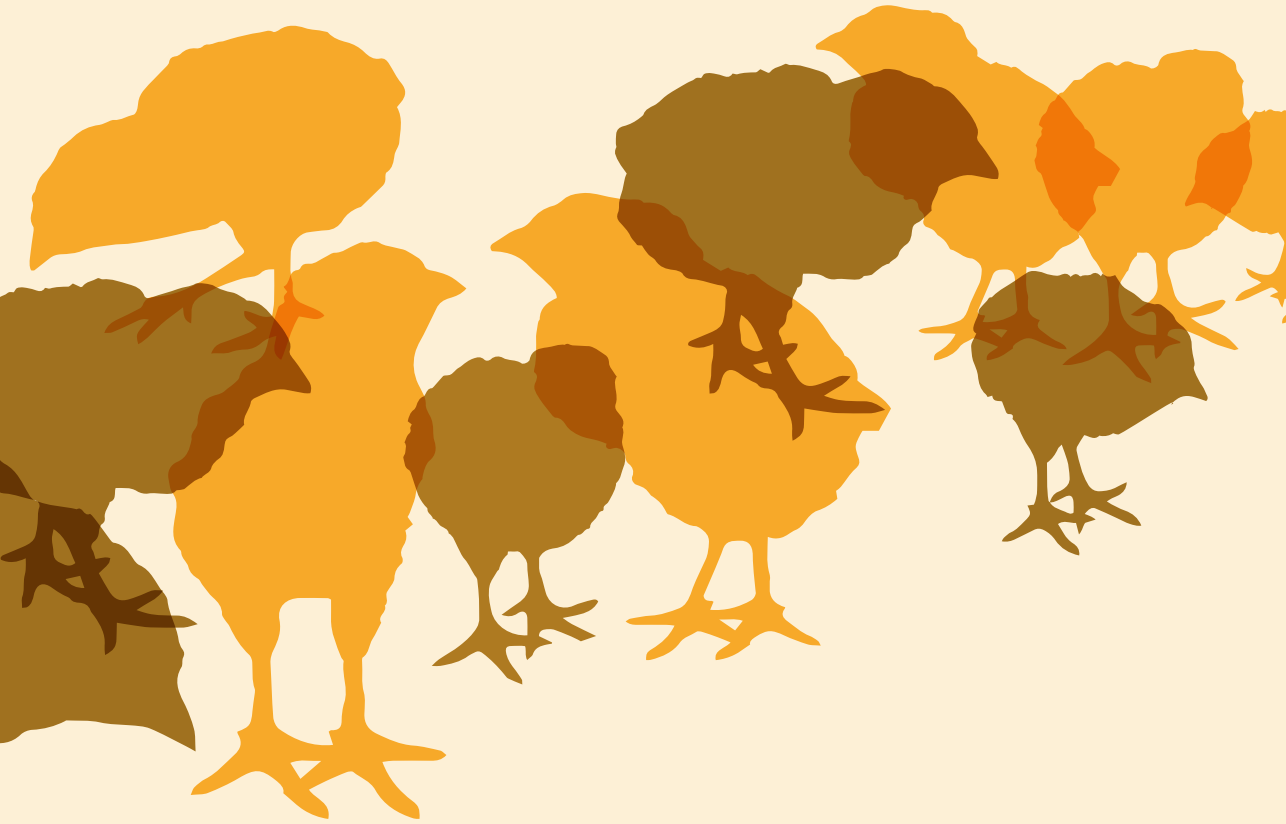
Interventions to reduce the spread of ESBL/pAmpC-producing *E. coli* in the broiler production pyramid



Anita Dame-Korevaar

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Interventies om de verspreiding van ESBL/pAmpC-producerende *E. coli* in de vleeskuikenketen te verminderen

(met een samenvatting in het Nederlands)

