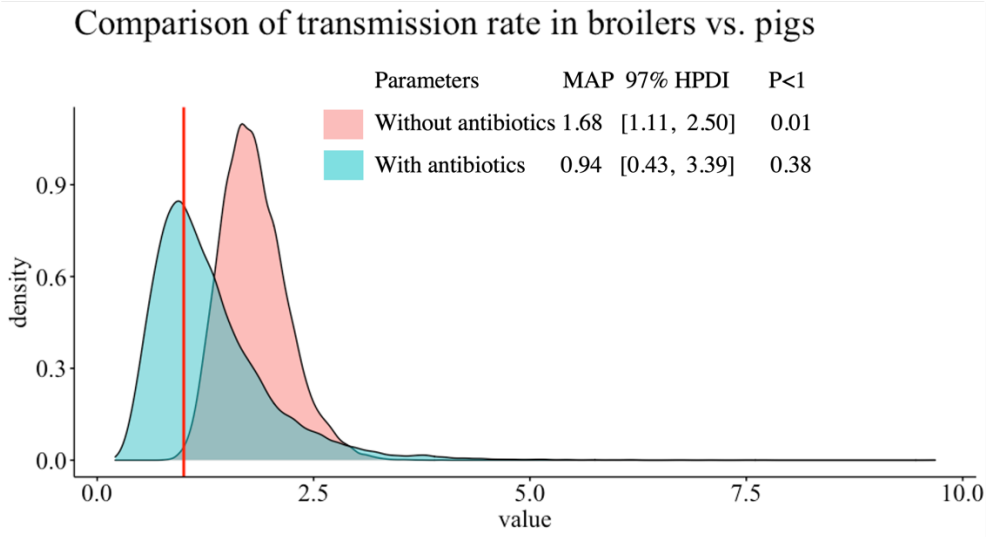

Figure 4.3- Posterior distributions of transmission rate ratio of broilers and piglets when treated and not treated with antibiotics.

4. 3. 7 Infectious period and reproduction ratio

The 97% HPDI of infectious periods of *E. coli* carrying resistance genes in broiler without antibiotic treatment are between 227 and 46,007 hours (9 – 1,917 days) (Table 4.2). In the group with antibiotic treatment, the HPDI of infectious periods for *E. coli* carrying resistance genes in broiler are between 868 and 56,678 hours (36 – 2,362 days) (Table 4.2). *E. coli* carrying $bla_{\text{OXA-162}}$ showed the highest MAP infectious period in broiler with antibiotic treatment, at 3,462 hours (144 days), compared to the lowest MAP infectious period of 475 hours (20 days) without antibiotic treatment. Antibiotic treatment increased the infectious period of *E. coli* carrying $bla_{\text{OXA-162}}$ by 6-fold (Table 4.3). Antibiotic treatment seemed to increase the infectious period of *E. coli* carrying $bla_{\text{CTX-M-2}}$ although with 0.25 probability of no effect. The 97% HPDI of the infectious periods is extremely wide for most

treatment groups in broilers due to the limited number of animals that stopped excreting the bacteria during the experiment.

Table 4.2- *Posterior distribution of infectious periods (hours) and reproduction ratio in different host species, resistance, and antibiotic treatment.*

Species	Resistance	Antibiotics	Infectious period (in hours); MAP [97% HPDI]	Reproduction ratio; MAP [97% HPDI]
Broiler	*bla*$_{\text{CTX-M-1}}$		2306 [1329, 46007]	92.38 [47, 2049]
	bla$_{\text{CTX-M-2}}$		1096 [586, 24905]	11.33 [5.4, 298]
	bla$_{\text{OXA-162}}$		475 [277, 1254]	3.46 [1.7, 11.92]
	catA1		1157 [462, 33248]	16.89 [7.64, 883]
	bla$_{\text{CTX-M-2}}$	Amoxicillin	2901 [950, 55906]	74.80 [35, 2560]
	bla$_{\text{OXA-162}}$	Amoxicillin	3462 [868, 50724]	16.71 [7.2, 717]
	catA1	Amoxicillin	2702 [893, 56678]	23.03 [10.4, 1270]
Piglet	*Fluoroquinolone resistance*		971 [617, 3468]	15.65 [8.40, 115.31]
	mcr-1		1065 [687, 4299]	18.19 [8.87, 206.98]
	mcr-1	Colistin	1043 [622, 4694]	27.70 [9.06, 383.53]

According to table 4.2, the 97% HPDI infectious period of *E. coli* carrying resistance genes in piglets without antibiotic treatment is between 617 to 4,299 hours (26 – 145 days). In the group with antibiotic treatment, the HPDI infectious periods of *E. coli* carrying resistance genes in piglets was similar, ranging from 622 to 4,694 hours (26 – 196 days) (Table 4.2). *E. coli* carrying fluoroquinolone resistance had the shortest MAP infectious period. The infectious period of *E. coli* carrying *mcr-1* was not affected by antibiotic treatment.

Table 4.3- Posterior distribution of the ratio of infectious period between antibiotic treatments (no antibiotic treatment vs. with antibiotic treatment) for different resistance in broilers and piglets.

Species	Resistance	Antibiotics	Ratio of Infectious period; MAP [97% HPDI]	Ratio of Infectious period; P<1	Ratio of reproduction ratio; MAP [97% HPDI]	Ratio of Infectious period; P<1
Broiler	*bla*$_{CTX-M-2}$	Amoxicillin	2.18 [0.16, 27.49]	0.26	3.08 [0.74, 82.75]	0.04
	bla$_{OXA-162}$	Amoxicillin	7.28 [1.60, 102.9]	0	3.72 [1.7, 123.2]	0.01
	catA1	Amoxicillin	1.05 [0.14, 30.45]	0.23	0.39 [0.11, 23.12]	0.37
Piglet	*mcr-1*	Colistin	0.91 [0.33, 2.93]	0.51	0.52 [0.20, 11.16]	0.34

The basic reproduction ratio (R_0) in broilers varies greatly between resistance and antibiotic treatments (Table 4.2). The 97% HPDI reproduction ratio in broiler is between 1.7 and 2,560 depending on the resistance and treatment. In broilers, the MAP R_0 (i.e. the reproduction without antibiotic treatment) is highest with 92.38 for *E. coli* carrying *bla*$_{CTX-M-1}$. *E. coli* carrying *bla*$_{OXA-162}$ with 3.46, has the lowest MAP R_0 among the groups without antibiotic treatment. However, both values are well above the threshold value 1. Conversely, *E. coli* carrying *bla*$_{OXA-162}$ with antibiotic treatment has the highest MAP reproduction ratio among the group with antibiotic treatment. Antibiotic use increased the reproduction ratio of *E. coli* carrying *bla*$_{CTX-M-2}$ and *E. coli* carrying *bla*$_{OXA-162}$ by three-fold but had inconclusive effect on the reproduction ratio of *E. coli* carrying *catA1* (Table 4.3).

The 97% HPDI R_0 of piglet is between 8.40 to 384. The 97% HPDI R_0 of all inoculations are overlapping. *E. coli* carrying fluoroquinolone resistance has with 15.65 the lowest MAP R_0. The effect of resistance genes and antibiotic treatment for piglets toward the R_0 is inconclusive, because of the large overlap in posterior distributions. The overall reproduction ratio in broilers without antibiotic treatment is two-fold of that of piglets without antibiotic treatment.

4. 4 Discussion

In our study we found a rapid transmission of *E. coli* carrying *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-2}$ compared to strains with other resistance genes. Notably, we found that amoxicillin increases the transmission of *bla*$_{CTX-M-2}$ by three-fold. Furthermore, we predict that *E. coli*

carrying resistance genes in broilers may have a wide range of infectious periods, potentially lasting a lifetime. Additionally, we observed that transmission of *E. coli* carrying resistance genes is faster between broilers than piglets in the absence of antibiotic treatment.

Our study indicates that in the absence of antibiotics the transmission rate of *E. coli* carrying resistance genes is higher among broilers than among piglets. We consider three possible explanations for this finding: the gut microbiome of animals, fitness costs of bacteria, and differences in experimental set-up between the studies.

First, the piglets were older than the broilers. The stability of the gut microbiome of piglets and broilers increases with the age of the animals (Guevarra et al., 2019; Ranjitkar et al., 2016). A stable gut microbiome has a preventive effect against resistant bacteria invasion (Lozupone et al., 2012; Sorbara and Pamer, 2019). Exogenous and potentially resistant bacteria will readily colonize an unstable gut microbiome (Kim et al., 2017; Rochegüe et al., 2021). Diverse bacteria species in a stable gut microbiome establish complex interactions to achieve homeostasis within the gut which results in a preventive effect against invasion of exogeneous bacteria (Awad et al., 2016; Lozupone et al., 2012; Rochegüe et al., 2021). In our meta-analysis, broilers were one to five day olds at the start of the experiment while piglets were at least seven weeks old. Young broilers of less than one week old typically have a volatile gut microbiome and are most vulnerable to *E. coli* colonization (Ranjitkar et al., 2016; Zhu and Joerger, 2003)(Chen et al., 2017; Guevarra et al., 2019; Zhou et al., 2021).

Secondly, specific resistance genes or the mobile elements with which these are associated could impose different fitness cost to *E. coli* thereby determining the transmission rate (Melnyk et al., 2015). Some genes are even associated with an improved fitness of bacteria without antibiotic treatement (Andersson, 2006; Borrell et al., 2013; Luo et al., 2005; Melnyk et al., 2015). Betalactamase producing genes (in our study $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-2}}$) are known to rapidly colonize host populations and diversify worldwide due to their highly mobilized genetic characters which suggests low fitness cost of these genes for the *E. coli* bacteria (Cantón et al., 2012; Palmeira et al., 2020).

Transmission rates per hour of *E. coli* carrying *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-2}$ genes in broilers were highest in our meta-analysis (Table 4.1). This indicates low fitness cost of *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-2}$ genes incurred to *E. coli*. While direct transmission experiment data involving *E. coli* carrying *bla*$_{CTX-M-1}$ among piglets is lacking, it is important to consider the consistently high prevalence of *bla*$_{CTX-M-1}$ *E. coli* in Dutch pigs (MARAN, 2020). This prevalence of *E. coli* carrying *bla*$_{CTX-M-1}$ among pigs suggests the potential for rapid transmission of *E. coli* carrying *bla*$_{CTX-M-1}$ exists between piglets as well. This observation, highlighted in national surveillance (2020), raises the possibility that piglet-to-piglet transmission of *E. coli* carrying *bla*$_{CTX-M-1}$ could occur at an accelerated rate. Although we did not have transmission experiment data of *E. coli* carrying *bla*$_{CTX-M-1}$ between piglets, we expect that *E. coli* carrying *bla*$_{CTX-M-1}$ would have a fast transmission rate in piglets as well. This would be in line with consistently high detection of *bla*$_{CTX-M-1}$ *E. coli* in Dutch pigs (MARAN, 2020).

Thirdly, variation of experimental settings, specifically housing, affects transmissibility of bacteria from the environment to the animal. In this meta-analysis, piglets were all housed in pens in a stable. Piglets inoculated with *E. coli* carrying fluoroquinolone resistance were housed on a slatted floor. Slatted floors may reduce transmission rate as part of excreted feces contaminated with resistant bacteria is sieved through these floors (Andraud et al., 2011). Though the type of floor was not mentioned in Mourand et al. (2018 and 2019), it is possible that their piglets were housed in a similar setting given both teams complied to same French regulation on animal welfare in experimentation (Mourand et al., 2019, 2018). This removal of feces through housing was not present in the experimental setting for broilers in isolators or pens without slatted floors (Ceccarelli et al., 2017; Dame-Korevaar et al., 2020) and could contribute to faster transmission rate between broilers than between piglets. Moreover, considering the distinction between SPF and conventional broiler chickens could further elucidate the observed transmission dynamics. The uncertainty highlighted by the wide-ranging probability distribution, resulting from evaluating the posterior distribution of transmission rate ratio of SPF broiler chickens and of conventional broiler chickens (ranging from 0.5 to 2.7), suggesting inconclusiveness in the effect of SPF and conventional bird to the transmission rates (Supplementary 4.7.6).

Based on the estimates for the infectious period, we conclude that *E. coli* carrying resistance genes can colonize broiler chickens for a lifelong period. However, the observations of the infectious period with an observed return to uncolonized state in our dataset were limited (7 %) due to censoring of 187 out of 204 excreting periods. In spite of the limited data, our parameteric survival model utilized both the observed data and prior information from literature to estimate the probable infectious period (Ragione et al., 2005; Fong and Lehmann, 2022; Kalbfleisch, 1978). While the estimated infectious period of 56,678 hours (6 years) for *E. coli* carrying resistance genes is biologically implausible for broiler chickens, this estimate could be interpreted as lifelong colonization that broiler chickens typically experience, which lasts for only 40-56 days until they are slaughtered. This conclusion is consistent with Ragione et al. (2005), which showed an extended colonization period of 35 to 156 days for nalidixic-resistant pathogenic *E. coli* in layer chickens. Moreover, studies by Conway & Cohan (2015) and Stromberg et al. (2018) have demonstrated the superior adaptability of commensal *E. coli* to colonize animal guts compared to pathogenic *E. coli*, which supports our estimated longer infectious period. Despite the wide range of uncertainty in our analysis, inclusion of weakly informative priors in our model still has benefits. By incorporating prior knowledge from literature, the model was able to make more informed estimates even in the presence of limited data and high censoring rates. The prior provides a regularization effect that helps to stabilize the estimates and prevent overfitting, which can lead to erroneous conclusions. Overall, the use of weakly informative priors can improve the accuracy and reliability of model estimates, even in situations where data are limited and uncertainty is high.

Our study estimated the infectious period of piglets to be between 25 and 195 days, which we believe is reflective of real-life situations. The HPDI credible interval surrounding this estimate was narrower compared to that of broilers due to the greater number of observed return to uncolonized state. Although only 19% of the observed infectious periods had an observed return to uncolonized state (18 out of 93), the fact that there were any observed return to uncolonized states at all suggests that return to uncolonized state may occur after the end of the transmission experiment, and this knowledge provided more weight to our estimate of the infectious period. Furthermore, our estimate of the infectious period in piglets was consistent with previous colonization studies of *E. coli* in pigs, which

reported colonization periods ranging from 1 to 5 months (Belloc et al., 2005; Johnson et al., 2015; Randall et al., 2018). However, animals in Belloc et al (2005), Johnson et al (2015), and Randall et al (2018) were excreting beyond the end of the experiment; meaning that the reported colonization period was the same length as the experiment itself. In contrast, our estimate narrowed down the infectious period in piglets to a more specific timeframe of up to 7 months. This duration which institutes an entire growth cycle in certain pig population, such as finisher pigs. Overall, our results suggest that our approach was able to produce a realistic estimate of the infectious period of piglets that can be useful for future research and control strategies.

The estimation of the R_0 is important in understanding the dynamics of infectious diseases. In our study, we estimated the R_0 for different resistance genes, antibiotic treatments, and animal species. To calculate R_0, we combined the estimates of the infectious period and transmission rate from our hierarchical models. We assumed that these parameters are completely independent, which might cause our estimates to be overdispersed. While the wide intervals for R_0 migth represent overdispersion, we still believe our estimates provide valuable insights into the dynamics of *E. coli* carrying resistance genes in broilers and piglets.

Our Bayesian meta-analysis effectively identified factors related to the transmission rate of *E. coli* carrying resistance gene with greater precision, despite the limited number of studies and small sample sizes. To mitigate uncertainties stemming from small datasets, we harnessed raw longitudinal data from each study (Individual Participant Data) and implemented a Bayesian probabilistic framework that is capable of incorporating both prior knowledge and data. Instead of relying on summarized statistics across various studies, we employed a subgroup (pen-level) within the hierarchical model to curtail between-study heterogeneity. This tactic enabled us to focus on a common analytical unit resulting in more informed and accurate estimates of the factors driving the transmission (Riley et al., 2010).

It is important to note that with a limited number of studies, traditional frequentist assume large sample sizes (asymptotic), and can result in underestimation of between-study

variance and overconfident confidence intervals (Mcneish, 2016). In contrast, Markov chain Monte Carlo (MCMC) in Bayesian approach explores the entire posterior distribution of the parameter and does not rely on asymptotic standard errors (Willams et al., 2018). As a result, Bayesian methods can provide more accurate estimates of between-study variance and are often recommended when dealing with meta-analyses of limited studies (Veroniki et al., 2014). Using this approach, we identified key factors contributing to the transmission dynamic of *E. coli* carrying resistance genes, including antibiotic treatment, resistance strain, and host species. The importance of each factor in determining the transmission dynamics of *E. coli* carrying resistance genes can vary depending on the specific resistance strain, animal species, and antibiotic treatment. For example, *E. coli* carrying resistance genes transfer faster in broiler chickens than in piglets, but only under no antibiotic treatment. Overall, our study highlights the complexity of the transmission dynamics of antibiotic resistant *E. coli* and emphasizes the need for comprehensive approach to mitigate the spread of antibiotic resistance. This multifaceted strategy could encompass interventions such as leveraging the animal's microbiome through probiotics to reduce transmission, implementing antibiotic stewardship to curtail antibiotic use, and exploring other variables that warrant further assessment. These combined efforts would work effectively toward controlling the propagation of antibiotic resistance.

The parameters identified in our study, such as the transmission rates and infectious periods of resistant *E. coli*, could be incorporated into more extensive simulation models. These models could aid in evaluating potential interventions to mitigate the spread of antibiotic resistance in livestock populations. Our study highlights the importance of rigorous analytical methods for small and limited data sets, which are necessary for accurately estimating these parameters and informing simulations.

The variation of transmission rate between resistance gene inoculation, antibiotic treatment, and animal species, highlighting the need for inclusion for additional transmission data. The uncertainty around infectious period estimates is also driven by unobserved return to uncolonized state due to short experimentation time. However, the Bayesian framework is flexible and can incorporate a wide range of data types and

structure including data from field experiments and observational studies, enabling estimation of differences between experimental and field settings. To illustrate, our Bayesian-meta analysis model can incorporate field data through the use of priors. By incorporating field data into the priors, we can adjust our estimates to better reflect the actual values in the field. Additionally, our hierarchical modeling approach can account for differences between experimental and field settings by including additional levels in the model, such as location or time, to capture the variability in the data. This allows for a more comprehensive and accurate representation of the transmission dynamics in real-world scenarios.

4. 5 Conclusion

We believe our results are useful for simulation modelling of transmission dynamics of resistant bacteria in piglets (7-8 weeks old) and broilers (less than one week old), especially because in the Bayesian framework we have obtained a posterior distribution that can be used to include the uncertainty of the parameter estimates in such simulation models.

4. 6 Acknowledgement

We are thankful for the fattening pigs longitudinal dataset provided by Gwenaelle Mourand's lab (ANSES, Laboratoire de Ploufragan-Plouzané, Ploufragan, France) and Mathieu Andraud's lab (ANSES, Fougères laboratory, Fougères Cedex, France). We appreciated the broilers dataset provided by Dr. Anita Dame-Korevaar (Utrecht University, Utrecht, the Netherlands) and Dr. Daniela Ceccarelli (Wageningen Bioveterinary Research, Lelystad, the Netherlands).

4. 7 Supplementary information

4. 7. 1 Systematic review following Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P, 2015).

4. 7. 1. 1 Eligible criteria

PICO protocol was drafted to specify the study characteristic wanted from the database.

- P (population or problem): ‘meat-producing livestock’ & ‘antimicrobial resistant bacteria’;
- I (intervention or exposure): transmission experiment.
- O (outcomes): Longitudinal data.

*C (comparison) is not included in the protocol

4. 7. 1. 2 Information sources and search strategy

First author (ND) performed the literature search in 2022. Pubmed and Google Scholar were the online database in which the search are performed. The search strategy encompassed three distinct groups of search strings. The initial set involved terms pertinent to meat-producing livestock, such as ‘meat-producing livestock’, ‘livestock’, ‘cow’, ‘veal calf’, ‘pig’, ‘broiler’, ‘broiler chicken’, and ‘chicken’ Concurrently, a second group consisted of terms related to antimicrobial-resistant bacteria, comprising ‘resistant bacteria’, ‘antibiotic resistance’, ‘ESBL’, ‘Extended-Spectrum β-Lactamase’, ‘carbapenemase-producing’, and ‘CPE’. A third group of terms centered around longitudinal data, including ‘longitudinal data’, ‘longitudinal study’, ‘transmission experiment’, and ‘transmission study’. These three groups of search strings were combined with the "OR" operator within their respective categories. Subsequently, to formulate a comprehensive search strategy, the combined sets of terms from the meat-producing livestock category, the antimicrobial-resistant bacteria category, and the longitudinal data category were interlinked using the "AND" operator.

4. 7. 1. 3 Selection criteria

To ensure a comprehensive evaluation of potential biases, consensus was initially reached among all coauthors regarding the inclusion criteria. Subsequently, the first author conducted a review of the selected literature. During this review process, our focus remains on the raw longitudinal data of transmission from each study, rather than

evaluating summary statistics. Following the first author's review, the final assessments were subjected to collective scrutiny by all coauthors.

The study employs the following criteria for inclusion purposes

1. **Longitudinal Data Collection:** The study protocol entails the collection the status of individual animals, involving a minimum of three distinct time points.

2. **Distinct Animal Categories:** The study protocol distinctly outlines two categories of animals: those inoculated with resistant bacteria and those without such inoculation (contact animals).

3. **Non-Pathogenic Bacteria:** The bacteria used for inoculation in animals must be non-pathogenic, such as *Escherichia coli*.

4. **Meat-Producing Animal Focus:** The subject animals under investigation are limited to meat-producing species, including broiler chickens, veal calves, fattening pigs, and piglets.

4. 7. 1. 4 Data collection and management

The primary responsibility for managing records and data lies with the first and last authors. When data is not readily available in a transferable format, such as a .CSV file or stored on GitHub, the first author manually extract the necessary information. Moreover, we proactively engage with the investigators of the reviewed studies to acquire data directly when raw data is not accessible online. The records collected during this process is securely stored in the university's designated drive, ensuring its confidentiality and accessibility to the research team. Additionally, we published the collected data on an online platform, specifically GitHub to enhance accessibility and transparent.

4. 7. 1. 5 Risk of bias assessment

The first and the second authors conducted a risk of bias assessment following the Cochrane risk-of-bias tool for randomized trials (RoB 2). The filled risk of bias of individual studies are attached as zip file title “filledriskofbias.zip”.

4. 7. 1. 6 Outcome

The excreting status (positive or negative for resistance markers) of individual animals was extracted at each sampling time point. For each individual animal, we extracted the pen information, the inoculation strain, any antibiotic treatments (yes/no), and the inoculation status (inoculated animal versus contact animal). Contact animals were classified as susceptible animals and could become cases, subsequently becoming infectious animals, whereas the inoculated animals could only become infectious but were not counted as cases.

The number of hours that an animal (contact and inoculated) excreted *E. coli* carrying resistance genes was extracted as an input for the infectious period (*D*). We assumed that all individuals would stop excreting the *E. coli* carrying resistance genes at the end of their infectious period (return to uncolonized state). Hence, to extract the infectious period, we counted hours from the first sampling time point that an animal is excreting (positive for resistance marker) until the first sampling time point that an animal stop excreting (negative for resistance marker). Only animals that exhibit at least two consecutive negative samples were considered to have undergone loss of colonization and potentially became infectious again. Animals that return to uncolonized state and became infectious again could have more than one infectious period.