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Table of contents

CHAPTER 1

General introduction

Antimicrobial resistance

The use of antimicrobial substances has a long history, ancient civilizations were already using natural compounds to treat infections. It lasted however until the 20th century before important steps were taken in the development of antibiotics (Gould, 2016). After the discovery of penicillin by Alexander Fleming in 1928 (Fleming, 1929), the era with antibiotics available to treat patients suffering from infections started in the early 1940s. However, already in 1942 bacterial strains resistant to penicillin where detected in patients. Thereafter, other classes of antibiotics have been discovered and clinically implemented, quickly followed by the development of resistance (Davies and Davies, 2010; Lobanovska and Pilla, 2017). Interestingly, antibiotic resistant mechanisms were observed in the lab before the general use of antibiotics (Abraham and Chain, 1940). Moreover, the more recent findings of antibiotic resistance genes present in natural microbial populations in soil samples from different locations (D'Costa et al., 2006), but also in ancient permafrost samples (D'Costa et al., 2011), suggest that antibiotic resistance is a natural occurring phenomenon, even before the general use of antibiotics. The extensive use of antibiotics in humans and animals has accelerated the development of antimicrobial resistance. Nowadays, antimicrobial resistance present in humans, animals, food and the environment is considered as a serious threat to global public health (World Health Organization, 2018). In poultry, the spread of vancomycin resistant enterococci in the 1990s has led to the awareness that antimicrobial resistance in farm animals might impact both human and animal health (Nilsson, 2012). In this thesis, the focus is on the spread of beta-lactam resistant bacteria in the broiler production chain.

ESBL/pAmpC- producing bacteria

Resistance against beta-lactam antibiotics in Enterobacteriaceae is predominantly caused by the production of enzymes able to hydrolyse the beta-lactam ring typical for this class of antibiotics (Li et al., 2007). Production of enzymes, grouped under the term "extended spectrum beta-lactamases" (ESBL) and AmpC beta-lactamases (AmpC), results in resistance against extended-spectrum cephalosporins (ESC) (Livermore, 2008; Jacoby, 2009). The genes encoding for the production of ESBL and plasmid mediated AmpC (ESBL/pAmpC) are in *E. coli* predominantly located on plasmids, mobile elements of DNA that can be transferred between bacteria of the same or different genera or kingdom (Bradford, 2001; Carattoli, 2009).

ESBL/pAmpC-producing bacteria are present in animals and humans (Ewers et al., 2012; Karanika et al., 2016), as well as in food (Cohen Stuart et al., 2012; Kluytmans et al., 2013; Randall et al., 2017) and in the environment (Huijbers et al., 2015a; Dorado-Garcia et al., 2018). In broilers, high prevalence and a high level of heterogeneity in ESBL-genes and plasmids are reported in several countries (Saliu et al., 2017; Dorado-Garcia et al., 2018).

ESBL/pAmpC-producing bacteria in the broiler production pyramid

Monitoring of antimicrobial resistance in livestock has started in the Netherlands in 1998. The dramatic increase in antimicrobial resistance together with the high antimicrobial usage led to concerns, followed by policy changes (Mevius and Heederik, 2014). As a result, total antimicrobial usage in livestock was reduced drastically: by 64% in 2018 compared to reference year 2009 (Netherlands Veterinary Medicines Institute (SDa), 2019). Since 2010, the prevalence of ESBL/pAmpC-producing *E. coli* in broilers reduced significantly (Hesp et al., 2019; MARAN, 2019), following the trend of reduced antimicrobial usage. However, ESBL/pAmpC-producing *E. coli* is still present in broilers (MARAN, 2019). Following the one health approach aiming to avoid emergence and spread of antibiotic resistance in humans and animals (World Health Organization, 2018), the prevalence of ESBL/pAmpC-producing bacteria should be further reduced.

Several studies are done on the potential risk of the presence of ESBL/pAmpC-producing bacteria in broilers for humans. Some studies indicate transmission of ESBL/pAmpC-producing bacteria via consumption of meat (Leverstein-van Hall et al., 2011; Kluytmans et al., 2013; Hijazi et al., 2016). However, others did not find an association (Leistner et al., 2013; Carmo et al., 2014) or strong epidemiological link between livestock or food reservoirs and people in the general population (Dorado-Garcia et al., 2018; Mughini-Gras et al., 2019). A recent Dutch study found that ESBL/pAmpC-producing *E. coli* carriage in the general human population was mostly attributed to human-human transmission (about 60%) and only 4.5% to the consumption of chicken meat (Mughini-Gras et al., 2019). Also, a quantitative risk assessment study indicated that, although the load of ESBL/pAmpC-producing *E. coli* in poultry meat was high (97%), as a result of heating during food processing consumption of poultry meat forms a relatively low contribution (18%) to the total ESBL/pAmpC-producing *E. coli* exposure of humans (Evers et al., 2017). On the other hand, direct contact could be a transmission route of ESBL/pAmpC-producing bacteria: people living or working on a farm are at increased risk of carrying ESBL/pAmpC-producing bacteria compared to people in the general population (Dierikx et al., 2013b; Huijbers et al., 2014; Huijbers et al., 2015b; van Hoek et al., 2016; Dorado-Garcia et al., 2018). Considering the complex links of ESBL/pAmpC-producing *E. coli* among different sources (Mughini-Gras et al., 2019) a one health approach is needed, and food production chains should be taken into account when aiming to avoid emergence and spread of antimicrobial resistance.

To understand the dynamics of spread of ESBL/pAmpC-producing bacteria, not only broilers and broiler meat, but the whole production pyramid should be taken into account. This pyramid consists of several levels, with a few purebred pedigree farms producing the Grand Parent Stock at the top of the pyramid. The Grand Parent Stock produce the Parent Stock, which produce the broilers, raised for meat production at broiler farms at the bottom of the production pyramid. ESBL/pAmpC-producing bacteria are observed at Grand Parent, Parent and broiler farms (Dierikx

et al., 2013a; Apostolakos et al., 2019). Control of ESBL/pAmpC-producing bacteria should therefore involve the whole production pyramid and ideally interventions are applicable at all levels of the broiler production pyramid.

Interventions

The process of colonization of birds with ESBL/pAmpC-producing bacteria involves several steps, as schematically presented in Figure 1. First, a susceptible bird is exposed to ESBL/pAmpC-producing bacteria, present in the environment of the bird. This bird picks up ESBL/pAmpC-producing bacteria, followed by either passage through the gut without colonization, or colonization and reproduction of the ingested ESBL/pAmpC-producing bacteria. This will be followed by excretion of ESBL/pAmpC-producing bacteria via the faeces into the environment. Subsequently, another susceptible bird in the population can be exposed to the excreted ESBL/pAmpC-producing bacteria and transmission can occur.

Interventions could act at different steps of this colonization process. First, interventions could aim to reduce the exposure of susceptible birds to ESBL/pAmpC-producing bacteria, thereby preventing uptake of the bacteria. This can be achieved by, for example, cleaning and disinfection of poultry houses between production rounds. However, these interventions were not always effective in preventing colonization (Daehre et al., 2018). ESBL/pAmpC-producing bacteria can be present in the environment in and around poultry houses (Laube et al., 2013; Zurfluh et al., 2014a; Zurfluh et al., 2014b; Huijbers et al., 2016; Daehre et al., 2018). Therefore, sufficiently reducing the exposure of birds to ESBL/pAmpC-producing bacteria is difficult. Secondly, interventions could aim to make the birds less susceptible to colonization upon exposure and reduce excretion of colonized birds (this thesis). Finally, spatial separation between excreting and susceptible birds might reduce transmission in a farm (this thesis).

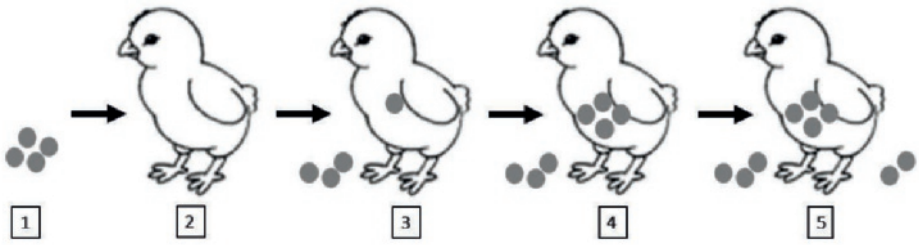

Figure 1 Spread of ESBL/pAmpC-producing bacteria. ESBL/pAmpC-producing bacteria are present in the environment (1) and form exposure to a susceptible bird (2). The susceptible bird picks up ESBL/pAmpC-producing bacteria (3), the bacteria colonizes and reproduces in the gut of the bird (4). The bird excretes ESBL/pAmpC-producing bacteria in the environment (5), forming exposure to another susceptible bird.