If the time of an animal's return to an uncolonized state is censored, indicating that the animal continues to excrete *E. coli* carrying resistance genes until the end of the experiment, we calculate the infectious period by measuring the time from the initial sampling time point, when the animal starts excreting (positive for the resistance marker), until the last observed sampling time point. In this context, we assume that the period of time during which an animal returns to an uncolonized state extends beyond the actual end of the experiment. This assumption about the time for an animal to return to an uncolonized state follows the gamma distribution, accounting for variations in dynamics among the subjects.

4. 7. 1. 7 Data synthesis

Before we apply the Bayesian hierarchical model, we adopted the Meta-analysis of Individual Participant Data technique to extract individual animal outcomes, such as excretion status across time points. These data were subsequently organized into pen clusters, facilitating analysis. Subsequently, the Bayesian hierarchical model was applied, incorporating the complete individual dataset, and treating pen clusters as random effects.

4. 7. 2 Decay rates from literatures

***Table 4.4**- Decay of Escherichia coli outside a live host. Abbreviations: fc: field capacity.*

ID	Temperature (°C)	Humidity	pH	Environment	Decay rate (per day)	Reference
1	Autumn			Lab	0.102	(Burrows and Rankin, 1970)
2	Autumn			Lab	0.287	(Burrows and Rankin, 1970)
3	4		7	Lab	0.686	(Kovács and Tamási, 1977)
4	January			Lab	0.109	(Rankin and Taylor, 1969)
5	26		7.4	Soil	0.896	(Klein and Casida, 1967)
6	10		7.4	Soil	0.195	(Klein and Casida, 1967)
7				Soil	0.115	(Mallmann and Litsky, 1951)
8			7	Soil	0.371	(Van Donsel et al., 1967)
9				Soil	0.143	(Mallmann and Litsky, 1951)
10		1/3 bar	6.16	Soil	0.473	(Tate, 1978)
11		saturated	6.64	Soil	0.839	(Tate, 1978)
12		100% fc	6.16	Soil	0.796	(Tate, 1978)
13		flooded		Soil	0.382	(Tate, 1978)
14	0			Inoculated water	0.192	(Mitchell, 1968)
15	10	60% fc		Swine manure-amended soil	0.22	(Rogers et al., 2011)
16	10	80% fc		Swine manure-amended soil	0.19	(Rogers et al., 2011)
17	25	60% fc		Swine manure-amended soil	0.40	(Rogers et al., 2011)
18	25	80% fc		Swine manure-amended soil	0.28	(Rogers et al., 2011)
19	10	60% fc		Beef manure-amended soil	0.17	(Rogers et al., 2011)
20	10	80% fc		Beef manure-amended soil	0.15	(Rogers et al., 2011)
21	25	60% fc		Beef manure-amended soil	0.33	(Rogers et al., 2011)
22	25	80% fc		Beef manure-amended soil	0.37	(Rogers et al., 2011)
23	Optimal condition to rear broilers			Broiler pen floor	0.0	(van Bunnik et al., 2014)

4. 7. 3 Prior distribution for Bayesian hierarchical inference for transmission rate and Bayesian parametric survival analysis for infectious period.

***Table 4.5**- Prior distributions applied in Bayesian model for transmission rate and parametric survival analysis.*

	Transmission rate		**Infectious period**		
	$\log(\bar{\beta})$	z_i	$\bar{a}$	σ_i	b
Broiler	Normal (-10, 10)	Normal (0, 1)	Normal (0,1)	Exponential(1)	Normal (0,1)
Piglets	Normal (-10, 10)	Normal (0,1)	Normal (0,2)	Exponential(1)	Normal (0,1)

4. 7. 4 R code and data.

R code and data for Bayesian hierarchical inference for transmission rate and Bayesian parametric survival analysis for infectious period are published in the following github URL:https://github.com/EgilFischer/BEWARE_TransmissionExperiments/tree/main/metaanalysis_PVM

4. 7. 5 R SIS model fitted to raw transmission experiment

Table 4.6- *Transmission rate per hour from different host species, status, mode of transmission, resistance genes, and antibiotic treatment. SIS model fitted to raw transmission experiment. MAP denotes Maximum a priori and 97% HPDI denotes 97% highest posterior density distribution.*

Species	Status	Resistance (genes)	Antibiotic	Number of animals	Inoculated dose (cfu/animal)	MAP (h^{-1})	97% HPDI	Reference
Broilers	Specific pathogen free animals	*bla*$_{CTX-M-1}$	No	20	$0.5{\cdot}10^6$	$2.6 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}, 8.7 \cdot 10^{-3}$	Ceccarelli et al., 2017
		bla$_{CTX-M-1}$	No	16	$0.5 \cdot 10^8$	$1.4 \cdot 10^{-3}$	$0.8 \cdot 10^{-3}, 3.0 \cdot 10^{-3}$	Ceccarelli et al., 2017
	Conventional	*bla*$_{CTX-M-1}$	No	10	$0.5 \cdot 10^1$	$0.4 \cdot 10^{-3}$	$0.1 \cdot 10^{-3}, 2.5 \cdot 10^{-3}$	Dame-Korevaar et al., 2020
		bla$_{CTX-M-1}$	No	30	$0.5 \cdot 10^2$	$2.4 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}, 4.8 \cdot 10^{-3}$	Dame-Korevaar et al., 2020
		bla$_{CTX-M-2}$	No	20	$0.5 \cdot 10^3$	$0.7 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}, 1.2 \cdot 10^{-3}$	Dankittipong et al., 2023
		bla$_{OXA-162}$	No	20	$0.5 \cdot 10^3$	$0.4 \cdot 10^{-3}$	$0.2 \cdot 10^{-3}, 0.6 \cdot 10^{-3}$	Dankittipong et al., 2023
		catA1	No	20	$0.5 \cdot 10^3$	$0.6 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}, 1.0 \cdot 10^{-3}$	Dankittipong et al., 2023
		bla$_{CTX-M-2}$	Amoxicillin	20	$0.5 \cdot 10^3$	$2.5 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}, 4.3 \cdot 10^{-3}$	Dankittipong et al., 2023
		bla$_{OXA-162}$	Amoxicillin	20	$0.5 \cdot 10^3$	$0.5 \cdot 10^{-3}$	$0.3 \cdot 10^{-3}, 1.1 \cdot 10^{-3}$	Dankittipong et al., 2023
		catA1	Amoxicillin	20	$0.5 \cdot 10^3$	$0.8 \cdot 10^{-3}$	$0.3 \cdot 10^{-3}, 3.3 \cdot 10^{-3}$	Dankittipong et al., 2023
Piglets	Specific pathogen free animals	*mcr-1*	No	10	$2.5 - 9 \cdot 10^8$	$0.6 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}, 1.1 \cdot 10^{-3}$	Mourand et al., 2019
		Fluoroquinolone	No	51	$1.0 \cdot 10^{10}$	$0.6 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}, 0.9 \cdot 10^{-3}$	Andraud et al., 2011
		mcr-1	Colistin	20	$2.5 - 9 \cdot 10^8$	$0.4 \cdot 10^{-3}$	$0.2 \cdot 10^{-3}, 0.7 \cdot 10^{-3}$	Mourand et al., 2019

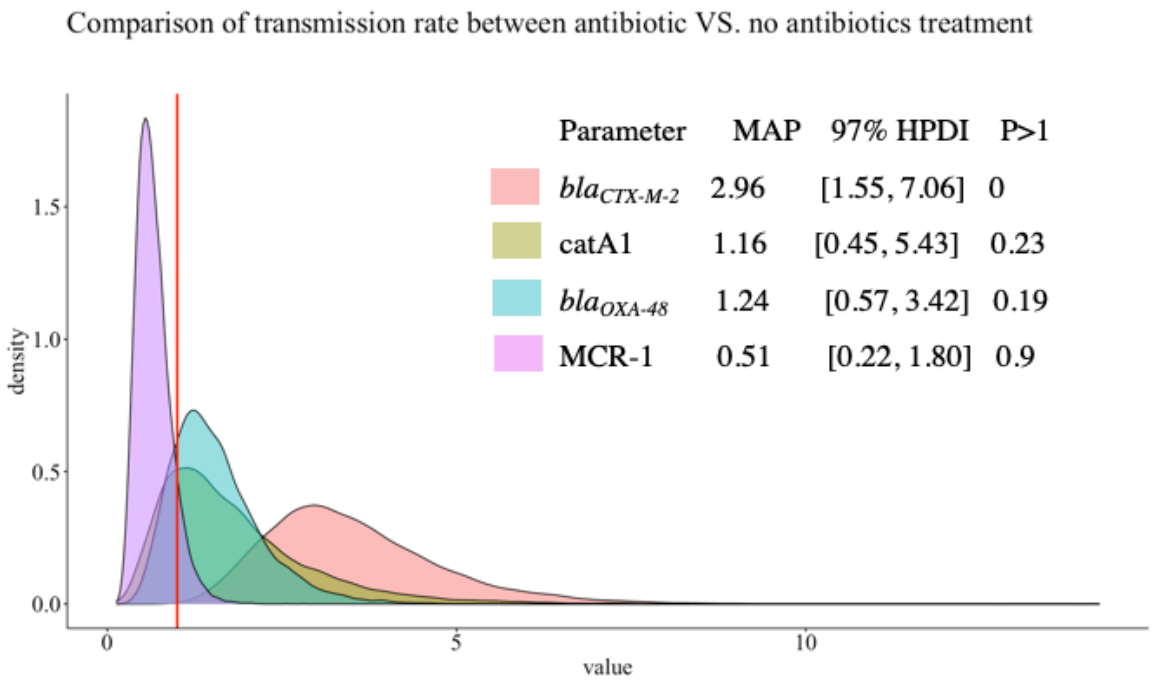

Figure 4.4- *Posterior distributions of the ratio of transmission between antibiotic treatments (no antibiotic treatment vs. with antibiotic treatment) for different resistance genes in broilers and piglets. Bayesian hierarchical model applied to raw transmission data.*

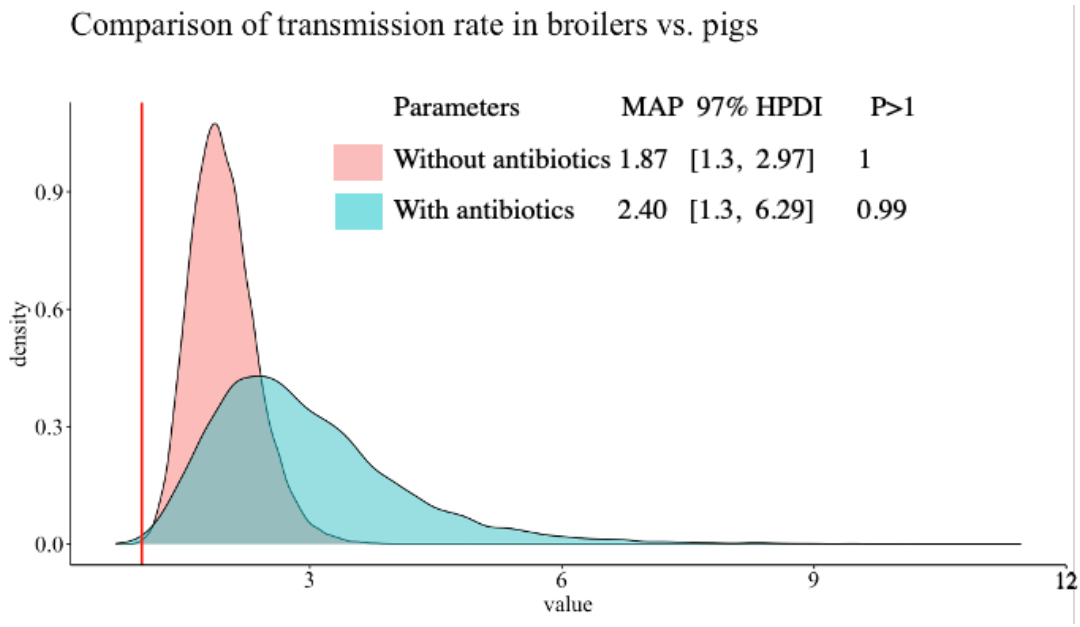

Figure 4.5- *Posterior distributions of transmission rate ratio of broilers and piglets when treated and not treated with antibiotics.*

4. 7. 6 Transmission rate per hour for E. coli carrying blaCTX-M-1 between broilers.

Table 4.7- *Transmission rate per hour for E. coli carrying $bla_{CTX\text{-}M\text{-}1}$ between broilers.*

Description	MAP (h^{-1})	97% HPDI	Probability of ratio higher than 1	Reference
Specific pathogen free animals	$2.1 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}, 3.7 \cdot 10^{-3}$		Ceccarelli et al., 2017
Conventional	$1.5 \cdot 10^{3}$	$0.7 \cdot 10^{-3}, 3.3 \cdot 10^{-3}$		Dame-Korevaar et al., 2020
Ratio SPF VS. Conventional	1.04	0.45, 2.52	0.301	

Chapter 5

Investigating a propagation of emerging carbapenemase-producing Enterobacteriaceae in Dutch broiler production pyramid through stochastic simulation

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Abstract

Simulating resistant bacteria transmission in livestock informs surveillance strategies for emerging threats like Carbapenem-resistant Enterobacteriaceae (CPE), aiding targeted surveillance and detecting CPE through active methods. We employed a simulation model to assess three potential scenarios for introducing CPE: 1) a single import of live animals, 2) the use of contaminated feed, and 3) multiple imports of live animals. Employing the SimInf package, we constructed a population model for broiler production, encompassing rearing farms, multiplier farms, hatcheries, and broiler farms. Subsequently, we introduced CPE and allowed it to spread throughout the population using the Susceptible-Colonized (Infectious)-Susceptible model. The model ran for 10 years with 100 runs.

In the single import scenario, 1-2 rearing and multiplier farms saw major outbreaks in all 100 runs, while the broiler farm experienced major outbreaks in only 10 out of 100 runs; in the feed scenario, major outbreaks occurred in rearing farms in 32 runs and in multiplier farms in 26 runs, with major outbreaks in broiler farms observed in all 100 runs; in the multiple import scenario, outbreaks in rearing and multiplier farms happened in all 100 runs, with these major outbreaks reaching the broiler farm in 91 out of 100 runs. CPE transmission from imported or colonized broilers is rapid but short-lived within the broiler population, contrasting with the sporadic and prolonged emergence of CPE from contaminated feed, resulting in lower cumulative probabilities of detection from imported or colonized animals (0-0.50) compared to contaminated feed (0.9-0.97) over a 10-year period. Sensitivity analysis indicated that key outcomes such as farm outbreaks, chicken colonization, and outbreak duration are highly correlated with age-associated reductions in transmission (ψ).

Keywords; Simulation model; Broiler production; Antibiotic resistant bacteria; SimInf; Transmission dynamics model

5. 1 Introduction

Carbapenem-resistant Enterobacteriaceae (CPE) pose a significant threat to public health due to their ability to resist the clinically important carbapenems. CPE emergence is particularly concerning because carbapenem-resistance genes are frequently located on plasmids, which are mobile genetic elements that can rapidly transfer between bacteria and facilitate the transmission of resistance between humans and animals (Anderson and Boerlin, 2020; Irrgang et al., 2020). Furthermore, CPE can arise from the use of other commonly administered antibiotics through co-resistance (ECDC, 2013; Levi et al., 2020).

Despite the prohibition of carbapenem use in livestock, on a global scale, the emergence of CPE in wildlife, companion animals, and livestock has been observed since 2009 (Kock et al., 2018: Anderson and Boerlin, 2020). This emergence raises concerns about the potential spillover of CPE into the livestock population in the Netherlands, which, in turn, could serve as a source of introduction to the human community through the consumption of CPE-contaminated meat (Mughini-Gras et al., 2019: Irrgang et al., 2020).

In recent decades extended-spectrum beta-lactamase (ESBL) producing bacteria have risen to high levels in Dutch poultry production. Although these levels have dropped considerably following a strong reduction in antibiotic use, this example shows the risk of plasmid-mediated resistance (MARAN report, 2022). Other plasmid-mediated resistant bacteria are also prevalent in broilers. CPE is plasmid-mediated and exhibits co-resistance with some of these resistant bacteria. This makes poultry a crucial target for surveillance efforts (EFSA, 2022).

Considering their significance in antimicrobial resistance (AMR), broiler production is prioritized for surveillance activities (EFSA, 2013; EFSA 2016; EFSA, 2022). The current national surveillance in the Netherlands tests a small proportion of the animal population in the slaughterhouse once a year (MARAN report, 2022). This protocol may not effectively detect emerging AMR at an early stage when curtailing its spread would still be feasible. For example, the detection of extended-spectrum beta-lactamases (ESBLs) in poultry occurred only after they had become widespread

(Leverstein-van Hall et al., 2011). Additionally, AMR may be introduced in farm types that are not included in current surveillance programs. This leaves a considerable proportion of the animal population exposed to colonization by newly emerging AMR strains before their presence has been discovered.

To gain a comprehensive understanding of the spread and persistence of CPE throughout the entire broiler production pyramid, it is imperative to systematically integrate the available information on CPE transmission dynamics. Meta-population models can be used to simulate the population dynamics of colonization within herds and between interconnected herds as has been demonstrated for exploring the transmission of bacterial diseases between broiler and pig farms (e.g. Furusawa et al., 2024; Saline et al., 2020; Sorenson et al., 2017). Such models can be useful to investigate population dynamics of CPE, but also to evaluate surveillance (Rosendal et al., 2020; Sykes et al., 2023; Tuominen et al., 2023).

We aim to quantify the consequences of CPE introduction into the Dutch broiler sector. Our main outcomes will be the number of farms experiencing an outbreak of CPE, the duration of the outbreaks, the number of contaminated batches and birds at slaughter, and the probability of detection with the current surveillance system. We investigate the two potentially most important routes of introduction, namely the import of live birds and contaminated feed (Dankittipong et al., 2022).

5. 2 Material and Method

5. 2. 1 Simulation model

We adapted an epidemiological simulation model for antibiotic resistance transmission within and between farms for a part of the poultry production pyramid (Furusawa et al., 2024). The model tracks flocks of broiler chickens and parent stock chickens (referred to as PS chickens) instead of farms, as farms may house multiple flocks of broiler chickens and PS chickens annually. We parameterized the model for CPE. In addition, we included the current Dutch CPE surveillance system in the model and performed a sensitivity and what-if analysis. We used a stochastic discrete-time simulation model in which the spread of CPE and population dynamics of rearing, multiplier farms, hatcheries, and fattening farms are explicitly simulated. The SimInf

package for R was used to implement the model. The initial number of PS chickens raised in rearing farms, obtained from Statistics Netherlands, served as the starting point (Statline, 2023). After each production round, the PS chickens were moved to multiplier farms. From there, the eggs moved through the hatchery, resulting in hatched broiler chickens that were subsequently sent to broiler farms.

The transmission dynamics were simulated for a period of 10 years (3,650 days) in each run, with a total of 100 iterations generated to assess the spread of CPE resulting from feed contamination and the import of live animals.

5. 2. 2 Epidemiological model

5. 2. 2. 1 Compartmental model and transition within compartment

The course of within-flock outbreaks of CPE was modeled with a stochastic Susceptible- colonized-Susceptible (SIS) compartmental model with environmental transmission (Table 5.1). The susceptible animals (S) are colonized by CPE in the environment at a colonization coefficient of CPE (β). The colonized animals (I) can recover and become susceptible animals (S) at a rate of recovery (γ). Please note that to be consistent with literature on SIR models, we use the symbol *I* for colonized animals, although CPE is not an infection.

Introduction of CPE occurs either by a constant rate of CPE colonization from feed (ω) specific for both parent (subscript ps) and broiler chickens (subscript b) or by the import of colonized chicks. After introduction, CPE spreads via the environment between chickens at time t at rate given by the contamination ($\varphi(t)$) and colonization coefficient of CPE (β). We assume an age-dependent decreasing susceptibility of chickens ($e^{-\psi(a)}$) mimicking the maturation of the gut microbiome (Table 5.2). The age-dependent susceptibility was fitted on observational data from broilers with only a short time span leading to resistance at higher age of parent stock (Dame-Korevaar et al., 2017; Dierikx et al., 2013; Huijbers et al., 2016). An alternative model, in which a minimum susceptibility (z) was assumed, was included such that the age-dependent susceptibility was $z - (z + 1)\, e^{-\psi(t)}$.

Contamination in the hatchery comes from the eggs laid by colonized parent broilers (EI). CPE-contaminated eggs are calculated from the number of eggs laid by colonized PS chickens multiplied with the probability of contamination of eggs by PS chickens(ν).

Table 5.11- *Transition states of the within-flock SIS compartmental model with environmental transmission and age-dependent transmission reduction (Furusawa et al., 2024). $\psi \cdot a$ = decrease of susceptibility with age (days); $\varphi(t)$ is the environmental contamination at time t. β is colonization coefficient; ω is the colonization rate through feed; type of bird k = ps (parent stock) or b (broiler); γ is recovery rate; b is egg laying rate, ν is probability of contamination of egg by PS chickens; subscript m is multiplier bird; h is hatching rate.*

Health state transition		Rate
$S \rightarrow I$	Colonization from susceptible parent stock or broiler bird (*S*) to colonized parent stock or broiler bird (*I*)	$(z-(z+1)\cdot e^{-\psi \cdot a}) \cdot (\varphi(t)\beta + \omega_k)$
$I \rightarrow S$	Recovery from colonized (*I*) to susceptible (*S*)	γ
$\rightarrow EE$	Production of uncontaminated eggs (*EE*)	$b(S_m + (1-l)I_m)$
$\rightarrow EI$	Production of contaminated eggs (*EI*)	$b \cdot I_m \cdot \nu$
$EE \rightarrow S$	Hatching of susceptible chickens from uncontaminated eggs (*EE*)	$h \cdot EE$
$EI \rightarrow S$	Hatching of susceptible chickens from contaminated eggs (*EI*)	$h\,(1-\mathrm{v}) \cdot EI$
$EI \rightarrow I$	Hatching of colonized chickens from contaminated eggs	$h \cdot \mathrm{v} \cdot EI$

5. 2. 2. 2 Transmission parameters

The rate of colonization from CPE-contaminated feed was calculated from the annual probability of colonization of a flock due to CPE contamination in feed (p_{feed}) estimated in a previous study (Dankittipong et al., 2023). We calculate the daily rate of introduction per animal as follows:

$$\omega = -\frac{\ln(1-p_{feed})}{365 \cdot N_{animal}} \qquad (1)$$

To determine the rate of colonization from feces in the environment ($\varphi(t)$), we modeled the excretion of viable CPE from colonized animals. We assumed that a colonized animal (I) at time *t* excretes CPE into the environment of its pen at a constant rate of (θ) units per day. The excreted CPE remains viable for transmission according to a survival rate of (ρ) per day (eq.2).

$$\varphi(t) = \rho\, \varphi(t-1) + \theta \cdot I \qquad (2)$$

We used the colonization coefficient of CPE (β) estimated from a transmission experiment of CPE in broiler chickens (Dankittipong et al. 2023a).