Factors influencing the process of colonization and transmission, and information on possible routes of transmission of ESBL/pAmpC-producing bacteria in the broiler production pyramid need to be studied first, in order to design the most effective intervention strategies to reduce ESBL/pAmpC-producing bacteria in the broiler production pyramid.

Aim and outline

The aim of this thesis was to get insight into the processes of colonization and transmission of ESBL/pAmpC-producing bacteria in the broiler production pyramid, using *E. coli* as reference bacteria, and to evaluate possible interventions aiming to prevent or reduce colonization and transmission of ESBL/pAmpC-producing bacteria in the broiler production pyramid. In Chapter 2, the dynamics of pAmpC-producing *E. coli* in a parent stock flock, their environment and offspring are described. Chapter 3 contains a review of possible transmission routes of ESBL/pAmpC-producing bacteria in the broiler production pyramid described in literature. In Chapter 4, the dose-response effect of two *E. coli* strains, carrying different ESBL/pAmpC-gene-plasmid combinations, is studied after challenge of specific pathogen free (SPF) and conventional broilers with different dosages. Interventions are tested in an animal model designed according to the findings described in Chapter 4. The tested interventions are competitive exclusion (supply of intestinal bacteria) and compartmentalization (subdividing the broiler flock), performed under controlled circumstances (Chapter 5, competitive exclusion), and semi-field circumstances (Chapter 6, competitive exclusion and compartmentalization). Chapter 7 contains the general discussion of the results described in this thesis.

CHAPTER 2

Dynamics of CMY-2 producing *E. coli* in a broiler parent flock

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Veterinary Microbiology (2017) 203:211-214.

Abstract

Extended-spectrum β-lactamase and plasmid mediated AmpC β-lactamase (ESBL/pAmpC) producing bacteria are resistant to extended-spectrum cephalosporins (ESC), and are present in all levels of the broiler production chain. We determined the prevalence, concentration, and persistence of ESBL/pAmpC-*Escherichia coli* in a broiler parent flock during the rearing and laying period. One-day old chickens were housed in four separate pens. Until week 33 no antibiotics or coccidiostatics were used. During rearing 57 chickens in each pen (*n*=228), and in the laying period two groups of 33 chickens were individually sampled (*n*=66). Environmental samples were taken from week 16 onwards. ESBL/pAmpC-*E. coli* presence was determined by selective culturing. In the samples of week 16-19 the concentration of ESBL/pAmpC-*E. coli* was determined. All ESC-resistant isolates found were positive for pAmpC gene bla_{CMY-2} located on IncA/C plasmids, in several *E. coli* MLST types. CMY-2-*E. coli* prevalence decreased from 91% (95% CI 86-94%) at day 7 (week 1) to 0% (95% CI 0-5%) in week 21. However, CMY-2-*E. coli* remained present in the environmental samples during the whole study. CMY-2-*E. coli* concentration varied between detection limit ($<10^3$) and $2{\cdot}10^4$ CFU/gram faeces. The sharp reduction of CMY-2-*E. coli* in this broiler parent flock in absence of antibiotics suggests a selective disadvantage of bla_{CMY-2} on IncA/C plasmids on animal level. The underlying mechanism should be studied further as this may provide new insights on how to reduce ESBL/pAmpC prevalence and transmission in the broiler production chain.