Our model includes that animal become less susceptible to colonization as they age (Dame-Korevaar et al., 2017; Dierikx et al., 2013; Huijbers et al., 2016; Schreuder et al., 2020). Our baseline model assumes an exponential decrease of susceptibility with a reduction rate (((a)), which is fitted on data from a broiler flock. This would lead to approximately zero susceptibility in older parent birds, which is not in agreement with the abovementioned studies. Therefore, an alternative model with a minimum susceptibility (z) was also simulated. The transmission reduction ($\psi(a)$) and a minimum susceptibility (z) were fitted according to the data of Huijbers et al (2016) using Approximate Bayesian Computation Sequential Monte Carlo (ABC-MC) (Furusawa et al., 2024).

***Table 5.12**- Input parameters for the CPE SIS model in broiler chickens.*

Values	Parameter description	Values (unit)	References
b(t)	The daily eggs laying rate by PS chickens in multiplier farm	0.12 (day^{-1})	*refer to footnote
ν	The probability of contamination of egg by PS chickens	0.004	Projahn et al., 2017
β	Colonization coefficient of CPE	0.0048 (day^{-1})	Projahn et al., 2017
h(t)	The daily hatching rate in the hatchery	0.05 (day^{-1})	Projahn et al., 2017
			Dankittipong et al., 2023
γ	The recovery rate	0.03 (day^{-1})	Archer and Lee Cartwright, 2017
$\varphi(t)$	The rate of colonization from feces in the environment		
$p_{feed_{ps}}$	The probability of colonization from feed to parent flock	0.05 ($year^{-1}$)	Dankittipong et al., 2023
p_{feed_b}	The probability of colonization from feed to broiler flock	0.23 ($year^{-1}$)	
ω_{ps}	The rate of colonization from CPE-contaminated feed to parent flock	$1.4 \cdot 10^{-8}$(day^{-1})	Dankittipong et al., 2022
ω_b	The rate of colonization from CPE-contaminated feed to broiler flock	$7.0 \cdot 10^{-8}$(day^{-1})	Dankittipong et al., 2022
θ	The rate of CPE excretion into the environment of animal's pen	2.70 (day^{-1})	
$N_{animal_{broiler}}$	The average number of broiler chickens in a broiler farm	10,000	
			Dankittipong et al., 2023
$N_{animal_{PS}}$	The average number of PS chickens in a rearing and multiplier farm	40,000	
ρ	The survival rate of excreted CPE	0.62 (day^{-1})	
ψ	Reduction in the probability of colonization with time	0.6 (day^{-1})	
z	The minimum susceptibility	0.10	Fitted to data of Huijbers et al., 2016

* The laying rate was arbitrary set low to maintain a stable population size at broiler farms as all laid eggs are sent to the hatcheries in the simulation model.

5. 2. 2 Population dynamics

5. 2. 2. 1 Farm structure, size, and type

The four farm types most important for the Dutch broiler production sector are considered: rearing farm (n=90), multiplier farm (n=200), hatchery (n=6), and broiler farm (n=780). All rearing farms and multiplier farms were assumed to house 40,000 animals. The numbers of farms were based on a publicly available national database, Statistics Netherlands (CBS) and experts in broiler rearing (Statline, 2023). The queries to retrieve the data are detailed in Supplementary 5.5.2. We simulated the movements of parent broiler chickens (PS chickens), eggs, and broiler chickens across a network of 181 farms. This enabled us to simulate the boiler production sector with the smallest number of farms, including all farm types in the production chain (Figure 5.1), which facilitated the computations. In the simulation, the numbers of farms included are rearing farm (n=16), multiplier farm (n=32), hatchery (n=1), and broiler farm (n=132). This selection was made because our primary interest was in single introductions and feed that would be present in all farm types except hatchery, and therefore, we focused on simulating the network around a single hatchery. We did not simulate all the farms in the broiler production sector because we assumed that the connections between rearing farms, multiplier farms, hatchery, and broiler farms form a closed network. As such, we assumed that the rest of the farms in the broiler production sector would exhibit the same closed network properties as this group of 181 farms. Additionally, for efficiency and expediency, we chose to concentrate our efforts on this unit of a closed network rather than simulating the entire country, as the outcomes would be analogous but on a larger scale.

5. 2. 2. 2 Production round

According to the production procedure, we simulated all-in-all-out production rounds in parent-rearing farms, parent multiplier farms, and broiler farms. Hatcheries are modeled as a continuous flow system. Transport and production are calibrated so that the numbers of animals, farms, and hatcheries match.

Parent-rearing farms are stocked every 150 days with 40,000 one-day-old parent broiler chickens (PS: Parent stock) that are raised for 140 days. After 140 days, these PS chickens are transported to one of the two multiplier farms connected to this farm

(Figure 5.1). After a downtime of 10 days, the parent-rearing farm will receive a new batch of one-day-old PS chickens.

In the multiplier farm, PS chickens lay eggs at a rate of *b(t)* per day that are transported to the hatchery. PS chickens are kept in a multiplier farm for a period of 280 days, after which they will be slaughtered at the age of 420 days (140 days in the rearing farm and 280 days in a multiplier farm) (Mostert et al., 2022) (Table 5.2). The 32 multiplier farms are transporting their eggs to a single hatchery on alternate days. In the hatchery, eggs are hatched at the rate of *h(t)*) per day.

The number of eggs in the hatchery and chicks in broiler farms will be subject to a stochastic process characterized by variations in hatching and laying rates in PS chickens (Table 5.2).

After hatching, one-day-old broiler chickens are transported to broiler farms. 3,000 to 89,000 one-day-old chickens are transferred to each of 6 different farms every 3 days. To illustrate, on Day 0, broiler chickens are delivered to broiler farms 1 to 6, while on Day 3, broiler farms 7 to 12 receive the broiler chickens, etc. After 66 days (completing 22 transport rounds), the hatchery will restart the transportation process to the same broiler farms, repeating the round. Slow-growing broiler chickens are reared in the Netherlands for 56 days.

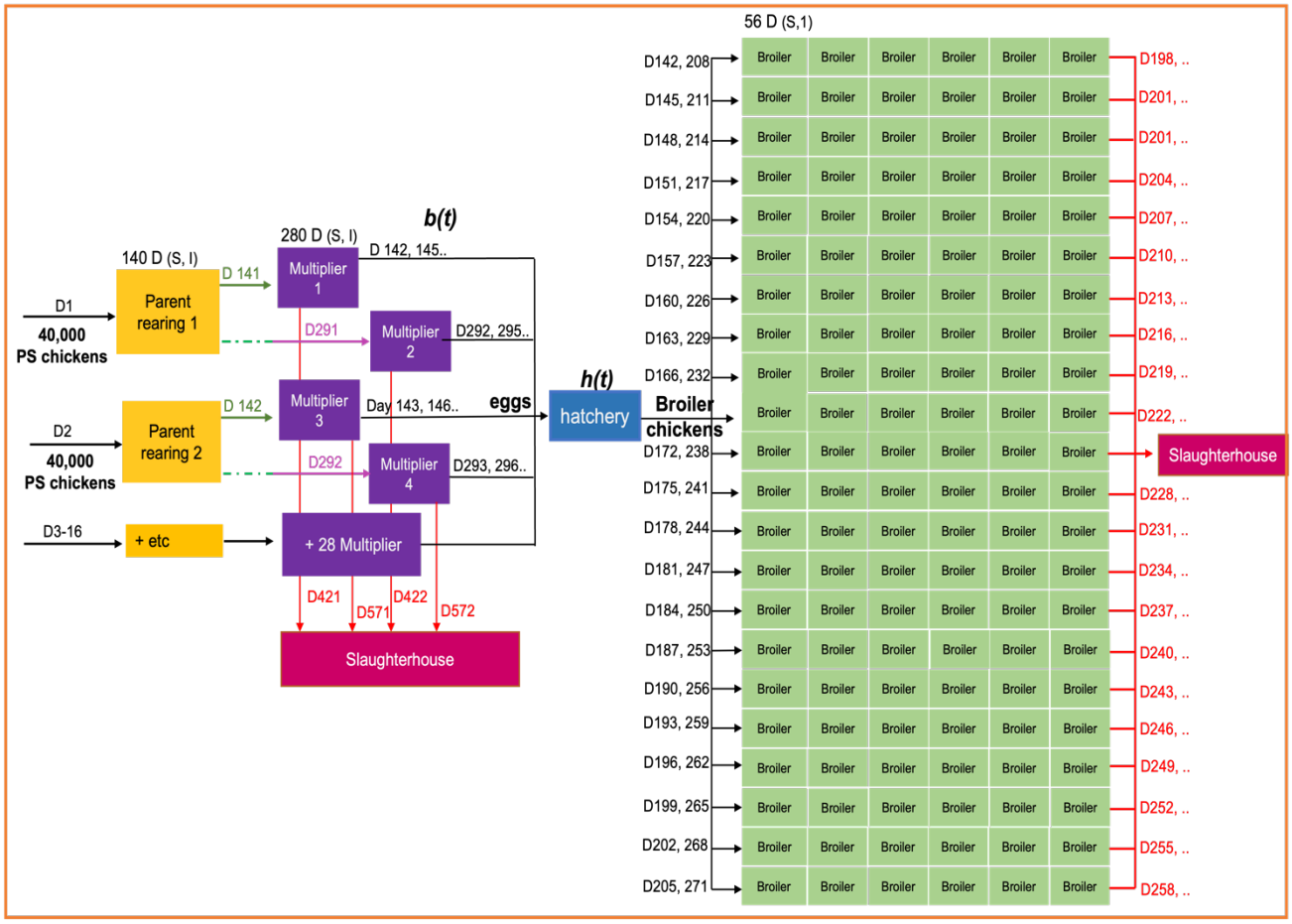


Figure 5.1- Farm structures in the broiler production. Arrows represent the flow of parent broiler chickens (PS chickens), eggs, and broiler chickens. Day and D indicate the day the movement occurs. B(t) is the parent broiler laying rate per day and h(t) is the egg hatching rate per day. The simulation covers a period of 4,000 days and begins with 40,000 PS (parent stock) chickens entering the rearing farms (yellow-colored boxes). These chickens are then transported to multiplier farms (purple-colored boxes). Eggs are transferred to the hatchery (blue-colored box) where they hatch. The resulting one-day-old broiler chickens are transported to broiler farms (green-colored boxes). Red-colored arrows indicate the movement of PS chickens and broiler chickens to slaughterhouses.

5. 2. 3 Probability of detection by slaughterhouse monitoring

Based on the current national surveillance protocol for CPE in broiler production (MARAN, 2020) and the simulated number of contaminated and slaughtered chickens, we calculated the probability of detecting CPE in a batch of broiler chickens sent to slaughterhouses (P_{det_i}). The probability is calculated by multiplying the probability that a batch of broiler was sampled during slaughterhouse surveillance with the probability that the batch of broiler *i* has at least one colonized broiler chick and would test positive for CPE if it is indeed colonized (P_{posdet_i}):

$$P_{det_i} = \left(\frac{sample_{NL}}{N_{broilerfarm} \cdot N_{broilerbatch}}\right) \cdot \left(1 - \left(1 - \frac{I_S}{N_S} Se\right)^n\right) \qquad (3)$$

The probability that a flock of broilers was sampled during annual surveillance is calculated by dividing the total number of broiler batches sampled annually ($sample_{NL}$) with the number of broiler batches raised per year. The probability that at least one broiler chicken in a flock of broiler chickens *i* is CPE positive and is sampled in the surveillance (P_{posdet_i}) is the fraction of broiler chickens in a batch being colonized by CPE at slaughter ($\frac{I_s}{N_s}$) and test sensitivity (Se). P_{posdet_i} varies depending on the route of introduction because it depends on the number of colonized broiler chickens at slaughter. Test sensitivity (Se) is based on the CPE screening report conducted by National Institute for Public Health and the Environment and *n* is the number of samples per farm (Wit et al., 2017: Statline., 2023). We assume a 100% specific test.

The number of samples per flock in the national surveillance was set to 10 samples per flock, following the sampling protocol outlined by the national surveillance for resistant bacteria in livestock (Expert).

Table 5.13: *Input to calculate the probability of detection CPE at slaughter.*

Input	Description	Value (unit)	Reference
$sample_{NL}$	Total number of broiler batches sampled per year in the national surveillance; each flock sampled came from a different broiler farm	300 (year^{-1})	MARAN, 2020; EFSA 2016
$N_{broilerfarm}$	Total number of broiler farms in the Netherlands	780	AVINED expert; Statline, 2022
$N_{broilerbatch}$	Total number of flocks of broilers raised in a broiler farm per year	5.5 (year^{-1})	Mostert et al., 2022: AVINED expert
$\frac{I_s}{N_s}$	Proportion of CPE-positive broiler chicken at slaughter age	proportions	Obtained from simulations
Se	CPE test sensitivity	0.85	Wit et al., 2017
n	Number of samples per flocks for CPE surveillance	10 (flocks)	MARAN, 2020; EFSA 2016; Expert

5. 2. 4 Outcomes

We evaluated the spread of CPE through five outcomes: (1) the number of farms that have a major outbreak of CPE (N_{farm}), (2) the number of animals colonized with CPE ($N_{col.}$), (3) the duration of CPE colonization on flocks (D), (4) the reoccurrences of

outbreaks in the same farm, and (5) the number of colonized animals sent to the slaughterhouse over the simulation period. For a farm to be classified as having a major CPE outbreak, the cumulative number of colonized birds must exceed 80. It should be noted that a major outbreak in a hatchery involves at least one contaminated egg, given that eggs are continually hatched and transferred to broiler farms.

5. 2. 4. 1 Introduction of CPE scenarios

We investigated the spread of CPE through the introductions from feed and import of live animals, which are the most likely sources of CPE introduction to broilers in the Netherlands (Dankittipong et al., 2022). Three introduction scenarios were simulated. In the first introduction model, we simulated introductions by colonized PS chickens in a single flock of import into a rearing farm (single import model), to model the consequences of rare introductions. To minimize the risk of stochastic fade out, we assumed each batch would include 40 CPE-colonized birds. For feed, a continuous exposure of CPE in rearing, multiplier, and broiler farms was simulated (feed model) according to the potential exposure estimated from the risk assessment. Lastly, the introduction of 20 colonized PS chickens in every flock of a rearing farm (multiple import model) was simulated to mimic the impact of a constant influx of colonized chickens. In the model, CPE was introduced after a one-year (warm-up) simulating period to the stable number in the population.

5. 2. 4. 2 Sensitivity analysis

Sensitivity analysis was used to assess the robustness of the model prediction to changes in model input and structure (Kirkeby, 2021). In the sensitivity analysis, we changed parameters one-at-a-time with a factor 0.1 and 2 (Table 5.4). All parameters were evaluated. We applied the sensitivity analysis to two models: with introduction from feed and with single introduction from import. The number of runs in the sensitivity analysis is 10 for each parameter combination. The key output variables used to assess the influence of each parameter included the total number of positive flocks, the total number of positive animals, the duration of the period of contagiousness, and the number of CPE-colonized broiler chickens at the slaughterhouse.

Table 5.14- *Parameters included in the sensitivity analysis. Parameter values displayed 10% of the original value, original value, and a 100% increase of the original value.*

Parameter changes	Farms	Parameter	Parameter values
Rate of exposure to CPE from feed	Rearing farm, Multiplier farm	ω_{ps}	$1.4 \cdot 10^{-10}, 1.4 \cdot 10^{-8}, 2.8 \cdot 10^{-8}$ (day^{-1})
	Broiler farm	ω_b	$7 \cdot 10^{-10}, 7 \cdot 10^{-8}, 1.4 \cdot 10^{-7}$ (day^{-1})
Proportion of colonized PS chicken in a batch of import	Rearing farm	Proportion of colonized PS chickens in one batch of import	0.0001, 0.001 (40 PS chickens), 0.002 (80 PS chickens)
Recovery rate	Rearing farm, Multiplier farm Broiler farm	γ	0.003, 0.03, 0.06 (day^{-1})
Reduction in susceptibility to transmission due to age	Rearing farm, Multiplier farm, Broiler farm	ψ	0.006, 0.6, 1.2 (day^{-1})
Minimum susceptibility	Rearing farm, Multiplier farm, Broiler farm	z	0.01, 0.10, 0.20
E. coli carrying CPE shedding rate	Rearing farm, Multiplier farm, Broiler farm	θ (day^{-1})	0.005, 0.5, 1
E. coli carrying CPE survival rate in the environment	Rearing farm, Multiplier farm, Broiler farm	ρ (day^{-1})	0.005, 0.05, 0.1
Time-dependent environmental transmission rate	Rearing farm, Multiplier farm, Broiler farm	β (day^{-1})	0.00048, 0.0048, 0.0096

5. 2. 4. 3 What-if analysis

In what-if analysis, we explored six different scenarios in broiler production, considering variations in farm characteristics, connections, and exposure to the risk of CPE (Table 5.5). Scenarios 1 to 6 were implemented for a model with introduction from single import and, additionally, Scenarios 5 and 7 were implemented for introduction by feed.

Table 5.15- *The changes made to the simulation model to investigate possible scenarios in the broiler production process.*

Scenario	Descriptions	Farm types adjusted	Model
Baseline	Single import model	All rearing farms	20 colonized PS chickens in one batch Zero colonization rate from CPE-contaminated feed in parent flock Slaughter age = 56 days
	Feed model	All farms	Zero colonized PS chickens in one batch $1.4 \cdot 10^{-8}$ colonization rate from CPE-contaminated feed in parent flock $7.0 \cdot 10^{-8}$ colonization rate from CPE-contaminated feed in broiler flock Slaughter age = 56 days
1	All rearing farms import PS chickens from outside of the Netherlands	All rearing farms	20 of colonized PS chickens imported to 16 rearing farms in every batches
2	All rearing farms import PS chickens from outside of the Netherlands half of the time	All rearing farms	40 of colonized PS chickens imported to 16 rearing farms in every other batches
3	Broiler farms imported additional batch of broiler chickens from hatchery outside the Netherlands once per year	All broiler farms	20 colonized broilers out of 20,000 broiler chickens are imported from outside NL to 132 broiler farms once per year
4	Broiler farms imported additional batch of broiler chickens from hatchery outside the Netherlands in every round	All broiler farms	4 colonized broilers out of 20,000 broiler chickens are imported from outside NL to 132 broiler farms 5.5 times per year

Scenario	Descriptions	Farm types adjusted	Model
5	The lifespan of chicken is shorter reflecting the slaughter age of conventional broiler chicken	All broiler farms	The slaughter age is at 42 days old
6	Broiler chickens in all broiler farms receive antibiotics.	All rearing farms, all multiplier farms, all broiler farms	The transmission rate of CPE increases to 0.048 in all farms
7	Localize the exposure to feed to 10% of broiler farms.	Broiler farms	The colonization rate from CPE-contaminated feed in broiler flock (ω_b) is $7 \cdot 10^{-8}$ in 13 broiler farms across the length of simulation, whereas 119 broiler farms have zero rate of exposure throughout the length of simulation

5. 3 Results

5. 3. 1 Introduction of CPE scenarios

As stated above, three introduction models were simulated: 1) single import of live animals, 2) use of contaminated feed (feed), and 3) multiple imports of live animals. The introduction from import was assumed in a single rearing farm, either once (single import) or multiple times in the same farm (multiple imports). The introduction of CPE into the broiler production pyramid from feed was assumed to be continuous in all farms. In the case of the hatchery, where there is a continuous flow of eggs and animals every two days, contamination was considered present if at least one positive egg is present. We compiled the median, 5th percentile, and 95th percentile of all outcomes in Table 5.6.

Table 5.16- *Outcome of three introduction models; 1) Single import of colonized PS chicken into a rearing farm, 2) multiple import of colonized PS chicken into the same rearing farm, and 3) contaminated feed in rearing, multiplier, and broiler farms.*

**N_{farm} is number of farms, N_{col} is number of colonized chickens OR number of contaminated eggs, D = duration of outbreak within a farm*

Introduction models*		Single import	Multiple import	Feed
N_{farm}	Rearing	1 [1, 1]	1 [1, 1]	0 [0, 2]
	Multiplier	1 [1, 1]	2 [2, 2]	0 [0, 2]
	Broiler	0 [0, 6]	13 [1, 25]	56 [45, 64]
N_{col}	PS chickens in rearing	39,932 [39,919, 39,945]	1,077,590 [1,077,478, 1,077,712]	0 [0, 1323]
	PS chickens in multiplier	793 [753, 836]	20,581 [20,362, 20,797]	0 [0, 63,867]
	Eggs in hatchery	10 [6, 15]	275 [249, 297]	0 [0, 16]
	Broiler chickens in broiler farms	0 [0, 390,269]	860,365 [63,096, 1,853,520]	4209142 [3,338,773, 5,333,092]
D	PS chickens in rearing	138 [138, 138]	138 [99, 138]	136 [133, 137]
	PS chickens in multiplier	236 [184, 278]	228 [166, 279]	198 [63, 261]
	Broiler chickens	54 [54, 54]	54 [54, 54]	52 [48, 54]
Prevalence at slaughter in colonized broiler	Broiler chickens	0.26 [0.25, 0.26]	0.26 [0.25, 0.26]	0.25 [<0.01, 0.26]
N_{farm} at slaughter	Broiler chickens	0 [0, 6]	13 [1, 25]	63 [53, 71]

5. 3. 1. 1 Major outbreaks in the simulations

When a rearing farm received a batch of 40 contagious PS chickens (single import), all runs resulted in a major outbreak in the rearing farm and the connected multiplier farms was always colonized. The presence of colonized PS chickens in the multiplier farms led to the production of a small number of contaminated eggs in all 100 runs. Despite the presence of contaminated eggs, the number of day-old colonized broiler chickens remains low in the hatchery due to the relatively small quantity of contaminated eggs and low probability of colonization of the chicks from the eggs. However, this small number of contaminated eggs can lead to secondary transmission

in the broiler farms. Contrary to other farm types, a small number of contaminated eggs of at least one is categorized as a major outbreak in Figure 5.2 as the eggs are moved from hatchery to broiler farms every two days, thus, the number of contaminated eggs would not accumulate to be as high as 80 contaminated eggs. The hatched eggs resulted in major outbreaks in broiler farms in 10 out of 100 runs, with the number of affected farms varying between 1 and 6 (Figure 5.2). Repeated outbreaks in the same farm were not observed in this scenario.

In the scenario of multiple import introductions, where 20 colonized PS chickens were introduced with each batch to the one specific rearing farm, it was observed that the rearing farm became colonized in all runs as well as the two multiplier farms connected to this rearing farm and their accompanying hatchery. Repeated outbreaks were observed in the rearing farm, aligned with the repeated introductions of colonized PS stock. In between introductions, the contamination faded out (Figure 5.3). In this scenario, major outbreaks in broilers occurred in 91 out of 100 runs with the number of colonized farms varying between 8 and 24 (Figure 5.2). In addition, repeated outbreaks occurred within the same broiler farm in 13 out of these runs, with the number of outbreaks ranging from 1 to 6. Most of the time, the hatchery received one contaminated egg per day, while for less than 10 percent of the time, they received 2 to 4 contaminated eggs. We regard at least one contaminated egg as a major outbreak. This pattern was consistent across all runs, indicating that the instances of recurrence were random chance events. In the simulation, each broiler farm supplied a total of 51 batches of broiler chickens to the slaughterhouse. Consequently, the majority of farms delivered a batch with colonized animals. In 13 runs, 1 to 6 farms delivered two batches with colonized birds.

Continuous exposure from feed resulted in major outbreaks in rearing farms in 32 runs and in multipliers in 26 runs. Major outbreaks in multiplier farms resulted in a major outbreak of at least one contaminated egg in a hatchery in 23 runs. In both farm types, the number of affected farms varied between 1 and 2. Recurring series of outbreaks within the same farm were observed in 10 of the runs in rearing farms, but not in the multiplier farms. All runs experienced series of major outbreaks in broiler farms, with the number of affected farms varying between 48 and 54. Repeated outbreaks in the same broiler farm occurred in all 100 runs, with most of these being limited to one

repeated outbreak affecting 2 to 18 farms, while 5 farms experienced two repeated outbreaks. Rarely, one farm would have 3 recurring outbreaks. Broiler farms exhibited an almost five-fold higher probability of colonization per year compared to other farm types (Table 5.4). This elevated probability is attributed to several factors, including a higher number of batches per year, younger chickens that are more susceptible to colonization, and the resulting increased likelihood of introduction through contaminated feed.

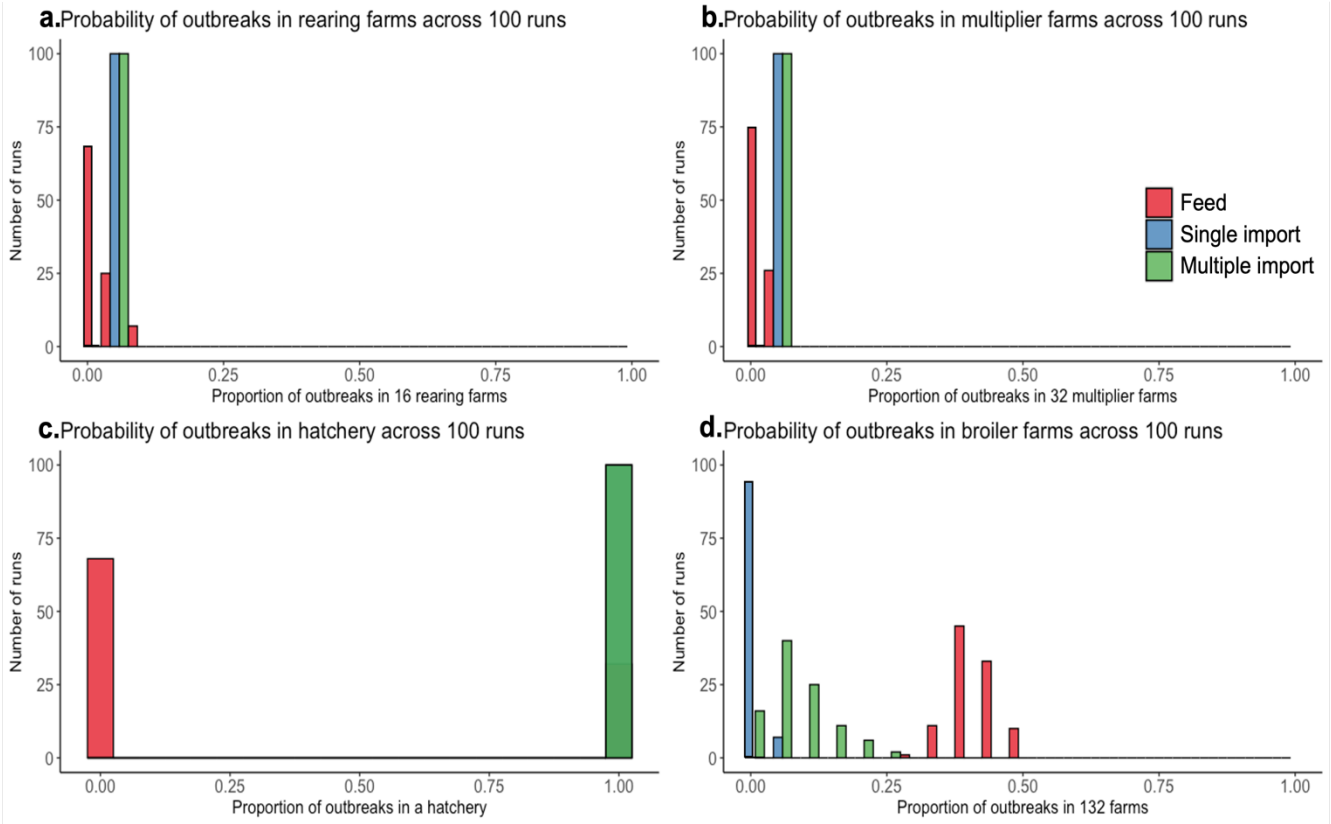


Figure 5.2- *Probability distribution of the proportion of farms experiencing a major outbreak and contamination (hatchery) across three introduction scenarios, feed, single import, and multiple imports. The simulations contain 16 rearing farms (a), 32 multiplier farms (b), 1 hatchery (c), and 132 broiler farms (d). The proportion of farms from each run is represented as one point. Thus, the width of each of the points on X-axis reflects the number of runs with the specific outcome on the Y-axis. The y-axis indicates the proportion of farms that experience major outbreaks and contamination of the hatchery.*

5. 3. 1. 2 Number of chickens colonized with CPE

The transmission dynamics of CPE spread in rearing farms and broiler farms follow a similar pattern of rapid spread, affecting 99% of the batch within 1 to 3 days after introduction, and subsequently declining gradually, irrespective of the source of

introduction. Both rearing and broiler chickens are relatively young (1 to 8 days) and are thus, very susceptible to colonization.

On a rearing farm, when CPE is introduced through 40 colonized rearing chickens, the number of colonized PS chickens rapidly peaks with number of 39,992 colonized chickens (99% of the PS chickens in the farm) after 3 days (Figure 5.3a). Afterward, the number of colonized PS chickens slowly declines due to recovery and a decreasing susceptibility with age. It reaches 800 to 900 chickens at the time of transport to the multiplier farm.

Next, the number of colonized PS chickens on the multiplier farm gradually declines from 800 to 400 due to further increasing age resistance or recovery (Figure 5.3b). Ultimately, the multiplier farm becomes free of CPE after 210 days (when the PS chickens are approximately 350 days old). According to the simulations, these colonized PS chickens produce only 6 to 15 contaminated eggs in the entire round (Figure 5.3c). Consequently, outbreaks in broiler farms are rare, occurring in only 10 out of 100 runs (as shown in Figure 5.2). However, when such outbreaks do occur, they spread rapidly and reach a maximum of colonized broiler chickens after 1 to 4 days throughout the entire broiler farm population, affecting between 60,000 to 88,000 chickens (99% of the chicken in a batch) (Figure 5.3d).

The introduction of CPE through multiple imports leads to similar dynamics within each of the farm types, although the occurrence of the outbreaks is more frequent due to repeated introductions into the rearing farm. Although the quantity of contaminated eggs increases to 22 to 248 eggs in this scenario, the number of colonized broiler farms remains relatively low (Figure 5.2c).

Contaminated feed results in more variability in the size of an outbreak in rearing farms and broiler farms than single or multiple import. In rearing farms, the peak of spread from feed introduction ranges from 20,000 to 29,492 chickens (50 to 70% of the population), which represents the 5th to 95th percentile of the population. On rare occasions, the peak can reach between 100 and 39,207 chickens (minimum of 0.25% to a maximum of 98.00%). The time to reach the peak varies from 7 to 14 days, where a longer time until the peak results in a lower peak, due to the age-dependent

susceptibility (ψ) assigned in all introduction models. A similar variation is observed in broiler farms, where the percentage of colonized animals ranges from 0.1% to 99%, and the time to peak ranges from 5 to 11 days. Importantly, in the feed scenario, only very young animals can become colonized in both broiler and rearing farms. Moreover, if the introduction of contaminated feed occurs on Day 8, the probability of a major outbreak is small, as only 0.2% of animals are colonized by that age. Overall, the dynamics of spread in the explored introduction scenarios exhibit variations but are all capable of causing major outbreaks in the flocks.

After the outbreak peaks, typically affecting 80-99% of the flock, there is a rapid surge in the number of colonized chickens, followed by a steady decline. This decline is consistent across all introduction routes due to the assumption of age-dependent susceptibility, which reduces the number of colonized animals as the production cycle progresses, thereby reducing CPE contamination in the farm environment. Following each production round, the entire flock of birds is relocated to a different farm, resulting in an average downtime of 10 days. During this downtime, the remaining CPE colonization in the environment, which has already been reduced due to the lower number of colonized animals, continues to decrease. Additionally, cleaning and disinfection procedures carried out during this downtime further eliminate any remaining CPE in the environment, effectively halting its spread to the upcoming batch.

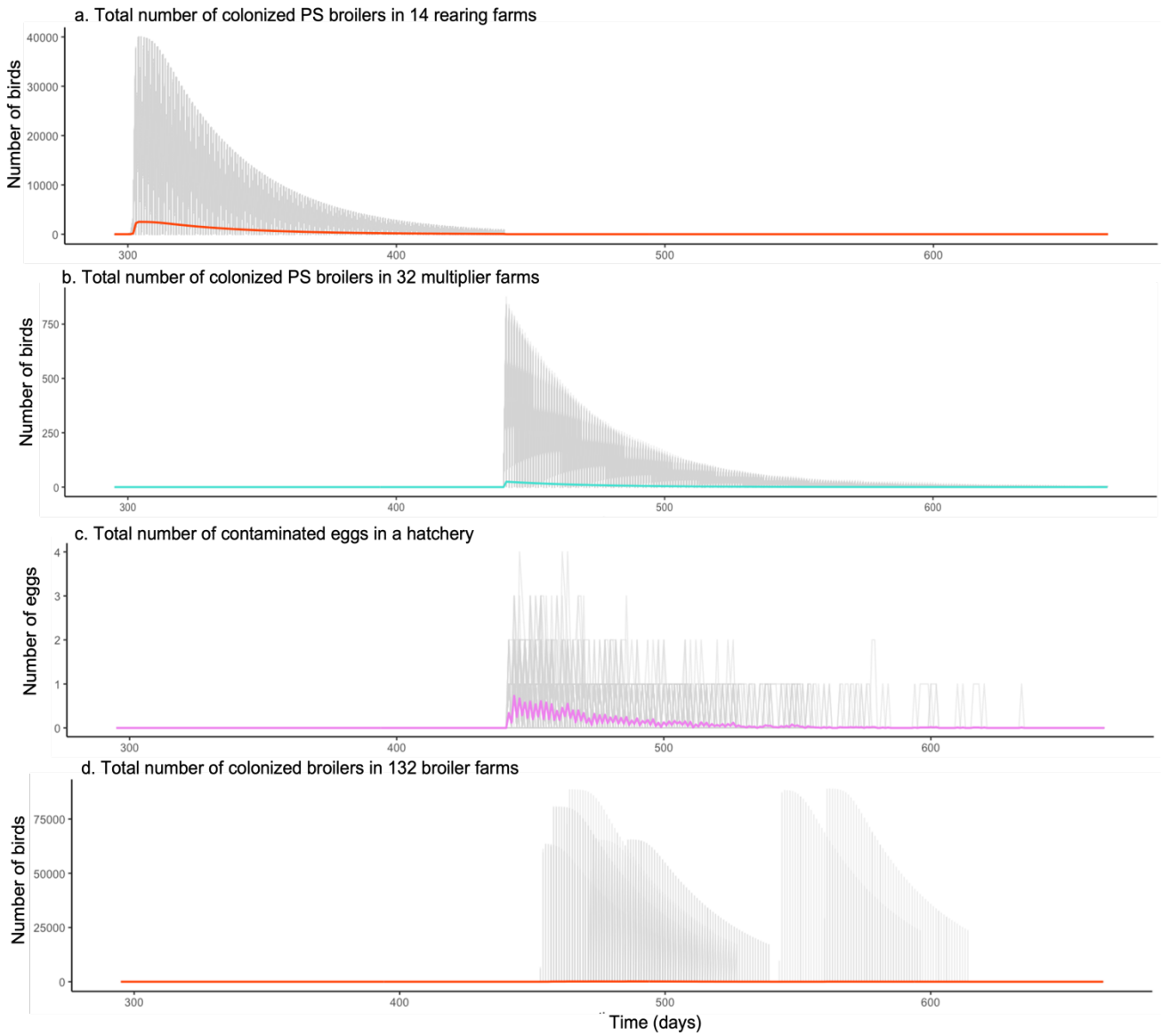


Figure 5.3- The average number of animals colonized with CPE introduced from a single import across all farms from 100 runs. Each plot shows the number of animals in each farm type from Day 300 to Day 665. The gray lines represent the number of colonized animals from individual runs (100 in total), while the color-coded lines indicate the average number of animals colonized within each farm type.

5. 3. 1. 3 Duration of CPE outbreaks in flocks

The duration of CPE outbreaks in rearing farms and broiler farms is approximately equal to the length of one production round, regardless of the route of introduction (Figure 5.4). For the import that is consistent with 1) the introduction by colonized animals at the start, either by live import (rearing farm) or colonized day-old chicks and 2) all-in-all out production system with 10 days downtime preventing spillover of colonization from one round to another. For feed, major outbreaks can only begin shortly after the start of a round due to the age-dependent susceptibility.

There is notable variation in the duration of colonization observed in multiplier farms compared to the length of their production round. In both single and multiple imports scenarios, the duration of colonization in multiplier farms ranges from 180 to 280 days (Figure 5.4). This extended duration is due to the longer raising period in multiplier farms compared to rearing farms allows for variations in the timing when the flocks become completely rid of colonized PS chickens. However, we suspect that if the rearing farm extended the PS chicken raising period to be as long as that in the multiplier farm, the duration of colonization would be longer in rearing farms due to the higher susceptibility to colonization (and recolonization) in younger PS chickens compared to those in multiplier farms.

In the feed scenario, a small variation in the duration of CPE outbreaks in multiplier farms was observed, with a range of up to 50 days. This occurrence is attributed to a small number of colonized PS chickens from the rearing farm that was still colonized when they reached the multiplier farm. The relatively small population of colonized animals leads to a faster recovery in the farm, resulting in the complete elimination of contamination within a shorter time frame.

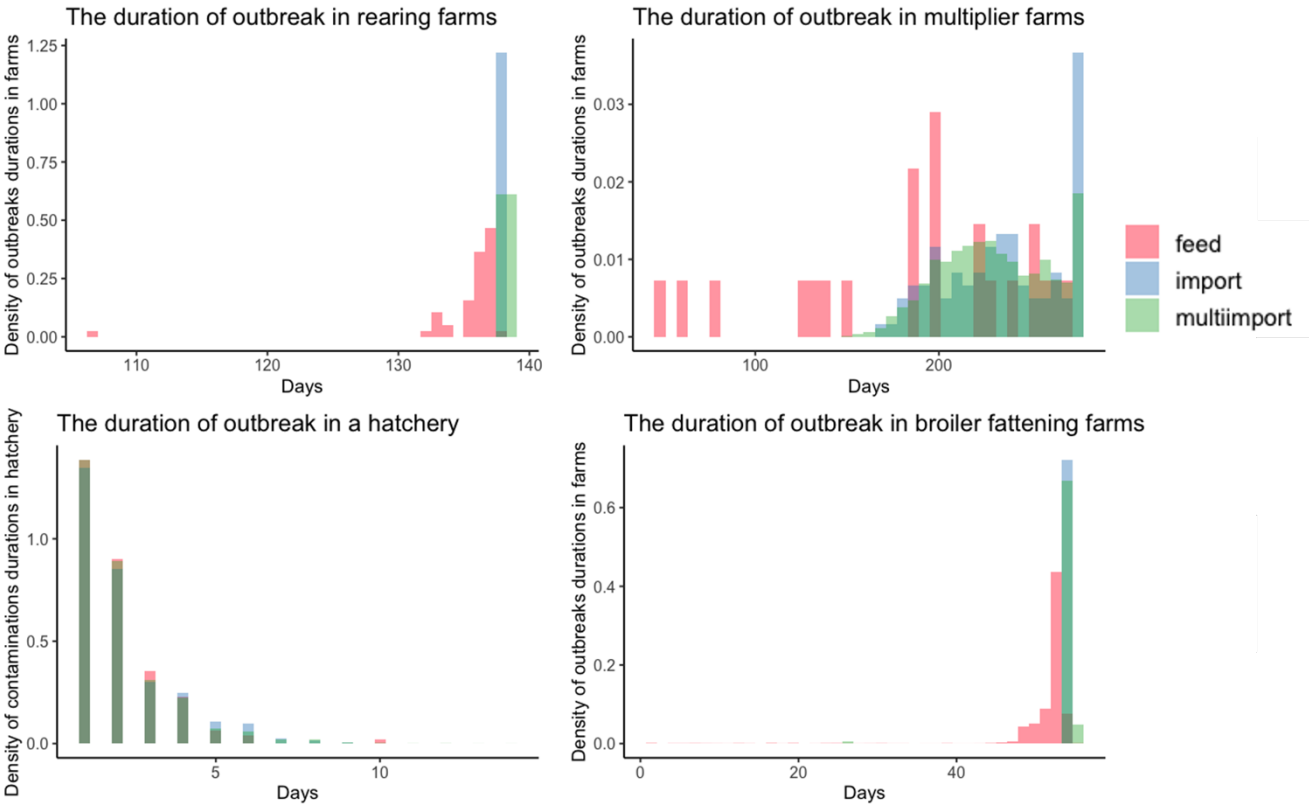


Figure 5.4- Distribution of the duration of CPE outbreaks in PS chickens, broiler chickens, and eggs contaminated with CPE. X-axis is the duration of outbreak in days and Y-axis is the number of durations counted from all 100 simulations. Blue- and green-colored bars show the number of colonized animals from single import and multiple import baseline models. Pink-colored bars show the number of colonized animals from the feed model.

5. 3. 1. 4 CPE colonization in broiler flocks bound to slaughterhouse

Across all three scenarios, broiler farms experiencing a CPE outbreak will remain colonized until the time of slaughter (Figure 5.4). However, the duration of the outbreak varies among sources due to differences in their introduction times. Outbreaks originating from feed have a slightly shorter duration because they are initiated during the middle of the production cycle, whereas those stemming from live imports begin at the beginning of the production cycle. With the reduction in colonization over time, the introduction from contaminated feed must have occurred before Day 8 to result in a major outbreak.

On the other hand, the number of colonized broiler chickens at slaughter is reduced to approximately 27% of the peak number observed during the spread of the outbreak. This reduction in colonization is consistent across introduction routes. Among the

scenarios, the feed introduction scenario shows the highest number of colonized broilers and broiler farms at the time of slaughter. This outcome is attributed to the fact that the feed introduction scenario results in a higher number of colonized flocks and broiler chickens compared to the other scenarios (refer to Sections 5.4.3 for more details).

Over the course of 9 years of simulations, a total of 6,702 flocks of broiler chickens from 132 broiler farms were sent to slaughterhouses. Among these batches, the number of colonized batches was highest in feed introduction scenario ranging between 52 and 92 batches during 9 years. In the single import scenario, outbreaks in batches of broiler chickens are rare, resulting in 1 to 6 batches of broilers sent to slaughterhouses in 9 years (Figure 5.5). However, when multiple introductions of CPE to a rearing farm occurred, the number of colonized batches sent to slaughterhouses increased significantly, ranging from 1 to 30 batches.

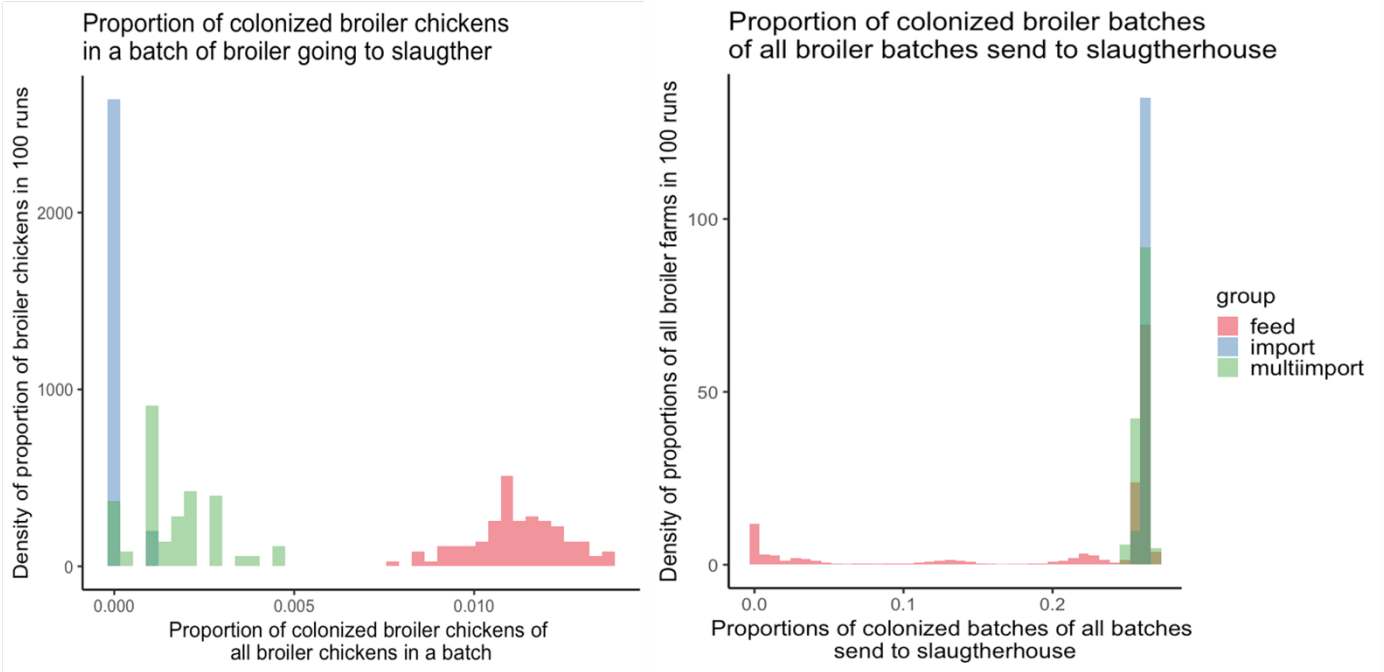


Figure 5.5- Left) The X-axis represents the proportion of broiler chickens colonized with CPE, relative to all broiler chickens in a batch. The Y-axis illustrates the frequency of these proportions across 100 simulations. Right) On the X-axis, the proportion of colonized batches from all broiler batches (6702 batches) sent to the slaughterhouse over a span of 9 years is displayed. The bars are color-coded: blue and green represent proportions from the single import and multiple import models, while red indicates the proportion of colonized animals from the feed model.

5. 3. 1. 5 Probability of detecting CPE outbreak with national surveillance

When considering the scenario of a single import into the rearing farm, only 10% of the runs resulted in one or a series of outbreaks in broiler farms, impacting 1 to 6 farms (6 flocks). Consequently, the model predicts that in 90% of the runs, the probability of detection will be 0, despite CPE having circulated in a rearing farm and multiplier farm. In these 1 to 6 colonized flocks, the probability that the colonized flock is being tested from national surveillance is 0.069. This probability represents the likelihood of the flock being selected for national sampling. Subsequently, the probability of detecting CPE within the colonized batch (P_{posdet_i}) is approximately 0.92. As a result, the probability that CPE-colonized batch of broilers will be detected in the surveillance (P_{det_i}) is 0.064.