Introduction

Extended-spectrum β-lactamase and plasmid mediated AmpC β-lactamase (ESBL/pAmpC) producing bacteria are resistant to extended-spectrum cephalosporins (ESC). In the Netherlands, 56.5% of the broilers at slaughter were carriers of ESBL/pAmpC-*Escherichia coli* in 2015 (MARAN, 2016). Although prevalence in poultry varies between farms (Blaak et al., 2015), ESBL/pAmpC producing bacteria are present in all levels of the broiler production chain (Dierikx et al., 2013a). The broiler production chain has a pyramidal structure, thus the presence of ESBL/pAmpC in the upper levels of the chain might influence the ESBL/pAmpC status of lower levels in the chain, e.g. through vertical transmission (Nilsson et al., 2014; Zurfluh et al., 2014b). To our knowledge reports on the dynamics of ESBL/pAmpC-*E. coli* in parent stock are lacking. The aim of this study is to determine prevalence, faecal concentration, and persistence of ESBL/pAmpC-*E. coli* in a broiler parent flock during the rearing and laying period.

Material and methods

Chickens

One-day old broiler parent stock chickens (*n*=3184) were housed in a rearing house of an experimental poultry farm in the Netherlands. The chickens were divided over four completely separated pens. Each pen housed 693 females and 103 males, separated by a fence. At week 20 all chickens were moved to the laying house. Two groups of 30 females and three males were selected from the four rearing pens, and randomly allocated to two separate pens. During the laying phase two females died and one lame male was replaced in pen 1 and one female died in pen 2.

Chickens received feed without antibiotics or coccidiostats. Feed and water were available *ad libitum* during the first seven days, thereafter feed was supplied based on body weight. Drinking water pipes were cleaned before entry of the chickens and thereafter weekly by acidifying the water using peracetic acid. Chickens received a microflora product (Aviguard®) at day of arrival. A standard vaccination and lighting scheme was applied.

Identification and Sampling

Rearing period

At day 7 (week 1), in each pen 57 females (minimal sample size to detect 5% prevalence) were selected randomly, and sampled by individual cloacal swabs. At week 12 per pen 57 females were randomly selected, tagged and individual cloacal swabs were taken. In week 16-19 the tagged females were sampled weekly and environmental samples were taken using bootsocks. In week 19 environmental samples were taken in the male pens.

Laying period

At week 20, the females and males were moved to the laying house. All females found ESBL/pAmpC-*E. coli* positive at least once during week 16-19 and a random selection of females being ESBL/pAmpC-*E. coli* positive in week 12 were selected and housed in two groups of 30 females. To each group three randomly selected and tagged males were added. All chickens were sampled individually in week 21, 24, 34 and 35. Environmental samples from the litter were taken at week 21, 24, 34 and 35 (morning) and 43, 45, 46, 47 and 49 (noon). In week 34 and 35 also environmental samples from the laying nests were taken, by hand wiping using bootsocks. In week 49 chickens were euthanized and cecal content was collected.

Antibiotic treatment

In week 33 the chickens in pen 1 were administered amoxicillin via the drinking water for five days (20 g/1000 kg live weight/day).

Follow up offspring

During week 34, 160 eggs were collected from both pens and disinfected with formaldehyde. Forty eggs were crushed, eggshells and egg content was mixed and analysed for ESBL/pAmpC presence, 120 eggs were incubated. After hatching, individual cloacal swabs were taken from the broilers at day of hatch, daily until day 7 and at days 14 and 21. Environmental samples were taken at the same days, starting the day after hatch. At day 21 broilers were euthanized and cecal content was collected.

Ethics

The animal procedures at Utrecht University were approved by the Animal Ethical Committee of Utrecht University (Utrecht, the Netherlands), in full compliance with all relevant legislation.

Analysis

ESBL/pAmpC-E. coli detection

Cloacal and cecal samples, eggs and bootsocks were selectively cultured (3 mL LB broth versus 400 mL LB, supplemented with 1 mg/L cefotaxime). After overnight incubation at 37 °C, 10 µL broth was inoculated on MacConkey plates supplemented with 1 mg/L cefotaxime and incubated overnight at 37 °C. Cloacal samples were analysed individually. Eggs and cecal samples were pooled per five, bootsocks were pooled per pen, except for bootsocks taken in week 16 and 43-49.