Consequently, the cumulative probability of detection over the studied period is 0 in 90% of the runs and for the other 10% varies between 0.022 and 0.064 when only one batch is contaminated in the entire run and 0.32 when 6 batches are contaminated. Thus, overall, the probability of detecting CPE after a single introduction with live birds is very low.

In the scenario with multiple imports into a rearing farm, the probability of detecting a CPE outbreak in a batch of broilers (P_{det_i}) is the same as in the single import scenario. Here, 91 of the 100 runs resulted in positive flocks of broilers, with the total number of positive farms varying between 8 and 24. Consequently, the cumulative probability of detection across the simulated period varied between runs (Figure 5.6: left plot). Nevertheless, overall the probability of detection is higher than in the single import scenario, with a maximum cumulative probability of detection ranging from 0 to 0.81, with the 50 percentile at 0.45. Although the probability of detection is higher in this scenario than in the single import, also here there is a considerable probability of missing CPE in the surveillance and time to detect is generally long.

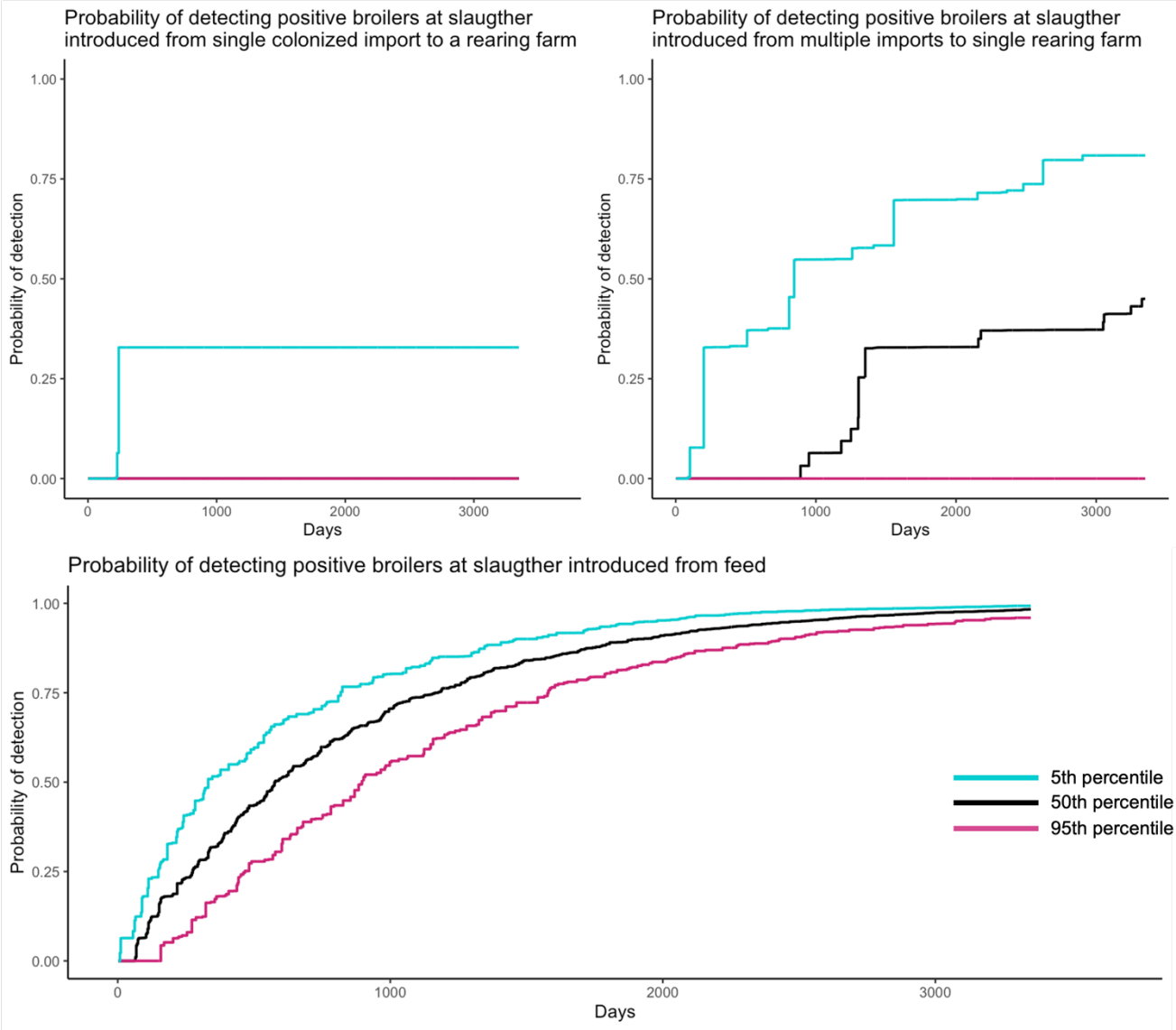


Figure 5.6- Cumulative probability of to detect CPE using Dutch National Surveillance Protocol. The top left plot displays the cumulative probability of detection introduced from a single batch of colonized PS chickens to a rearing farm. The top right plot displays multiple batches of colonized import to a single rearing farm. The bottom plot displays the cumulative probability of detection introduced from contaminated feed. Black line represents the median, turquoise line represents the 5th percentile, and pink line indicates the 95th percentile probability of detection on a specific day. The right plot displays the cumulative probability of detection introduced from feed.

The continuous exposure of feed leads to different dynamics in the detection probability for import scenarios (Figure 5.6). The series of outbreaks originating from feed are quite similar in the runs due to ongoing exposure to contaminated feed in all broiler farms. The cumulative probability of detection ranges from 0.95 to 0.99, with the 50 percentile at 0.98. The time it takes to detect contaminated batches with a cumulative probability of 0.95 ranges from 1,837 to 2,935 days and a median of 2,365 days after the first broiler chickens are contaminated.

5. 3. 2 Sensitivity analysis

In the feed model, we identified the strongest correlation between the age-associated reduction in transmission (ψ) to all output variables (Table 5.6; Supplementary 5.5.3). Decreasing the reduction in susceptibility to transmission due to age (ψ) (resulting in higher probability of colonization at higher age) resulted in a significant increase in all output variables, as all animals were equally susceptible to CPE introduction. Doubling the reduction in susceptibility to transmission due to age (ψ) dramatically reduces all the output variables (Table 5.6).

Furthermore, the rate of colonization from CPE contamination in feed on farms (ω_{ps} & ω_b) demonstrated a notable correlation with the output related to broiler farms, although its impact was less pronounced than the reduction in transmission (ψ). When the rate of colonization from CPE contamination in feed on broiler farms (ω_b) doubled, the number of broiler farms with outbreaks increased by 56 percent. Conversely, a 90% reduction in the rate of colonization from CPE contamination in feed on farms (ω_b) eliminated the number of broiler farms experiencing outbreaks in other words, no outbreaks of CPE.

Across all outcomes, the colonization coefficient of CPE (β) showed a relatively minor influence on the results. Further sensitivity analysis showed that the other parameters - rate of CPE excretion into the environment of the animal's pen (θ), rate of excreted CPE survival (ρ) and minimum susceptibility (z) – also had limited effects on the outcomes.

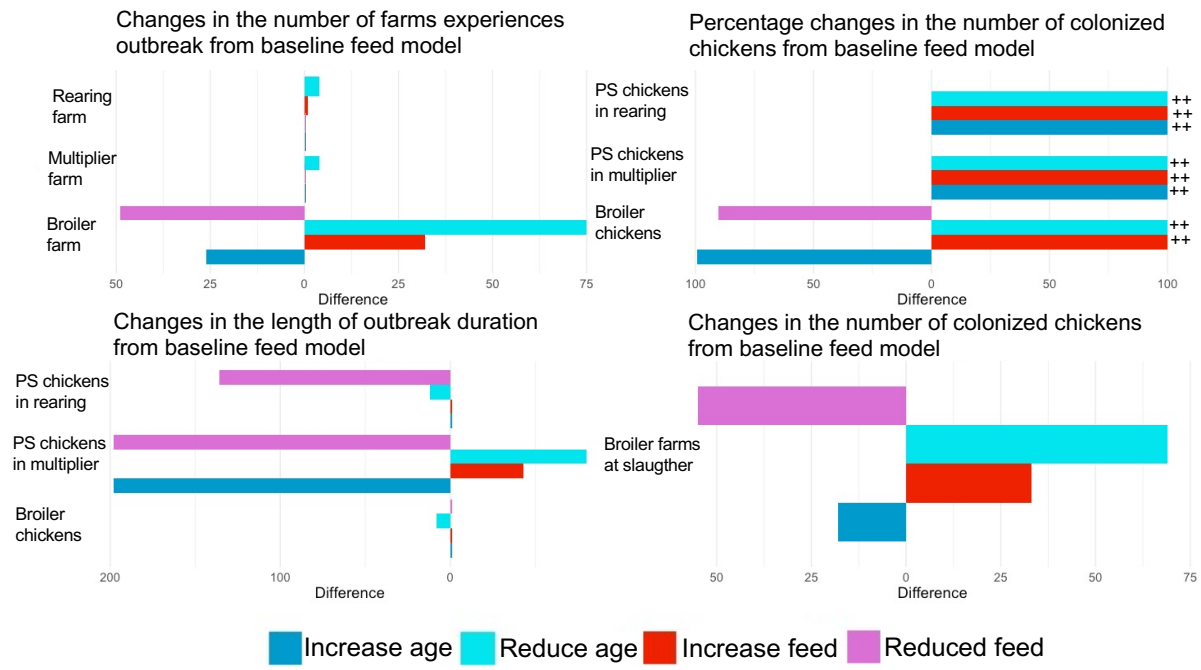


Figure 5.7- *Sensitivity analysis results in feed Scenario. Two parameters with significant correlation are included, the reduction in susceptibility to transmission due to age (ψ) and the rate of colonization from CPE contamination in feed on farms (ω_{ps} & ω_b). Top left tornado plot depicts the changes in number of colonized farms from baseline feed introduction model. Top right tornado plot shows the changes in number of colonized chickens in percentage from baseline feed introduction model. Bottom left tornado plot displays the days changes in the duration of outbreak in each farm type from baseline feed introduction model. Bottom right tornado plot shows the changes in the number of colonized broiler farms at slaughter time. The tornado plots display median number of mentioned outcome. The ++ sign in the percentage changes in the number of colonized chickens from baseline feed model means the changes is more than 100 percent.*

In the single import model, the recovery rate (γ) showed the strongest correlation with the number of broiler farms experiencing series of outbreaks compared to other parameters. Additionally, the reduction in transmission (ψ) also exhibits a moderate but notable correlation to the number of broiler farms experiencing series of outbreaks. Doubling both parameters did not result in an increased number of broiler farms with outbreaks. However, a tenfold reduction in the recovery rate (γ) and the reduction in transmission (ψ) led to a significant increase in the number of affected broiler farms, ranging from 89 to 118 farms (recovery rate) and 5 to 29 farms (reduction transmission), respectively. We see the same strong correlation of the recovery rate (γ) and the reduction in transmission (ψ) to the output of CPE-positive broilers at the slaughterhouse, the number of colonized broiler farms, and the prevalence of colonized broiler farms.

5. 3. 3 What-if analysis

5. 3. 3. 1 Import of colonized birds: Scenarios 1 to 4

The increased number of imports of contagious PS chickens to multiple rearing farms has a significant impact on the outbreak in broiler farms, resulting in a higher number of colonized broilers at slaughter (Table 5.6). The more frequently contagious imports occur in rearing farms, the greater the number of broilers that become colonized.

The import of one-day-old broiler chickens from hatcheries outside the Netherlands would be a major risk of CPE introduction in broiler farms. Even a single import of contagious one-day-old broiler chickens per broiler farm per year has resulted in the colonization of all broiler farms, irrespective of the import frequency. This highlights the high susceptibility of broiler farms to a major outbreak from just one import event.

While the colonization of broiler farms remains constant regardless of the import frequency, the number of colonized broiler chickens at slaughter is directly proportional to the frequency of import. This means that the more frequent the contagious imports, the higher the number of broiler chickens that become colonized at the time of slaughter. To illustrate, both Scenario 3, with one imported batch of colonized broiler chickens per farm per year, and Scenario 4, with all imported broiler chicken batches being colonized per farm per year, result in the same total number of contagious broiler chickens imported from the hatchery per farm per year. Despite this consistency, the increased frequency of import in the fourth scenario leads to a higher overall number of colonized broiler chickens, reinforcing the correlation between import frequency and colonization rate.

Lastly, an increased number of imports raises the probability of detection based on national surveillance efforts due to the increased number of colonized chickens at slaughter. In the single import model, the probability of detection did not exceed 0.33. However, when the import of colonized PS chickens and broiler chickens increases, the probability of detection reached 0.95. The import of colonized broiler chickens to broiler farms significantly impacts the spread, leading to a probability of detection of 0.95 within 55 days after the broiler chickens enter the broiler farms. Other scenarios

may take a longer time to achieve 0.95 probability of detection, but will not exceed 2 years.

Table 5.7- *The results of a what-if analysis, including the number of broiler farms with major outbreaks at slaughter, the number of colonized broiler chickens at slaughter, the frequency of outbreaks in all broiler farms, and the day that the cumulative probability of detection will reach 0.95. * NA days means the cumulative probability of detection did not reach 0.95 within 9 years.*

What-if analysis for single import model				
Scenarios	Number of farms with outbreak of CPE at slaughter	Number of broiler chickens colonized at slaughter	Frequency of outbreak in broiler farms	Days that the cumulative probability of detecting contaminated batch reached 0.95*
Baseline	0 [0;6]	0 [0, 101814]	1 [1,1]	NA
All batches to rearing farms have contagious PS chickens	106.5 [96.9, 121]	5.21 E06 [4.57 E06, 6.64 E06]	2 [1, 5]	82 [19, 244]
Alternate batches to rearing farms have contagious PS chickens	70 [50.4, 94.95]	1.80 E06 [1.34 E06, 3 E06]	1 [1, 3]	528 [213, 809]
Every year, a batch to broiler farms have contagious chickens	132 [132, 132]	29.08 E06 [29.1 E 06, 29.2 E06]	8 [8, 8]	91 [89, 91]
Every batch to broiler farms have contagious chickens	132 [132, 132]	177.9 E06[177.6 E06, 178.4 E06]	49 [42, 53]	56 [56, 56]
Broilers are raised for 42 days	0 [0, 4.849]	0 [0, 113,080]	1 [1, 1]	NA
The environmental transmission rate is 10-fold due to antibiotics	0 [0, 6]	0 [0, 129,057]	1 [1, 1]	NA

What-if analysis for feed model

	Number of farms with outbreak of CPE at slaughter	Number of broiler chickens colonized at slaughter	Frequency of outbreak in broiler farms	Days that the cumulative probability of detecting contaminated batch reached 0.95*
Baseline	56 [45, 64]	1.4 E06 [9.02 E05,1.43 E06]	1 [1,2]	754 [357,1063]
Broilers are raised for 42 days	53.5 [46.9, 61.85]	1.6 E06 [1.3 E06,1.8 E06]	1 [1,3]	671 [389, 734]
The environmental transmission rate is 10-fold due to antibiotics	58.5 [54, 63.55]	1.67 E06 [1.44 E06, 2 E06]	1 [1,2]	313 [577, 789]
The exposure to feed in broiler farms is limited to 13 farms (10%)	4 [2.45, 8]	765 [392, 1871]	1 [1,3]	NA

5. 3. 3. 2 Reducing raising time in broiler farm and administering antibiotics: Scenarios 5 and 6

When broiler chickens are slaughtered at the age of 42 days instead of 56 days, both the single import model and the feed model show no significant changes in output (Table 5.6). However, the total number of colonized broiler chickens at slaughter was slightly higher when the raising time was reduced to 42 days, as the animals tend to lose their colonization as they age.

Similarly, in the 6th scenario, where the environmental transmission rate was increased by tenfold, simulating the effect of antibiotic treatment on transmission (β), the what-if analysis reveals slight increases in the number of colonized broiler chickens at slaughterhouses.

5. 3. 3. 3 Localizing the exposure to feed to 10% of broiler farms

To test an alternative assumption in our analysis, we explored a scenario where we do not assume CPE homogeneously spreads to all feed mill in all farms. Instead, we examined the impact of localized introduction of CPE to a specific subset of feed mill in broiler farm. When the exposure to feed was restricted to 13 farms, several key outputs exhibited notable declines. The number of broiler farms experiencing major

CPE outbreaks decreased from 46 to 2 farms, even though the number of colonized broiler farms varied, and in some runs, it closely resembled the results of the feed model in terms of major outbreaks. The number of colonized broiler chickens at slaughter saw a substantial reduction of 99.99% due to the reduction in the number of colonized broiler farms at slaughter. This significant reduction in colonized broiler chickens effectively reduced the cumulative probability of detection, which ranged between 0.002 and 0.0147, and never reached a probability of 0.95 during the simulation. Consequently, this small probability of detection implies that broiler farms with contaminated feed, producing colonized broiler chickens, may remain undetected.

5. 4. Discussion

The transmission simulation model presented in this study shows remarkable differences in dynamics between import of colonized animals and use of contaminated feed. Single imports are expected not to result in contaminated batches of broilers in most cases, despite circulation of CPE in rearing and multiplier PS birds. However, if contaminated broiler batches do arise, they have a low probability of being detected by the Dutch national AMR surveillance program. On the other hand, when multiple imports are involved, the probability of detection increases, and can reach a 0.8 (95^{th} percentile) over a 10-year period. In the case of contaminated feed, the proportion of contaminated broilers is at its highest, as is the cumulative probability of detection reaches 0.99.

A crucial assumption in our model is the age-dependent susceptibility. Our sensitivity analysis has demonstrated that the model's outcomes are particularly sensitive to this parameter. Even slight reductions in this parameter can lead to significant changes in outcomes, ranging from nearly 0 to high values.

When a farm is initially exposed to a small number of live animals through imports, CPE rapidly spreads to almost the entire flock (98-99%) within three days, regardless of whether it is a rearing or broiler farm. On the contrary, the transmission through contaminated feed is less efficient in terms of spreading within a farm. In cases of major outbreaks in rearing and broiler farms, it takes 7-14 days to reach its peak,

affecting 25%-98% of the animals in the farm. As animals age, their susceptibility to CPE colonization decreases. Therefore, the critical factor influencing the impact of the introduction lies in the timing of contagious chickens introduced into the batch. According to the model used here, colonizations by contaminated feed resulting in a major outbreak can only take place in animals younger than 8 days. While the model assumed homogeneous mixing of all animals throughout the barn, the number of colonized live animals is likely to be reduced. However, the exposed barn will still experience a major outbreak and could make its way to the slaughterhouse.

In the import scenario, a single batch of 40 colonized PS chickens and broiler chickens are introduced to the farm at Day 1of a round, facilitating rapid spread throughout 99% of the batch. Conversely, when colonization occurs through exposure to contaminated feed, a delay ensues, leading to a slower transmission rate as other broilers mature. For instance, if one PS chicken ingests CPE-contaminated feed and becomes colonized on Day 3, CPE can spread to approximately 72% of the batch. However, if a PS chicken becomes colonized on Day 8 through feed exposure, it will not result in a major outbreak, affecting only 0.2% of the batch.

The dynamics of CPE outbreaks in the broiler production system are influenced by various factors, including farm structure and biosecurity measures, leading to different outbreak characteristics. The broiler production system operates on an all-in-all-out basis, wherein all colonized animals must leave the farm before a new batch of animals arrives. As a result, outbreaks in rearing, multiplier, and broiler farms tend to die out between two production rounds. The downtime between batches effectively prevents continuous exposure to CPE contamination to the following batch of animal entering the same farm (Reu et al., 2019; Luyckx et al., 2015).

A single introduction into the rearing farm remains contained within that specific rearing farm and its corresponding multiplier farm because all animals are transported to the same multiplier farm. Although the same batch of PS chickens may supply eggs to a hatchery for a long period of up to 280 days, only a few contaminated eggs will be transported to the hatchery and even fewer broiler chickens will be colonized after hatching. Thus, outbreaks in broiler farms are rare and remain contained within one round. We refer to this outbreak characteristic as sporadic outbreaks.

Continuous or frequent exposure to CPE is crucial to achieve widespread and continuous CPE outbreaks in a significant number of farms. This assumes that transmission occurs primarily through direct animal contact with food and other animals. We refer to this continuing series of outbreaks as endemic outbreaks. Endemic outbreaks of CPE occur when farms are exposed continuously or frequently to CPE, especially during the early phase of the production round.

In a multiple imports model, compared to the single import model, the occurrence of outbreaks in all farm types is notably more frequent, which results in endemic outbreaks in broiler farms. Rearing farms transport to two multiplier farms in alternate rounds, leading to annual outbreaks in both connected multiplier farms. The doubled number of multiplier farms with repeated outbreaks results in a higher number of contaminated eggs in the hatchery. Consequently, the continuous influx of contaminated eggs from both multiplier farms, combined with the greater number of contaminated eggs, leads to all 100 runs experiencing outbreaks in at least one broiler farm.

Still, colonization due to multiple imports remained contained in less than 20% of the total broiler farms (8 to 24 broiler farms) in the simulation. The reason is that horizontal between-farm spread does not occur in our model. Feed, on the other hand, resulted in colonization of 30 to 40% of the broiler farms. This is due to the continuous exposure to feed in all farms.

Looking at the current information available from EU and national surveillance (EFSA, 2022; MARAN, 2022), a single contaminated import is a likely scenario. Detection of CPE in broiler production at slaughter has been rare in the EU national surveillance, with only 0.04% or 3 positive samples out of 8,530 (EFSA, 2014). This reflects our scenario for contamination in imported PS chickens, where we only see sporadic outbreaks in broilers with a very small probability of detection in the current surveillance program. Consequently, these few outbreaks might remain undetected in the national surveillance. The multiple import scenario is currently unlikely, as no CPE cases have been detected in Dutch livestock. However, it serves to predict what would happen if a source farm abroad were to become endemically colonized.

However, if the feed scenario as simulated here would be true, we would expect to detect outbreaks by the current Dutch surveillance. Although the presence of *E. coli* in feed has been demonstrated (GMP+, 2020), no data regarding contamination with E. coli carrying CPE are available. CPE contamination in feed may occur through cross-contamination and from the environment (Filippitzi et al., 2016; DaSalva et al., 2019). The sensitivity analysis shows that the model is sensitive to the degree of feed contamination; reducing the exposure rate of feed in farms by 90% reduces the number of broiler farms with CPE outbreaks at slaughter (Section 5.3.2) considerably.

The absence of detection in the current situation might have four possible explanations. Firstly, the concentration of CPE in the feed may be lower than assumed here. Secondly, the dose needed for colonization could be higher in real life. Both reasons will lead to a lower probability of birds being colonized. Thirdly, farms exposed to contaminated feed might be limited to only a few farms, such that detection is less probable because the flock is not sampled at slaughter. Lastly, there is no CPE contamination in feed at all. This last explanation reflects the outline of the Dutch broiler production system, which effectively limits the transmission of colonized chickens to a few farms.