ESBL/pAmpC-E. coli and E. coli concentration

Swabs used in week 16-19 in pen 1 and 2 were weighed before and after sampling to determine the amount of faeces collected. Swabs were suspended in 1 mL saline solution and tenfold dilution series were made to quantify the colony-forming units (CFU) of ESBL/pAmpC-*E. coli* and total *E.*

coli per mL, using MacConkey plates with and without 1 mg/L cefotaxime. Based on the amount of faeces on the swabs CFU/gram faeces was calculated.

Typing

From week 12 onwards, in at least one isolate of every sampling moment, ESBL/ pAmpC genes were typed by PCR and sequencing (Dierikx et al., 2010). Plasmids were characterized by transformation (Dierikx et al., 2010) and PCR-based Replicon Typing (PBRT) (Diatheva, Italy). Selection of transformants was performed on LB agar containing 1 mg/L cefotaxime. *E. coli* genotyping was performed by MLST (Wirth et al., 2006). MLST patterns were analysed using Bionumerics version 6.1.

Results and discussion

At day 7 (week 1) prevalence of ESBL/pAmpC-*E. coli* ranged between pens from 89-93% (Figure 1). All isolates carried the pAmpC gene bla_{CMY-2} on IncA/C plasmids (Figure 2). Overall CMY-2-*E. coli* prevalence showed a remarkable decrease, from 91% (range 89-93%) at day 7 to 46% (32-70%) in week 12, 11% (0-30%) in week 16, 16% (0-53%) in week 17, 3% (0-9%) in week 18 and 1% (0-2%) in week 19, without intervention. During the laying period (week 21, 24) no positive cloacal swabs were found. All 44 typed isolates carried bla_{CMY-2} and 22 samples were also carrying bla_{TEM-1}. The predominant *E. coli* sequence type (ST10, 28 samples) was found in all pens, suggesting clonal spread. The bla_{CMY-2}-IncA/C combination was found in different *E. coli* STs suggesting plasmid spread.

The high prevalence in week 1 might be the result of vertical transmission from the grandparent flock or other sources of contamination at the hatchery or during transport. The grandparent flock had been treated with antibiotics in the weeks prior to production of the parent stock. Unfortunately, no data about the ESBL/pAmpC prevalence in this flock is available. Despite the high prevalence at day 7, CMY-2-*E. coli* was not able to persist in the chickens. Other studies in poultry have also shown a decreasing prevalence of antibiotic resistant bacteria, with and without the use of antibiotics (Diarra et al., 2007; Baron et al., 2014; Huijbers et al., 2015b). However, most of these studies report limited reduction. Factors as ageing (Lu et al., 2003), diet (Amerah et al., 2011), litter (Torok et al., 2009), probiotics (Nakphaichit et al., 2011), disease (Stanley et al., 2012) and stress (Burkholder et al., 2008) might influence the microbiota composition and thus the potential of CMY-2-*E. coli* to persist in the gut. Until week 33 no antibiotics were used, resulting in no selective advantage to CMY-2-*E. coli*. After applying amoxicillin in week 33 in one of the pens, 2/31 chickens became positive in week 34, in the non-treated pen prevalence was 0%. However, one week later, 1/32 chickens was positive in the non-treated pen, whereas no positive samples were found in the treated pen (Figure 1).