A shift of target in sampling focus is advisable to enhance the probability of detecting potential outbreaks. Directing sampling efforts toward rearing farms is a prudent approach, given that these farms represent the point of introduction for CPE carried by live imports. This introduction, often resulting in sporadic outbreaks, carries a higher risk of escaping detection when it reaches broiler farms. Focusing on rearing farms, where live import birds initially enter the production cycle, enables prompt CPE identification before further spread. If available, surveillance should be specifically targeted at rearing farms that import PS chickens from outside the Netherlands. Additionally, rearing farms constitute a relatively smaller subset of the total farms within the industry compared to broiler farms, making comprehensive coverage achievable with fewer samples.

Sensitivity analysis has identified key parameters that greatly influence the persistence of CPE within the broiler population. An important variable with considerable uncertainty is the rate at which susceptibility reduces with age (ψ). The decline in

susceptibility assumed here was linked to the decreasing trend of resistant extended-beta-lactamase-producing Enterobacteriaceae (ESBL) in broiler farms (Dame-Korevaars et al., 2020). Nevertheless, as no dynamics of CPE have been observed within a broiler farm, it could be worthwhile to explore this for CPE. In the case of young broiler chickens, their gut is susceptible to colonization by various bacteria (Ballou et al., 2016). As they age, their gut exhibits a less diverse but stable and complex bacterial composition, enabling the animals to become more resilient against the invasion of exogenous bacteria (Ballou et al., 2016; Awad et al., 2016; Rochegüe et al., 2021; Kim et al., 2017). This protective effect of a stable gut microbiome against ESBL is observed in broiler chickens (Dame-Korevaars et al., 2020). Furthermore, recent experiments involving CPE and ESBL transmission among one-day-old broiler chickens have shown a similar trend in bacterial composition, particularly as the chickens grow older. Both CPE and ESBL groups tend to converge toward similar bacterial complexity after 14 days in the experiment (Dankittipong et al., 2023). This convergence in gut composition could indicate the presence of a stable gut microbiome that acts preventively against the colonization of resistant bacteria.

The preventive effect of reduction in transmission rate may change in the presence of antibiotics. In Dankittipong et al. (2023) a specific antibiotic (amoxicillin) was administered to evaluate the impact of antibiotics on transmission. They found that broilers treated with amoxicillin exhibited a more diverse gut microbiome, indicating an opportunity for various bacteria species to grow after the disruption of the gut by the antibiotics. Although the bacteria composition in both amoxicillin-treated and untreated groups eventually converged, we believe that stability was achieved primarily because amoxicillin was only present for a short time period and quickly degraded.

Studies by Rama et al. (2016) and Fairchild et al. (2005) also examined the effects of conventional antibiotics and tetracycline on antibiotic-resistant bacteria in 4-week-old broilers. They found that older broilers consistently maintained antibiotic-resistant bacteria due to continuous disruption of the gut microbiome by antibiotics. Transitioning to a simulation, a what-if analysis scenario was introduced where the transmission rate was artificially increased tenfold, mimicking antibiotic use. The outcome demonstrated a 14% increase in the number of broiler chickens at slaughter, although the number of colonized flocks at slaughter did not exhibit a significant rise.

This outcome therefore suggested a restricted impact of antibiotics treatment to the acceleration of CPE spread. A reference to a One Health study (Rahman and Hollis, 2023) adds weight to the argument, suggesting that a 1% increase in antibiotic usage corresponds to a modest 0.2 to 0.4% escalation in resistance in animals. In conclusion, sensitivity analysis and what-if analysis reveal the complex interplay between age-related reduction in transmission rates, antibiotic treatments, and their collective impact. It asserts that the preventive efficacy of reducing transmission rates with age is not absolute and should be carefully evaluated in conjunction with antibiotic treatments.

This simulation study extends our comprehension of the emergence of resistant bacteria in broiler production and assesses the probability of detection. The simulation highlights the necessity of accurately estimating highly influential variables, such as the contamination and distribution of CPE in animal feed, as well as the reduction in the probability of colonization over time. The foremost action is monitoring the feed to establish whether CPE is present. After aligning important variables with the real situation, the model can be readily extended to investigate other surveillance programs, including sampling other types of poultry and varying sampling sizes and frequencies.

Recognizing that the transmission dynamics of CPE introduced through feed could be a major factor in its introduction and spread, it is imperative to gain a clear understanding of CPE contamination levels in feed and the precise CPE dosage required to initiate colonization. Routine collection of commensal bacteria in feed and CPE dose-response experiments will improve our transmission model accuracy in broiler production. For example, we currently lack knowledge about CPE's specific entry point into the feed mill system. Unlike imported livestock, where efforts can be focused on specific farm types that receive imports (rearing farms), once we ascertain the concentration and distribution of CPE in feed, we can pinpoint the timing and types of farms most likely to produce a higher number of colonized broilers. Additionally, feed serves as a source of CPE for other meat-producing animals like pigs, making it essential to obtain clear data on CPE concentration in feed (Dankittipong et al., 2022). This information helps assess the risks associated with antibiotic-resistant bacteria in

the feed supply chain and develop targeted strategies for animal health and food safety.

By investigating two different routes of CPE introduction, import of live birds and contaminated feed, and assessing their impact on bacterial spread, this study is a steppingstone toward building an active surveillance strategy for the early detection of emerging CPE colonization in the broiler population. As a first step, the current Dutch surveillance program was assessed regarding its potential to detect CPE incursions at an early stage. Ultimately, this research will contribute to improved control and prevention measures in the broiler production system.

5. 5. Conclusion

The transmission simulation outcome shows that single imports lead to a small number of contaminated broiler batches and have a low detection probability. On the other hand, multiple imports significantly increase detection probability, reaching 0.8 over 10 years. Contaminated feed results in the highest proportion of contaminated broilers, with a cumulative detection probability of 0.99.

Age-dependent susceptibility is a crucial parameter in colonization; small reductions can drastically alter outcomes to zero colonization. CPE introduction from colonized imports spreads rapidly within farms, achieving nearly complete flock colonization within three days. Transmission through contaminated feed is slower but will propagate to complete flock colonization within 7-14 days, depending on exposure timing. The broiler production system's all-in-all-out basis helps contain outbreaks to individual rounds, preventing continuous exposure.

Thus, accurate quantification of age-related susceptibility is required. Moreover, investigating the presence of CPE in feed and conducting dose-response experiments will improve transmission model accuracy.

5. 6 Supplementary information

5. 6. 1 Decay rate from literatures

Table 5.8- *Decay of Escherichia coli outside a live host. Abbreviations: fc: field capacity.*

ID	Temperature (°C)	Humidity	pH	Environment	Decay rate (per day)	Reference
1	Autumn			Lab	0.102	(Burrows and Rankin, 1970)
2	Autumn			Lab	0.287	(Burrows and Rankin, 1970)
3	4		7	Lab	0.686	(Kovács and Tamási, 1977)
4	January			Lab	0.109	(Rankin and Taylor, 1969)
5	26		7.4	Soil	0.896	(Klein and Casida, 1967)
6	10		7.4	Soil	0.195	(Klein and Casida, 1967)
7				Soil	0.115	(Mallmann and Litsky, 1951)
8			7	Soil	0.371	(Van Donsel et al., 1967)
9				Soil	0.143	(Mallmann and Litsky, 1951)
10		1/3 bar	6.16	Soil	0.473	(Tate, 1978)
11		Saturated	6.64	Soil	0.839	(Tate, 1978)
12		100% fc	6.16	Soil	0.796	(Tate, 1978)
13		Flooded		Soil	0.382	(Tate, 1978)
14	0			Inoculated water	0.192	(Mitchell, 1968)
15	10	60% fc		Swine manure-amended soil	0.22	(Rogers et al., 2011)
16	10	80% fc		Swine manure-amended soil	0.19	(Rogers et al., 2011)
17	25	60% fc		Swine manure-amended soil	0.40	(Rogers et al., 2011)
18	25	80% fc		Swine manure-amended soil	0.28	(Rogers et al., 2011)
19	10	60% fc		Beef manure-amended soil	0.17	(Rogers et al., 2011)
20	10	80% fc		Beef manure-amended soil	0.15	(Rogers et al., 2011)
21	25	60% fc		Beef manure-amended soil	0.33	(Rogers et al., 2011)
22	25	80% fc		Beef manure-amended soil	0.37	(Rogers et al., 2011)
23	Optimal condition to rear broilers			Broiler pen floor	0.0	(van Bunnik et al., 2014)

5. 6. 2 Queries to get demographic data from CBS

Queries to retrieve import livestock of interest from cbs.nl

URL: https://opendata.cbs.nl/statline/portal.html

- Click “Kies thema” at the top of the page
- Click the following options internationale handel> handel; goederen> goederensoorten, landen per jaar> natuur, voeding en tabak; jaar
- Click "Preview data" then Select the following animal species from the drop down “goederensoorten natuur, voeding en tabak”

  0102291000: Cattle, live, with a weight of <= 80 kg (excl. pure-bred breeding cattle)

  0102900500: Cattle/ domestic animals/live weighing <= 80 kg (excl. pure-bred breeding animals)

  0103100000: Pure-bred breeding pigs

  0103911000: Pigs/ domestic animals/ live pigs weighing <50 kg (excl. pure-bred breeding animals

  0103921100: Sows /domestic animals /live ..."who have farrowed at least once, weighing> = 160 kg (excl. pure-bred breeding animals)

  0105111900: Female breeding chicks of chickens/ poultry/weighing <= 185 g (excl. those of laying breeds)

  0105119900: Roosters and chickens/ poultry/ weighing <= 185 g (excl. those of laying breeds and excl. female and breeding chicks)
- Specify imported animals by select drop down “Onderwerp” > Invoerhoeveelheid
- Specify countries of import (European Member states in our analysis) by select drop down “Landen”

5. 6. 3 Number of farms with major outbreak

Table 5.9- *Output for the sensitivity analysis in one import baseline*

		Number of farms with outbreak of CPE		Number of chickens colonized with CPE		Duration of outbreak		Frequency of outbreak per farm	
Parameter	Farm	n.farm	difffarm	n.animal	difffarm	Days	difffarm	Times	difffarm
gamma_d	rearing	1[1,1]	0[0,0]	39862.5[39846.9,39879.5]	-69.5[-72.0999999999985,-65.5500000000029]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
gamma_0	rearing	1[1,1]	0[0,0]	40000[40000,40000]	68[81,54.9499999999971]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
agedecay_d	rearing	1[1,1]	0[0,0]	35799.5[34608.65,36186.7]	-4132.5[-5310.35,-3758.35000000001]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
agedecay_0	rearing	1[1,1]	0[0,0]	40239.5[40217.4,40263.8]	307.5[298.400000000001,318.75]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
min_d	rearing	1[1,1]	0[0,0]	39933[39912.25,39943.85]	1[-6.75,-1.19999999999709]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
min_0	rearing	1[1,1]	0[0,0]	39932.5[39921.25,39947.65]	0.5[2.25,2.59999999999854]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
theta_d	rearing	1[1,1]	0[0,0]	39929.5[39920.45,39941.75]	-2.5[1.44999999999709,-3.30000000000291]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
theta_0	rearing	1[1,1]	0[0,0]	39933[39912.25,39943.85]	1[-6.75,-1.19999999999709]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
rho_d	rearing	1[1,1]	0[0,0]	39968.5[39961.45,39976.2]	36.5[42.4499999999971,31.1499999999942]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
rho_0	rearing	0[0,0]	-1[-1,-1]	36.5[30.45,43.1]	-39895.5[-39888.55,-39901.95]	138[116,138]	0[-22,0]	1[1,1]	0[0,0]
beta_d	rearing	1[1,1]	0[0,0]	39987[39982,39992.55]	55[63,47.5]	138[138,138]	0[0,0]	1[1,1]	0[0,0]
beta_0	rearing	0[0,0]	-1[-1,-1]	42[36,50.55]	-39890[-39883,-39894.5]	134.5[97.6,138]	-3.5[-40.4,0]	1[1,1]	0[0,0]
gamma_d	multiplier	0[0,0]	-1[-1,-1]	14[11,18.1]	-779[-742.95,-817.9]	49.5[30.45,77.05]	-186.5[-154.45,-200.95]	1[1,1]	0[0,0]
gamma_0	multiplier	1[1,1]	0[0,0]	40000[40000,40000]	39207[39246.05,39164]	278[278,278]	42[93.1,0]	1[1,1]	0[0,0]
agedecay_d	multiplier	1[1,1]	0[0,0]	625.5[585.95,653.8]	-167.5[-168,-182.2]	236[193.1,278]	0[8.200002,0]	1[1,1]	0[0,0]
agedecay_0	multiplier	1[1,1]	0[0,0]	40471[40446.85,40515.55]	39678[39692.9,39679.55]	278[278,278]	42[93.1,0]	1[1,1]	0[0,0]

		Number of farms with outbreak of CPE		Number of chickens colonized with CPE		Duration of outbreak		Frequency of outbreak per farm	
Parameter	Farm	n.farm	difffarm	n.animal	difffarm	Days	difffarm	Times	difffarm
min_d	multiplier	1[1,1]	0[0,0]	788.5[766.25,817.65]	-4.5[12.3,-18.35]	234[181.55,274.85]	-2 [-3.349999,-3.150000000000003]	1[1,1]	0[0,0]
min_0	multiplier	1[1,1]	0[0,0]	782[764.25,825.15]	-11[10.3,-10.85]	229.5[187.25,278]	-6.5[2.3499,0]	1[1,1]	0[0,0]
theta_d	multiplier	1[1,1]	0[0,0]	790[725.55,821.55]	-3[-28.4000000000001,-14.45]	238.5[189.15,278]	2.5[4.25,0]	1[1,1]	0[0,0]
theta_0	multiplier	1[1,1]	0[0,0]	788.5[766.25,817.65]	-4.5[12.3,-18.35]	234[181,274.85]	-2 [-3.349999,-3.150000]	1[1,1]	0[0,0]
rho_d	multiplier	1[1,1]	0[0,0]	826[769.15,854.65]	33[15.1999999999999,18.6500000000001]	238.5[216.45,273.5]	2.5[31.55,-4.5]	1[1,1]	0[0,0]
rho_0	multiplier	0[0,0]	-1[-1,-1]	1[0,2.55]	-792[-753.95,-833.45]	44[6.2,81.6]	-192 [-178.7,-196.4]	1[1,1]	0[0,0]
beta_d	multiplier	1[1,1]	0[0,0]	842.5[823.85,885.6]	49.5[69.9,49.5999999999999]	214.5[193,272.15]	-21.5 [8.0999999999999,-5.85000000000002]	1[1,1]	0[0,0]
beta_0	multiplier	0[0,0]	-1[-1,-1]	0.5[0,2.1]	-792.5[-753.95,-833.9]	23[6,50]	-213[-178.9,-228]	1[1,1]	0[0,0]
gamma_d	hatchery	0[0,0]	0[0,0]	0[0,0]	-10[-6,-15]	0[0,0]	-2[-1,-5]	0[0,0]	-11[-11,-11]
gamma_0	hatchery	1[1,1]	1[1,1]	3511[3430.05,3564.1]	3501[3424.05,3549.1]	283[280,285]	281[279,280]	285[285,285]	274[274,274]
agedecay_d	hatchery	1[1,1]	1[1,1]	9.5[5.8,14.65]	-0.5[-0.2,-0.350000000000003]	1[1,2.85]	-1[0,-2.15]	13[13,13]	2[2,2]
agedecay_0	hatchery	1[1,1]	1[1,1]	3484[3423.15,3581.6]	3474[3417.15,3566.6]	281.5[281,284]	279.5[280,279]	286[286,286]	275[275,275]
min_d	hatchery	1[1,1]	1[1,1]	12[8.35,16.1]	2[2.35,1.1]	2[1,3]	0[0,-2]	13[13,13]	2[2,2]

min_0	hatchery	1[1,1]	1[1,1]	9.5[5,12]	-0.5[-1,-3]	1[1,3.1]	-1[0,-1.9]	15[15,15]	4[4,4]
		Number of farms with outbreak of CPE		Number of chickens colonized with CPE		Duration of outbreak		Frequency of outbreak per farm	
Parameter	Farm	n.farm	difffarm	n.animal	difffarm	Days	difffarm	Times	difffarm
theta_d	hatchery	1[1,1]	1[1,1]	11[6.8,13]	1[0.8,-2]	1[1,4.75]	-1[0,-0.25]	25[25,25]	14[14,14]
theta_0	hatchery	1[1,1]	1[1,1]	12[8.35,16.1]	2[2.35,1.1]	2[1,3]	0[0,-2]	13[13,13]	2[2,2]
rho_d	hatchery	1[1,1]	1[1,1]	10[5.45,15.65]	0[-0.550000000000001, 0.6499999999999997]	1[1,4]	-1[0,-1]	15[15,15]	4[4,4]
rho_0	hatchery	0[0,0]	0[0,0]	0[0,0]	-10[-6,-15]	0[0,0]	-2[-1,-5]	0[0,0]	-11[-11,-11]
beta_d	hatchery	1[1,1]	1[1,1]	12.5[6.9,19.55]	2.5[0.9,4.55]	1[1,5.3]	-1[0,0.299999]	27[27,27]	16[16,16]
beta_0	hatchery	0[0,0]	0[0,0]	0[0,0]	-10[-6,-15]	0[0,0]	-2[-1,-5]	0[0,0]	-11[-11,-11]
gamma_d	broiler	0[0,0]	0[0,-6]	0[0,0]	0[0,-390269.95]	0[0,0]	-54[-54,-54]	0[0,0]	-1[-1,-1]
gamma_0	broiler	112[101.9,119.55]	112[101.9, 113.55]	13163438.5[12275957,14732323]	13163438.5[12275957.1, 14342053.15]	54[54,55]	0[0,1]	2[1,3]	1[0,2]
agedecay_d	broiler	0[0,3.2999]	0[0,-2.70000]	0[0,132811.8]	0[0,-257458.15]	0[0,0]	-54[-54,-54]	1[1,1]	0[0,0]
agedecay_0	broiler	105.5[92.5,124.4]	105.5[92.5, 118.4]	13031605[10270825,16141983]	13031605[10270825.45, 15751713.1]	54[54,55]	0[0,1]	1[1,3]	0[0,2]
min_d	broiler	0[0,3.74999]	0[0,-2.25000]	0[0,304316.7]	0[0,-85953.2500000003]	54[54,54]	0[0,0]	1[1,1]	0[0,0]
min_0	broiler	0[0,0]	0[0,-6]	0[0,0]	0[0,-390269.95]	0[0,0]	-54[-54,-54]	0[0,0]	-1[-1,-1]
theta_d	broiler	0[0,6]	0[0,0]	0[0,507826.35]	0[0,117556.4]	54[54,54]	0[0,0]	1[1,1]	0[0,0]
theta_0	broiler	0[0,3.7499]	0[0,-2.2500000]	0[0,304316.7]	0[0,-85953.2500000003]	54[54,54]	0[0,0]	1[1,1]	0[0,0]
rho_d	broiler	0[0,6]	0[0,0]	0[0,390928.9]	0[0,658.95000000007]	54[54,54]	0[0,0]	1[1,1]	0[0,0]
rho_0	broiler	0[0,0]	0[0,-6]	0[0,0]	0[0,-390269.95]	0[0,0]	-54[-54,-54]	0[0,0]	-1[-1,-1]
beta_d	broiler	0[0,6]	0[0,0]	0[0,440411.85]	0[0,50141.9]	54[54,54]	0[0,0]	1[1,1]	0[0,0]

beta_0	broiler	0[0,0]	0[0,-6]	0[0,0]	0[0,-390269.95]	0[0,0]	-54[-54,-54]	0[0,0]	-1[-1,-1]

***Table 5.10**- Output for sensitivity analysis from continuous exposure to feed.*

		Number of farms with outbreak of CPE		Number of chickens colonized with CPE		Duration of outbreak		Frequecy of outbreak per farm	
Farm	para	n.farm	difffarm	n.animal	difffarm	Days	difffarm	Times	difffarm
rearing	gamma_d	0[0,1]	0[0,-1]	0[0,33918.15]	0[0,-30052.65]	136.5[136.05,136.95]	0.5[48.85,-0.049]	1[1,1]	0[0,-1]
rearing	gamma_0	1[0,1.55]	1[0,-0.450]	15047.5[0,40038.55]	15047.5[0,-23932.25]	136.5[131.05,137]	0.5[43.85,0]	1[1,1]	0[0,-1]
rearing	agedecay_d	0[0,1]	0[0,-1]	0[0,128.15]	0[0,-63842.65]	137[137,137]	1[49.8,0]	1[1,1]	0[0,-1]
rearing	agedecay_0	14[11.9,15]	14[11.9,13]	1123005[740053.7,1511038.6]	1123005[740053.7,1447067.8]	73[13,131]	-63[-74.2,-6]	1[1,2]	0[0,0]
rearing	min_d	0[0,1.55]	0[0,-0.450]	0[0,27260.05]	0[0,-36710.7500000001]	135.5[135,136.85]	-0.5[47.8,-0.15000]	1[1,2]	0[0,0]
rearing	min_0	0[0,1.55]	0[0,-0.450]	0[0,53174.7]	0[0,-10796.1]	136.5[50.15,137]	0.5[-37.05,0]	1[1,1]	0[0,-1]
rearing	omega_ps_d	0.5[0,2]	0.5[0,0]	1450[0,74241.6]	1450[0,10270.8]	137[134.35,137]	1[47.15,0]	1[1,1]	0[0,-1]
rearing	omega_ps_0	0[0,0]	0[0,-2]	0[0,0]	0[0,-63970.8]	NA	NA	NA	NA
rearing	omega_b_d	0.5[0,1]	0.5[0,-1]	2244.5[0,39257.2]	2244.5[0,-24713.6]	136[96.75,137]	0[9.55,0]	1[1,1]	0[0,-1]
rearing	omega_b_0	0[0,1]	0[0,-1]	0[0,18463.85]	0[0,-45506.95]	134.5[134,135.85]	-1.5[46.8,-1.1500]	1[1,1]	0[0,-1]
rearing	theta_d	0[0,1]	0[0,-1]	0[0,39652.9]	0[0,-24317.9]	135.5[132.45,136.85]	-0.5[45.25,-0.1500]	1[1,1]	0[0,-1]
rearing	theta_0	0[0,1.55]	0[0,-0.450]	0[0,27260.05]	0[0,-36710.7500000001]	135.5[135,136.85]	-0.5[47.8,-0.150]	1[1,2]	0[0,0]
rearing	rho_d	0[0,1]	0[0,-1]	0[0,39652.9]	0[0,-24317.9]	135.5[132.45,136.85]	-0.5[45.25,-0.150000]	1[1,1]	0[0,-1]
rearing	rho_0	0[0,0]	0[0,-2]	0[0,1]	0[0,-63969.8]	12[5.7,12]	-124[-81.5,-125]	1[1,1]	0[0,-1]
rearing	beta_d	0[0,1.55]	0[0,-0.450]	0[0,61727.05]	0[0,-2243.75000000004]	135.5[133.3,136.85]	-0.5[46.1,-0.15000]	1[1,1]	0[0,-1]
rearing	beta_0	0[0,0]	0[0,-2]	0[0,1]	0[0,-63969.8]	8.5[1.75,46.7]	-127.5[-85.45,-90.3]	1[1,1]	0[0,-1]
multiplier	gamma_d	0[0,0]	0[0,-2]	0[0,21]	0[0,-1350.8]	57[57,57]	-141[-6.6,-204.4]	1[1,1]	0[0,0]
multiplier	gamma_0	1[0,1.55]	1[0,-0.450]	15047.5[0,40038.55]	15047.5[0,38666.75]	278[278,278]	80[214.4,16.6]	1[1,1]	0[0,0]
multiplier	agedecay_d	0[0,0]	0[0,-2]	0[0,3]	0[0,-1368.8]	NA	NA	1[1,1]	0[0,0]

multiplier	agedecay_0	29[26.45,30]	29[26.45,28]	2,587,216.5[2190395.05, 3090142.55]	2587216.5[2190395.05,3088770.75]	208[11.4,279]	10[-52.2,17.6]	1[1,3]	0[0,2]

		Number of farms with outbreak of CPE		Number of chickens colonized with CPE		Duration of outbreak		Frequecy of outbreak per farm	
Farm	para	n.farm	difffarm	n.animal	difffarm	Days	difffarm	Times	difffarm
multiplier	min_d	0[0,1.1]	0[0,-0.900]	0[0,554.749]	0[0,-817.050000000001]	186[139.2,249]	-12[75.6,-12.4]	1[1,1]	0[0,0]
multiplier	min_0	0[0,1]	0[0,-1]	0[0,806.75]	0[0,-565.05]	270[270,270]	72[206.4,8.60000]	1[1,1]	0[0,0]
multiplier	omega_ps_d	0[0,2]	0[0,0]	28.5[0,1526.6]	28.5[0,154.8]	241[172.75,270.25]	43[109.15,8.8500]	1[1,1]	0[0,0]
multiplier	omega_ps_0	0[0,0]	0[0,-2]	0[0,0]	0[0,-1371.8]	NA	NA	NA	NA
multiplier	omega_b_d	0.5[0,1]	0.5[0,-1]	57[0,777]	57[0,-594.8]	233[189.8,267.2]	35[126.2,5.800]	1[1,1]	0[0,0]
multiplier	omega_b_0	0[0,1]	0[0,-1]	0[0,416.6499]	0[0,-955.150000000001]	104.5[41.95,167.05]	-93.5[-21.65,-94.35]	1[1,1]	0[0,0]
multiplier	theta_d	0[0,1]	0[0,-1]	0[0,850.4]	0[0,-521.4]	243[215.1,270.9]	45[151.5,9.500]	1[1,1]	0[0,0]
multiplier	theta_0	0[0,1.1]	0[0,-0.900]	0[0,554.74]	0[0,-817.050000000001]	186[139.2,249]	-12[75.6,-12.4]	1[1,1]	0[0,0]
multiplier	rho_d	0[0,1]	0[0,-1]	0[0,850.4]	0[0,-521.4]	243[215.1,270.9]	45[151.5,9.5000]	1[1,1]	0[0,0]
multiplier	rho_0	0[0,0]	0[0,-2]	0[0,0]	0[0,-1371.8]	NA	NA	NA	NA
multiplier	beta_d	0[0,1.55]	0[0,-0.450]	0[0,1343.55]	0[0,-28.2500000000009]	238[213.7,274]	40[150.1,12.6]	1[1,1]	0[0,0]
multiplier	beta_0	0[0,0]	0[0,-2]	0[0,0]	0[0,-1371.8]	NA	NA	NA	NA
hatchery	gamma_d	0[0,0]	0[0,-1]	0[0,0.549999]	0[0,-17.5]	NA	NA	NA	NA
hatchery	gamma_0	1[0,1]	1[0,0]	291[0,3490.15]	291[0,3472.1]	3.5[1,282.7]	1.5[0,278.7]	285[285,285]	NA
hatchery	agedecay_d	0[0,0]	0[0,-1]	0[0,0]	0[0,-18.05]	NA	NA	NA	NA
hatchery	agedecay_0	1[1,1]	1[1,0]	142046[111451,175690.05]	142046.5[111451.6,175672]	1270[6,2729.4]	1268[5,2725.4]	3498[3498,3498]	NA
hatchery	min_d	0[0,0.5499]	0[0,-0.450	0[0,9.249999]	0[0,-8.800000]	1[1,3.4]	-1[0,-0.600000]	NA	NA
hatchery	min_0	0[0,1]	0[0,0]	0[0,13.85]	0[0,-4.2]	1.5[1,7.94999]	-0.5[0,3.949999]	NA	NA
hatchery	omega_ps_d	0[0,1]	0[0,0]	0.5[0,15.65]	0.5[0,-2.4]	1[1,5.7]	-1[0,1.7]	NA	NA
hatchery	omega_ps_0	0[0,0]	0[0,-1]	0[0,0]	0[0,-18.05]	NA	NA	NA	NA

hatchery	omega_b_d	0[0,1]	0[0,0]	0.5[0,12.3]	0.5[0,-5.75]	1[1,3.1]	-1[0,-0.89999]	12[12,12]	NA
hatchery	omega_b_0	0[0,0.5499]	0[0,-0.450]	0[0,3.7499]	0[0,-14.3]	2[2,2]	0[1,-2]	NA	NA

		Number of farms with outbreak of CPE		Number of chickens colonized with CPE		Duration of outbreak		Frequecy of outbreak per farm	
Farm	para	n.farm	difffarm	n.animal	difffarm	Days	difffarm	Times	difffarm
hatchery	theta_d	0[0,1]	0[0,0]	0[0,13]	0[0,-5.05]	1[1,6.4]	-1[0,2.4]	NA	NA
hatchery	theta_0	0[0,0.5499]	0[0,-0.450]	0[0,9.2499]	0[0,-8.8000]	1[1,3.4]	-1[0,-0.60000]	NA	NA
hatchery	rho_d	0[0,1]	0[0,0]	0[0,13]	0[0,-5.05]	1[1,6.4]	-1[0,2.4]	NA	NA
hatchery	rho_0	0[0,0]	0[0,-1]	0[0,0]	0[0,-18.05]	NA	NA	NA	NA
hatchery	beta_d	0[0,1]	0[0,0]	0[0,16.85]	0[0,-1.2]	1[1,3.2]	-1[0,-0.800000]	NA	NA
hatchery	beta_0	0[0,0]	0[0,-1]	0[0,0]	0[0,-18.05]	NA	NA	NA	NA
broiler	gamma_d	54.5[49.9,58.55]	-9.5[-4.05,-13.5]	4128699[3897449,4853409]	-713826[44088.75,-1119129.95]	53[48.4,54]	1[0.3999,0]	1[1,2]	0[0,-1]
broiler	gamma_0	72.5[49.8,122.1]	8.5[-4.15,50.05]	6020096[4071360,17521295.4]	1177570.5[217999.95,11548756.25]	54[50,55]	2[2,1]	2[1,4]	1[0,1]
broiler	agedecay_d	30[22.45,34.55]	-34[-31.5,-37.5]	28856.5[23678,33396]	-4813669[-3829681.75,-5939142.3]	53[51,54]	1[3,0]	1[1,2]	0[0,-1]
broiler	agedecay_0	132[132,132]	68[78.05,59.95]	371123761[329729015,394318695]	366281236[325875654.6,388346156.45]	40[4,55]	-12[-44,1]	36[28,42]	35[27,39]
broiler	min_d	58[55.45,66.55]	-6[1.5,-5.5]	4955534[4093881,5699845.15]	113008.5[240520.65,-272694]	53[48,54]	1[0,0]	1[1,3]	0[0,0]
broiler	min_0	57.5[46.85,61]	-6.5[-7.100,-11.05]	4487914.5[3627141.4,4946723]	-354611[-226219,-1025816.1]	52[49,54]	0[1,0]	1[1,2]	0[0,-1]
broiler	omega_ps_d	60.5[51.35,66]	-3.5[-2.6,-6.05]	4829238[4163785,5708883]	-13287.5[310425.4,-263655.3]	53[48.9,54]	1[0.9000,0]	1[1,3]	0[0,0]
broiler	omega_ps_0	56.5[49.45,61.1]	-7.5[-4.5,-10.95]	4447061[4080253,4928957]	-395464.5[226892.7,-1043582]	53[48,54]	1[0,0]	1[1,2]	0[0,-1]
broiler	omega_b_d	87[83.45,92.75]	23[29.5,20.7]	8490749[7877223,9099954]	3648223.5[4023862.65,3127415]	53[48.8,54]	1[0.80000,0]	1[1,3]	0[0,0]

broiler	omega_b_0	0[0,0]	-64[-53.95,-72.05]	0[0,0]	-4842525.5[-3853360.4,-5972539.15]	NA	NA	NA	NA
broiler	theta_d	57[50.9,67]	-7[-3.05,-5.05]	4960308.5[3984092,5814099.45]	117783[130731.6,-158439.7]	52[48,54]	0[0,0]	1[1,2]	0[0,-1]

		Number of farms with outbreak of CPE		Number of chickens colonized with CPE		Duration of outbreak		Frequecy of outbreak per farm	
Farm	para	n.farm	difffarm	n.animal	difffarm	Days	difffarm	Times	difffarm
broiler	theta_0	58[55.45,66.55]	-6[1.5,-5.5]	4955534[4093881.05,5699845.15]	113008.5[240520.65,-272694]	53[48,54]	1[0,0]	1[1,3]	0[0,0]
broiler	rho_d	57[50.9,67]	-7[-3.05,-5.05]	4960308.5[3984092,5814099]	117783[130731.6,-158439.7]	52[48,54]	0[0,0]	1[1,2]	0[0,-1]
broiler	rho_0	0[0,0]	-64[-53.95,-72.05]	0[0,0]	-4842525.5[-3853360.4,-5972539.15]	NA	NA	NA	NA
broiler	beta_d	58[49.45,64.55]	-6[-4.5,-7.5]	5278657[4538179.2,6455275.25]	436131.5[684818.8,482736.1]	52.5[48.85,54]	0.5[0.85,0]	1[1,2]	0[0,-1]
broiler	beta_0	0[0,0]	-64[-53.95,-72.05]	0[0,0]	-4842525.5[-3853360.4,-5972539.15]	NA	NA	NA	NA

Chapter 6

General Discussion

The research described in this thesis endeavored to assess the probability and frequency of introducing Carbapenemase-Producing Enterobacteriaceae (CPE) into the Dutch meat-producing animal population and its consequential spread. The overarching goal was to inform risk-based surveillance design. Identifying the animal population most at risk and the source of risk are crucial from the start when assessing emerging microorganisms. Key findings from the risk assessment in Chapter 2 indicate that feed and imported livestock represent critical sources of CPE introduction. Among the broiler, veal calf, and pig production sectors, broiler farms are predicted to have the highest per farm probability of CPE introduction. On the other hand, the total number of farms with CPE introduction was predicted to be the highest at fattening pig farms.

Transmission parameters need to be quantified to assess the consequences of introducing CPE. The CPE transmission experiment (Chapter 3) and the meta-analysis (Chapter 4) quantified such parameters. In Chapter 3, it was demonstrated that CPE transmits at half the rate of Extended-Spectrum Beta-Lactamase (ESBL)-carrying Enterobacteriacae and 30% of the rate of *catA1*-carrying *E. coli*. When treated with amoxicillin, broilers transmitted CPE-resistant bacteria at a 70% faster rate compared to the group without amoxicillin treatment. A comparison of resistant *E. coli* transmission between host species (Chapter 4) indicated that the transmission of resistant *E. coli* in groups not treated with antibiotics was nearly twice as high in broilers than in piglets. Furthermore, in a transmission experiment, the transmission rate of ESBL-resistant bacteria in broilers treated with amoxicillin increased by up to 300% compared to that in broilers without antibiotic treatment.

A robust surveillance design requires a thorough understanding of the transmission dynamics of emerging CPE across the livestock population following its introduction. Simulating the dynamics of CPE transmission helps to identify where risk-based surveillance should be targeted and to assess the probability of detecting CPE through active surveillance. Chapter 5 shows that the propagation of CPE from imported colonized broilers and contaminated feed within a flock is explosive, but colonization does not persist within the broiler population. However, while the risk of exposure from

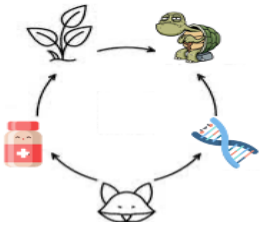
contaminated feed per flock is lower compared to imports, it poses a prolonged risk extended across multiple production rounds (flocks) within a farm. Consequently, under the current surveillance system, the probability of detecting CPE from imported colonized animals is low, ranging between 0 and 0.50 over a 10-year simulation period. However, the cumulative probability of detecting CPE from contaminated feed ranged from 0.90 to 0.97 over the same duration.

In terms of surveillance strategy, the existing active surveillance in broiler, pig, and veal calf production, MARAN, which is based on the EFSA livestock surveillance for antimicrobial resistance, serves as a good starting point. However, a critical limitation for early detection of emerging CPE identified in the current surveillance system is its limitation to slaughterhouses, which is compounded by inadequate resources in laboratory settings for CPE detection and reporting. The risk assessment and simulation study indicate that sample collection efforts in earlier stages of animals' lives and other farm types, including parent broiler farms and farrow-to-finish farms, are needed to enhance the probability of early detection.

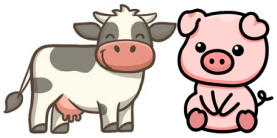
The subsequent topics outline essential ingredients for developing a surveillance program for early detection of CPE.

Investigating the presence of CPE in animal feed.

Expanding knowledge related to imported veal calves.



Exploring the impact of specific resistance genes, antibiotics treatments, and age on the transmission of CPE.



Practical applications of CPE transmission dynamics in broiler production compared to veal calf and pig production.

Investigating the role of feed in CPE introduction is a necessary step

Investigating the presence of CPE in animal feed is critical for evaluating the risks of introduction in Dutch livestock. The risk assessment in Chapter 2 consistently identified feed and imported livestock as high-risk sources, impacting various farm types. However, the risk assessment may have overestimated the risk of CPE from feed due to the lack of available information. The simulation study in Chapter 5 demonstrated that the continuous introduction of CPE through feed could lead to the sustained presence of CPE, resulting in subsequent flocks in broiler farms producing colonized animals. Under the current surveillance protocol, this level of colonization would likely have been detected. In the simulation, which included only 25% of the broiler population, detection typically takes six years. Considering the entire broiler population and assuming similar exposure to contaminated feed of the entire broiler population, detection with a 0.95 probability would occur within 1-2 years. Given the lack of CPE detection in the current surveillance, the results of the simulation indicate a lower probability of CPE contamination in feed than identified in Chapter 2. Consequently, more insight into the CPE contamination in feed is warranted to improve the risk assessment. Such a study should involve collecting feed samples at every stage of the production process, from the ingredients at the feed mills to the arrival on the farm, to determine the contamination of CPE. The study design can draw inspiration from the Finnish risk assessment and cost-benefit analysis of Salmonella in feed and animal production, estimating the true prevalence of Salmonella at various points in the feed production chain and pig production (Evira, 2018). This methodology is feasible for the CPE study in the Netherlands, given the existing Salmonella monitoring system in compliance with EU legislation for Salmonella in feedstuffs for food-producing animals (Yassin et al., 2015).

Apart from the lack of information regarding the presence of CPE in feed, the quantity of CPE ingested through contaminated feed, if present, significantly influences the probability of CPE introduction. Limited information on bacterial distribution in animal feed and associated exposure dosage (dose-response model) present additional challenges to the risk assessment. If CPE is found in the feed, these knowledge gaps could be

cleared as well. This will improve precision in calculating the probability of animal exposure to CPE from feed.

Addressing the lack of CPE surveillance in veal calves in the EU through national studies

The risk assessment identified veal calf farms as having the highest exposure risk to CPE from imported livestock (Chapter 2). The increased risk associated with veal calf farms is linked to the restricted sample collection within the EU. Currently, EFSA surveillance in live veal calves operates on a voluntary basis, allowing Member States with lower veal calf populations to opt out of surveillance (EFSA, 2022). It should be noted that some Member States have a small or non-existent veal calf industry. Veal calves were examined in only 10 out of the 28 EU Member States and 3 partner states (EFSA, 2017). Consequently, the heightened risk indicated in the introduction is highly uncertain, arising from the lack of samples from live veal calves in 18 Member States. For those Member States, data from CPE tests on meat were converted to CPE prevalence in veal calves based on ESBL information. Three proposals could be considered to reduce the uncertainty of the result. Firstly, CPE surveillance may be conducted on calves on dairy farms, given that veal calves are typically transferred from dairy farms at a young age (EFSA, 2017; EFSA, 2016). During this period, veal calves are still susceptible to colonization of CPE (Hordijk et al., 2013; Horton et al., 2011). Secondly, a sample of imported calves could be tested. Thirdly, the exposure risk may be derived from the adult cattle in the dairy farms.

Understanding the effect of resistance gene, antibiotic treatment, age and animal species on CPE transmission

Antibiotic treatment accelerates the transmission of the majority of *E. coli* carrying resistance genes. This was also observed in the experiment of Chapter 3, where amoxicillin notably accelerated the transmission of all three *E. coli* carrying resistance genes: $bla_{CTX-M-2}, bla_{OXA-162}, catA1$. Field data further supports the link between antibiotic usage and resistance prevalence on farms (Holmer et al., 2019; Alexander et al., 2009; Alali et al., 2009; Burow et al., 2019; Dantas Palmeira and Ferreira, 2020).

Antibiotics have a major effect on destabilizing the gut microbiome of animals, as evidenced by microbiome analyses in Chapter 3 and corroborated by multiple studies (Duan et al., 2022; Ramirez et al., 2020; Neuman et al., 2018).

Numerous studies have highlighted widespread antibiotic usage in meat-producing farms (Bosman et al., 2022; Lekagul et al., 2019; Hordijk et al., 2013). While antibiotic regimens may vary between farms based on livestock type, assessing overall antibiotic usage per farm or per meat-producing animal can aid in risk-based surveillance by identifying high-risk farm types. Such input for risk-based surveillance can be retrieved from antibiotic usage data of specific farm types from literature can be used (e.g. Hordijk et al. (2013), Dorado-Garcia et al. (2016), and Dierikx et al. (2013)), or from national databases like SDa in the Netherlands (SDa, 2023), offering a comprehensive view across farm types.

A reanalysis by Furusawa et al (2024) of data from an observational study conducted at an organic broiler farm (Huijbers et al., 2016) suggested age-dependent transmission coefficients, indicative of a maturing gut microbiome, which could account for the decline in prevalence during a production round (Furusawa et al., 2024). Dame-Korevaar et al. (2017) also found that the prevalence of bla_{CMY} is highest at the beginning of the rearing period (one week old), gradually declining as the animals age, reaching a 10 percent prevalence at week 15 indicative of an age effect.

The age-related dynamics in gut microbiome stability are pivotal in the susceptibility of animals to colonization by all bacteria, including antibiotic-resistant bacteria. Broiler's gut microbiome develops to a more stable state between day 14 to day 35 (Jurburg et al., 2019; Kers et al., 2022; Callaway et al., 2008; Dame-Korevaar et al., 2020). The resistance to colonization as the gut microbiome becomes stable in broilers is further supported by observational studies conducted by Friese et al. (2013), Laube et al. (2014), Daehre et al. (2018), and Dame-Korevaar et al. (2020).

The meta-analysis encompassed various resistance genes harbored by *E. coli*, including crucial globally distributed plasmid-mediated colistin resistance genes, such as the *mcr-*

1 gene (Wang et al., 2018). It unveiled a consistently slower transmission rate of *mcr-1* in 8-week old piglets compared to the transmission rate of ESBL in young broiler chickens (less than a week old). Piglets possess distinct gut microbiome and gut physiology, and are raised in different rearing systems compared to broilers, which could affect the transmission of resistance genes. Nonetheless, age could exert a similar effect in protecting against colonization at older ages in piglets. In the continuous production system in swine husbandry, sows move from the breeding room to the gestation barn and then to the farrowing unit. Similarly, piglets move from the farrowing unit to the weaned piglet compartment and then to the finishing section/farm. Hansen et al. (2013) and Burow et al. (2019) sampled from different age groups within farrow-to-finish farms and found the highest prevalence of resistant bacteria in suckling piglets and the lowest in fattening pigs. Apart from age, the high prevalence in piglets may be also be attributed to antibiotic treatment, as young piglets are the primary recipients of antibiotic treatments (Dewulf et al., 2022; Lekagul et al., 2019). Dong et al. (2023), utilizing sequencing data from 3,000 pig samples, showed that the gut microbiome of pigs begins to stabilize from day 50 onward, indicating that age may have a significant impact (Dong et al., 2023).

The examination of veal calves by Brunton et al. (2014) unveiled a consistent and rapid surge in ESBL colonization within animal pens approximately six days after introduction, succeeded by a gradual decline within two weeks to levels below 1% prevalence. Complementary investigations by Horton et al. (2011), Hordijk et al. (2013), and Hoyle et al. (2004), focusing on veal calves aged between 1 and 20 weeks, emphasized the prolonged presence of ESBL colonization. These studies noted a swift increase in colonization during weeks 5 to 7, diminishing to a low prevalence at 20 weeks of age, with variations depending on the specific antibiotics employed. The peak of the prevalence always occurred before 10 weeks of age (Brunton et al., 2014; Horton et al., 2011; Hordijk et al., 2013; Hoyle et al., 2004). Inspection of the gut microbiome reveals similar temporal dynamics of the gut microbiome as in pigs and broilers, suggesting the preventive capacity of a stable gut microbiome against resistant bacteria colonization. The bacterial composition in the gut becomes less heterogeneous and converges to a more

generalized composition among veal calves from week 7 to week 13 (Du et al., 2023; Massot et al., 2020).

In summary, susceptibility to colonization of *E. coli* carrying resistance tends to decrease as animals age, although the age at which colonization slows down varies across different species. In broilers, based on an experimental study by Dame-Korevaar (2020), a critical period for extensive colonization occurs within the first week of the animals' lives. In veal calves, major outbreaks may occur before week 10, with a likelihood of occurrence decreasing with age. For pigs, the peak susceptibility of colonization is estimated to be from 1 to 6 weeks of age. I propose focusing targeted surveillance on animals in their early life stages. This includes conducting surveillance on veal calves within 1-2 weeks of entering the barn, young parent broiler chickens at the start of multiplier farms, and young broiler chickens in broiler fattening farms within the first week.

Resistant genes undoubtedly influence the transmission rate. For instance, in the CPE transmission experiment between broilers, the transmission rate of *E. coli* strains carrying CPE ($bla_{oxa-162}$) consistently lags behind by at least 50% compared to those carrying $bla_{CTX-M-1}$ and *catA1* (Chapter 3). Conversely, *E. coli* carrying ESBL genes ($bla_{CTX-M-1}$) consistently demonstrates the highest transmission rate. The lower transmission rate of CPE is likely attributable to both the resistance genes themselves and the specific bacterial strain carrying them. Despite the absence of experimental data comparing transmission of different beta-lactamase genes (bla) within the flock, field observations show a higher prevalence of $bla_{CTX-M-1}$ in humans and animals than other beta-lactamase-producing resistance genes spreading across farm, community, and hospital settings (Madec et al., 2023; Rana et al., 2022; Wei et al., 2021), implying a higher transmission rate of $bla_{CTX-M-1}$.

The transmission of resistant bacteria described in this thesis reveals a complex interplay of factors extending beyond the direct impact of antibiotics. While antibiotics accelerate transmission, their impact toward transmission rate is entangled with other factors such as resistance genes. Effectively addressing this complex web of factors influencing

transmission requires a One Health approach, which considers resistance genes, bacterial strains, animal species, and animal age. Given the practical limitations in conducting exhaustive experiments for each individual strain, a well-designed transmission experiment could be a viable option. This study, inoculating animals with a bacterial strain that is successful in the field but hosting varying resistance genes (achieved through genetic modification), will precisely demonstrate the specific effects of resistance genes on the transmission of resistant bacteria. Alternatively, statistical inference techniques can be used on available longitudinal field data, such as the study on ESBL in veal calf farms by Hordijk et al. (2013), employing the principles of Bayesian inference. A complication of the longitudinal data collected by Hordijk et al. (2013) is the sampling intervals, which were relatively long at 20-22 days with a total of five sample time points per calf. However, a transition occurs between the first and second sampling points, as veal calves are relocated from individual baby boxes to larger pens, with further movements between pens thereafter. This transition significantly limits the longitudinal data, reducing the number of samples available for analyzing transmission events to only four. Bayesian Hidden Markov Models (BHMMs) offer a promising dataset analysis method. These models, as explained by Mo et al. (2020), Shen et al. (2017), and Kirchherr et al. (2023), work by figuring out the “hidden” state based on the order of observed data over time. In the context of the veal calf dataset, characterized by extended unobserved states due to sampling intervals, BHMMs can adeptly capture the underlying dynamics. Through the inference of hidden states based on its relationship to the observed data (Liu and Song, 2021), BHMMs offer a robust framework for comprehending the intricate temporal patterns in the dataset.

Comparing risk factors for CPE introduction and transmission in broilers to fattening pigs and veal calves

The complexity of CPE transmission between meat-producing animals has been explored across this thesis. Although Chapter 5 focuses solely on simulating CPE transmission dynamics in broilers, the overarching objective is to develop an active surveillance protocol applicable to other meat-producing animals as well, such as pigs and veal calves. To achieve this, key factors including age, risk of CPE introduction, and antibiotic

treatment influencing the introduction and spread of CPE in broiler production were revisited and compared with those in other meat-producing livestock production.

Expanding beyond the age and antibiotic effects, factors such as **production management procedures**, particularly downtime (the resting period where no animals are present in the farm, allowing for cleaning), can prevent the carry-over of CPE to subsequent flocks (Newell et al., 2011; Course et al., 2021; Chin et al., 2009). However, Course et al. (2021) demonstrated that the elimination of *E. coli* and Salmonella varies with the cleaning method (dry vs. wet), floor type (wood vs. concrete), length of downtime cycle, and the frequency of disinfection (Course et al., 2021).

The “all-in-all-out” production procedure stops the CPE outbreaks in the broiler production (Chin et al., 2009). Although CPE introductions from livestock imports initially show rapid spread within broiler rearing farms and broiler farms (when importing broiler chicks from abroad), these outbreaks cease at the end of each production cycle due to a downtime of 7 to 14 days (EFSA, 2020: Sommer et al., 2016). Furthermore, the cleaning procedures conducted during downtime further diminish the survival chances of *E. coli* in livestock farms (Biocheck, 2024). Similarly, in veal calf farms, calves of the same age are raised together and moved to the slaughterhouse batchwise, allowing for a barn downtime of approximately nine days (Damiaans et al., 2019).

While fattening pig farms have an all-in-all out system, pig production in farrow-to-finish farms does not apply all-in-all-out at the farm level. Except for breeding sows, it is, however, applied at the level of rooms within the farm. This continuous flow production system increases the likelihood of persistent environmental contamination by CPE at pig farms. Additionally in pig production, the intensive production system exemplified in a study of 37 pig farms in Spain revealed an important correlation between resistance in bacteria and the production system (Mencía-Ares et al., 2021).

In their investigation of CPE cases on a pig farm in Germany, Fischer et al. (2012) isolated *E. coli* carrying bla_{VIM} from the same pig farm three months after the first isolation. The

persistence of *E. coli* CPE, despite the ban on carbapenems in livestock, may be attributed to the continuous production system, where newly susceptible pigs are born at regular intervals. Due to the absence of an “all-in-all-out” system for pigs at the farm level, limited internal biosecurity measures may lead to ongoing transmission of CPE. This is exemplified by multiple instances of CPE cases across various pig farms in Germany from 2011 to 2013 (Rochanski et al., 2018). Occasional cases of CPE have also been identified in farrow-to-finish farms in Germany (Irrgang et al., 2020). A detailed examination of CPE prevalence in farrow-to-finish farms in the United States revealed a prevalence of 18 percent in suckling pigs and at least 20 percent in sows across multiple sampling occasions (Mollenkopf et al., 2018). Environmental samples collected by investigators revealed contamination with CPE in at least 60 percent of all barns, suggesting continued excretion of CPE by breeding sows and gilts.

By synthesizing the critical factors of age, management practices, introduction routes, and antibiotic treatment, informed predictions can be made regarding CPE introduction and spread in other meat-producing animals.

Assimilating relevant factors into the probability of CPE colonization at the end of the production cycle.

The factors mentioned above were integrated and interpreted by qualitatively assessing them using the ECDC’s infectious disease threat prioritization tool, alongside the ‘Usage of Antibiotics in Agricultural Livestock in the Netherlands’ report (ECDC, 2017; SDa, 2023), supplemented by my own expertise. I organized the factors into four key stages that influence the dynamics of emerging CPE transmission: introduction, transmission within the flock, transmission between different production cycles within the same farm, and the presence of colonized animals at the end of production. This structured approach facilitates a comprehensive assessment of the likelihood of CPE colonization at the conclusion of the production cycle.

Firstly, the introduction and colonization of farms from contaminated feed and imported colonized animals deserve attention. The estimated risk of CPE colonization from

contaminated feed is high across all farm types, while the risk from imported colonized animals is elevated primarily in veal calf farms and comparatively low in other farm types.

Secondly, within-flock transmission constitutes the subsequent stage of emerging disease dynamics. Antibiotic usage levels are a crucial factor exacerbating transmission within flocks. According to the SDa report, broiler and pig farms show a lower level of persistent antibiotic usage compared to rose and white veal calf farms (SDa, 2023).

Thirdly, the production procedure influences transmission between different production cycles within the same farm. The ‘all-in-all-out’ system adopted by broiler and veal calf farms is expected to mitigate risk more effectively than pig production systems, where ‘all-in-all-out’ practices in farrow-to-finish farms occur only at the compartment level for the young animals, potentially allowing CPE to persist within the farm environment.

Lastly, older animals possess a more stable gut microbiome, making them less susceptible to colonization. Across all livestock types, animals are typically slaughtered after their gut microbiomes have matured, resulting in a lower anticipated prevalence of CPE. Consequently, surveillance at farms prior to slaughter may offer a heightened likelihood of detecting emerging CPE infections, particularly when the number of affected farms is small.

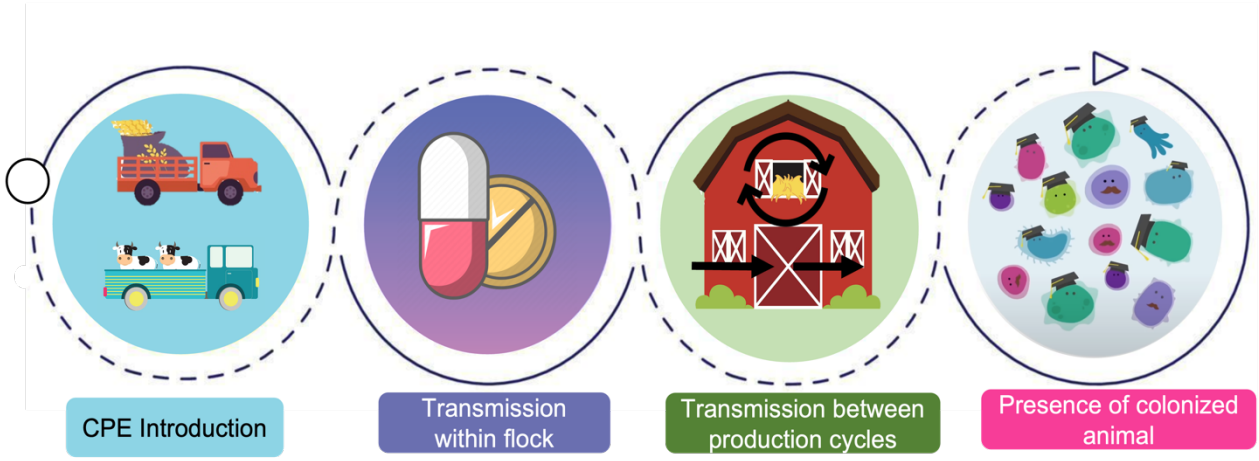


Figure 6.1- *Summary of factors influencing the probability of CPE colonization at the end of the production cycle in four overarching steps of emerging disease transmission dynamics: CPE introduction, transmission within the flock, transmission between production cycles in the same farm, and the presence of colonized animal at the end of production.*

The comparative analysis outlined above offers valuable insights into assessing the probability of CPE colonization at the conclusion of the production cycle. Since each farm type exhibits differences in livestock demographics, exposure to CPE sources, antibiotic treatment, and production procedures, a one-size-fits-all assessment of risk is not feasible. Nevertheless, the probability of CPE colonization can be evaluated based on the four overarching steps of emerging infection transmission dynamics.

In broilers, the risk of introduction is high from feed but low in subsequent steps of emerging disease transmission dynamics. Pigs face introduction risk from feed, compounded by within-flock transmission risks in continuous farrow-to-finish and breeding pig systems. Young piglets, most susceptible to colonization, require stringent internal biosecurity measures for risk reduction. Conversely, mature sows and gilts in continuous systems possess more stable gut microbiomes, potentially mitigating

colonization. Veal calves encounter high introduction risks from both feed and imported animals, with elevated transmission risks within flocks due to antibiotic treatment. Despite a high probability of colonization, veal calves would experience reduced colonization at the end of the production cycle due to a mature gut microbiome.

Future direction

There is an urgent need for a surveillance system capable of swiftly detecting emerging drug-resistant bacteria before they propagate extensively across farms. Nevertheless, the practicality and feasibility of expanding surveillance protocols must be considered. Such expansion would entail significant costs and additional expertise and resources, including microbiological diagnostic proficiency and robust data infrastructure (Do et al., 2023; Cornaglis et al., 2004). Reevaluating the system's effectiveness, factoring in future public health and societal costs, becomes crucial, particularly in a scenario where antibiotics may lose efficacy in treating both human and animal infections. This reassessment should guide efforts toward a sustainable and proactive surveillance strategy.

Our foremost task in laying a solid groundwork for designing an active surveillance protocol is to quantify CPE contamination in feed. This can be done by establishing an ongoing comprehensive study of the presence and distribution of CPE in feed. In addition, an investigation into young calves intended for the veal sector to establish the prevalence of CPE is important. Next, it is imperative to precisely quantify the age-related effects on transmission in pigs and veal calves. This can be achieved by either modeling the effect using existing literature or conducting transmission experiments in these animals.

Subsequently, the findings from such studies can be integrated into the existing simulation model used in Chapter 5, which can be extrapolated to other species, including veal calves and pigs. Critical parameters influencing dissemination can be leveraged from the simulation to design active surveillance strategies to pinpoint the most practical strategy for early detection.

Based on my research findings into CPE transmission dynamics, I propose redirecting current monitoring efforts from slaughterhouses to on-farm surveillance as a first step towards risk-based surveillance. More specifically, in on-farm surveillance, the emphasis should be on monitoring young animals, as they are more likely to be colonized if CPE is present on a farm.

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Summary

Samenvatting

The worldwide emergence of bacterial resistance to clinically important last-resort drugs such as Carbapenems across various environments, including communities, wildlife, and livestock, has prompted the surveillance of resistant bacteria in meat-producing animals. This thesis aims to gather crucial knowledge on these emerging resistant bacteria to support the design of an active surveillance protocol. This protocol intends to detect emerging resistance in meat-producing animals at an early stage when the cases are still limited.

In **Chapter 2**, the project commenced with a quantitative risk assessment focusing on the introduction of Carbapenemase-Producing Enterobacteriaceae (CPE) into livestock. Quantitative Risk Assessment estimates the likelihood and impact of potential harm (OIE, 2004). This project employs a stochastic quantitative risk assessment, representing input variables as probability distributions to address significant information gaps related to CPE dynamics within Dutch livestock.

This quantitative risk assessment model expresses the assessment process in a mathematical framework, presenting inputs and outputs as single numbers (deterministic) or distributions (stochastic) (OIE, 2004). It facilitates a comprehensive understanding of potential hazards and their interactions with the vulnerable population, accommodating uncertainties related to CPE introduction and its dynamics within Dutch livestock. The process commences by clearly defining the hazard, identifying the vulnerable population, and connecting the hazard to the population using scenario trees. Once the conceptual framework for the hazard and its movement toward the population is established, available information quantifies the probability of the hazard connecting with the population of interest (OIE, 2004). This provides a quantitative probability that a hazard, such as CPE-resistant bacteria, will occur in livestock and predicts when it might happen.

Chapter 3 details a transmission experiment comparing ESBL with CPE in broiler chickens, addressing limitations in precisely determining the moment of transmission due to limited statistical power. Transmission experiments are pivotal, typically quantified in controlled environments (Velthuis et al., 2007; Hu et al., 2017). Transmission experiments typically involve introducing the bacteria to a group of inoculated seed animals, which are

then reintroduced to a group of animals without the inoculated bacteria (challenge animals). The subsequent spread through the population is recorded (Hu et al., 2017).

Challenges exist due to logistical and ethical constraints, limiting the pinpointing of the moment of transmission. Collecting biological samples is a primary method, but precision may vary. Ethical and logistical constraints also limit the number of animals, affecting statistical power and potentially hindering the detection of significant effects (Cauchemez et al., 2004; Hu et al., 2017; Sedar et al., 2020; Festing and Altman, 2002; Button et al., 2013; Van de Schoot et al., 2015). To address these limitations, the project incorporates Bayesian statistics in the analysis, utilizing prior knowledge from other resistant bacteria transmission experiments, such as ESBL between broilers (Dame-Korevaars et al., 2018). Bayesian analysis effectively amalgamates observed data and prior knowledge, adapting the analysis to yield more realistic outcomes, even with limited samples (Van de Schoot et al., 2015).

Utilizing the longitudinal data obtained from Chapter 3, we conducted a Meta-analysis of Individual Patient Data (IPD) from five publications detailing the transmission experiment of *E. coli* carrying ESBL, *mcr-1,* and fluoroquinolone resistance in piglets and broiler chickens. In **Chapter 4**, These raw longitudinal data were analyzed using a Bayesian hierarchical model. The resulting transmission rates of different bacteria carrying resistance genes, animal hosts, and antimicrobial treatments provided clearer insights into the transmission dynamics of resistant bacteria and the impact of various relevant factors.

Simulation modeling in **Chapter 5** is a valuable tool for comprehending the spread of antibiotic-resistant bacteria and evaluating intervention strategies (Skyes et al., 2023; Schulz et al., 2018; Sorenson et al., 2017; Salines et al., 2020; Mercat et al., 2022; Dohoo et al., 2016; Faverjon et al., 2019). It serves as a critical approach to assess the transmission dynamics of antibiotic-resistant bacteria and appraise intervention programs, especially when real-world constraints, whether ethical or economic, hinder comprehensive studies. This transmission dynamic simulation model is particularly adept at analyzing the intricate mechanisms underlying the dissemination and persistence of

resistant bacteria within livestock populations (Lanzas et al., 2011; Sorenson et al., 2017; Schulz et al., 2018). The simulation tool is crucial for understanding the behavior of emerging antibiotic-resistant bacteria, offering pivotal insights that contribute to knowledge in surveillance efforts and improving our understanding of these complex dynamics within livestock populations.

De wereldwijde opkomst van bacteriële resistentie tegen klinisch belangrijke laatste redmiddelen zoals carbapenems in verschillende omgevingen, inclusief gemeenschappen, wilde dieren en vee, heeft geleid tot toezicht op resistente bacteriën in vleesproducerende dieren. Dit proefschrift heeft als doel cruciale kennis te vergaren over deze opkomende resistente bacteriën om de ontwikkeling van een actief surveillancesysteem te ondersteunen. Dit protocol is bedoeld om opkomende resistentie in vleesproducerende dieren in een vroeg stadium te detecteren, wanneer de gevallen nog beperkt zijn.

In Hoofdstuk 2 begon het project met een kwantitatieve risicoanalyse gericht op de introductie van Carbapenemase-Producerende Enterobacteriaceae (CPE) in vee. Kwantitatieve risicoanalyse schat de waarschijnlijkheid en impact van potentiële schade (OIE, 2004). Dit project maakt gebruik van een stochastische kwantitatieve risicoanalyse, waarbij invoervariabelen worden weergegeven als waarschijnlijkheidsverdelingen om significante informatie hiaten met betrekking tot CPE-dynamiek binnen Nederlands vee aan te pakken.

Dit kwantitatieve risicoanalysemodel drukt het beoordelingsproces uit in een wiskundig kader, waarbij invoer- en uitvoerwaarden worden gepresenteerd als enkele getallen (deterministisch) of verdelingen (stochastisch) (OIE, 2004). Het faciliteert een uitgebreid begrip van potentiële gevaren en hun interacties met de kwetsbare populatie, waarbij onzekerheden met betrekking tot CPE-introductie en de dynamiek ervan binnen Nederlands vee worden geaccommodeerd. Het proces begint met het duidelijk definiëren van het gevaar, het identificeren van de kwetsbare populatie en het verbinden van het gevaar met de populatie met behulp van scenariobomen. Zodra het conceptuele kader

voor het gevaar en de beweging ervan naar de populatie is vastgesteld, kwantificeert beschikbare informatie de waarschijnlijkheid dat het gevaar verbinding maakt met de populatie van belang (OIE, 2004). Dit levert een kwantitatieve waarschijnlijkheid dat een gevaar, zoals CPE-resistente bacteriën, in vee zal voorkomen en voorspelt wanneer dit zou kunnen gebeuren.

Hoofdstuk 3 beschrijft een transmissie-experiment waarin ESBL wordt vergeleken met CPE in vleeskippen, waarbij beperkingen worden aangepakt in het nauwkeurig bepalen van het moment van transmissie vanwege beperkte statistische kracht. Transmissie-experimenten zijn cruciaal, meestal gekwantificeerd in gecontroleerde omgevingen (Velthuis et al., 2007; Hu et al., 2017). Transmissie-experimenten omvatten typisch het introduceren van de bacteriën bij een groep geïnoculeerde zaaddieren, die vervolgens worden heringevoerd in een groep dieren zonder de geïnoculeerde bacteriën (uitdagingsdieren). De daaropvolgende verspreiding door de populatie wordt geregistreerd (Hu et al., 2017).

Uitdagingen bestaan vanwege logistieke en ethische beperkingen, waardoor het moeilijk is om het moment van transmissie nauwkeurig vast te stellen. Het verzamelen van biologische monsters is een primaire methode, maar de precisie kan variëren. Ethische en logistieke beperkingen beperken ook het aantal dieren, wat de statistische kracht beïnvloedt en mogelijk het detecteren van significante effecten belemmert (Cauchemez et al., 2004; Hu et al., 2017; Sedar et al., 2020; Festing en Altman, 2002; Button et al., 2013; Van de Schoot et al., 2015). Om deze beperkingen aan te pakken, integreert het project Bayesiaanse statistieken in de analyse, waarbij gebruik wordt gemaakt van eerdere kennis uit andere transmissie-experimenten met resistente bacteriën, zoals ESBL tussen vleeskippen (Dame-Korevaars et al., 2018). Bayesiaanse analyse combineert effectief geobserveerde gegevens en eerdere kennis, waardoor de analyse wordt aangepast om realistischere resultaten te leveren, zelfs met beperkte monsters (Van de Schoot et al., 2015).

Met behulp van de longitudinale gegevens die in Hoofdstuk 3 zijn verkregen, hebben we een meta-analyse van individuele patiëntgegevens (IPD) uitgevoerd van vijf publicaties

waarin het transmissie-experiment van *E. coli* met ESBL, *mcr-1* en fluoroquinolonresistentie bij biggen en vleeskippen werd beschreven. In **Hoofdstuk 4** werden deze ruwe longitudinale gegevens geanalyseerd met behulp van een Bayesiaans hiërarchisch model. De resulterende transmissiesnelheden van verschillende bacteriën met resistentiegenen, dierlijke gastheren en antimicrobiële behandelingen gaven duidelijker inzicht in de transmissiedynamiek van resistente bacteriën en de impact van verschillende relevante factoren.

Simulatiemodellering in **Hoofdstuk 5** is een waardevol instrument voor het begrijpen van de verspreiding van antibioticaresistente bacteriën en het evalueren van interventiestrategieën (Skyes et al., 2023; Schulz et al., 2018; Sorenson et al., 2017; Salines et al., 2020; Mercat et al., 2022; Dohoo et al., 2016; Faverjon et al., 2019). Het is een cruciale benadering om de transmissiedynamiek van antibioticaresistente bacteriën te beoordelen en interventieprogramma's te evalueren, vooral wanneer reële beperkingen, of die nu ethisch of economisch zijn, uitgebreide studies belemmeren. Dit transmissiedynamische simulatiemodel is bijzonder geschikt voor het analyseren van de ingewikkelde mechanismen die ten grondslag liggen aan de verspreiding en persistentie van resistente bacteriën binnen veepopulaties (Lanzas et al., 2011; Sorenson et al., 2017; Schulz et al., 2018). Het simulatie-instrument is cruciaal voor het begrijpen van het gedrag van opkomende antibioticaresistente bacteriën, en biedt essentiële inzichten die bijdragen aan kennis in toezichtinspanningen en ons begrip van deze complexe dynamiek binnen veepopulaties verbeteren.

About the author

Natcha is a veterinary quantitative epidemiologist, who has extensive experience with mathematical modelling, risk assessment and statistics, focusing on antimicrobial resistance. She holds a Bachelor degree of Biomedical Science from Mahidol University International College, Thailand, and a Masters of Science in Ecology, Evolution, and Conservation from Imperial College London, London. In her current post-doctoral position is at the Ghent University, Natcha is the lead researcher for the AB-ChangeR1 project which investigates the linkage between genotypic and phenotypic antimicrobial resistance and antimicrobial usage. Natcha has achieved numerous accolades, including a Merit Award for her bachelor's project and a Distinction Award for her Master's thesis.

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Dankittipong, N., Broek, J. V. D., de Vos, C. J., Wagenaar, J. A., Stegeman, J. A., & Fischer, E. A. J. (2024). Transmission rates of veterinary and clinically important antibiotic resistant Escherichia coli: A meta-analysis. *Preventive veterinary medicine*, 225, 106156. https://doi.org/10.1016/j.prevetmed.2024.106156

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Galletino, ND