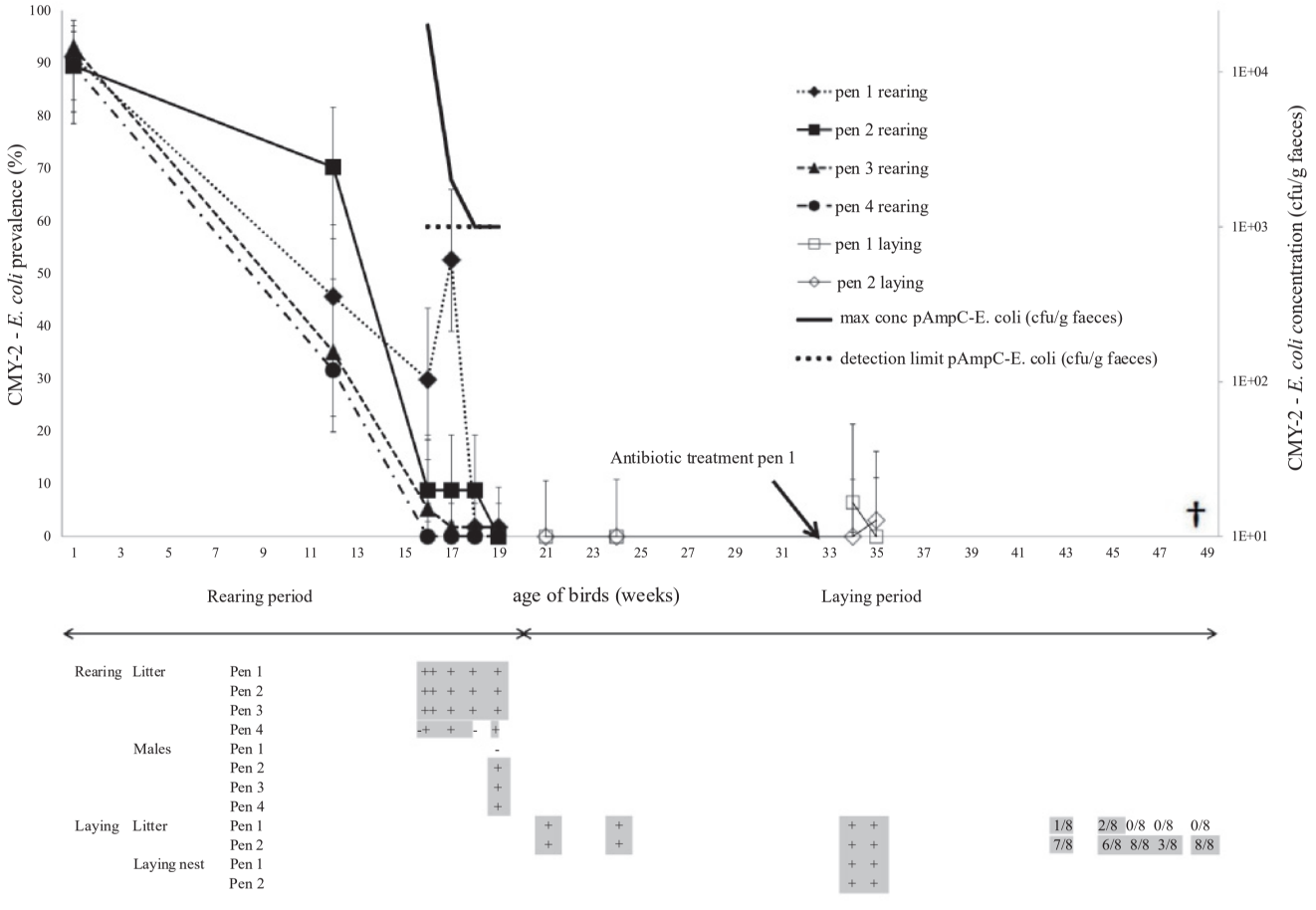

Figure 1 CMY-2-*E. coli* prevalence (%), concentration (CFU/g faeces) and presence in the environment in a broiler parent flock during the rearing and laying period.

The inability to persist on animal level might be due to an unsuccessful combination of CMY-2-*E. coli* on plasmid IncA/C. In European broiler meat, CMY-2-*E. coli* is often found in combination with plasmids IncI1 or IncK (Borjesson et al., 2013b; Egervarn et al., 2014). The low occurrence and the observed decrease in this study may suggest that plasmid IncA/C is less able to conjugate and spread in bacterial populations as was previously described for *Salmonella* (Poole and Crippen, 2009).

The decreasing prevalence was also represented by decreasing concentrations of CMY-2-*E. coli* in faeces. The maximum concentration of CMY-2-*E. coli* observed decreased from $2 \cdot 10^4$ CFU/gram faeces in week 16, to $1 \cdot 10^3$ (detection limit) in weeks 18 and 19 (Figure 1). During week 16-19 the total *E. coli* counts remained between 10^4 and $>10^8$ CFU/gram faeces.

Contrary to the decreasing prevalence and CMY-2-*E. coli* concentration in the faeces, almost all environmental samples (90/116) were part of a positive pen (Figure 1). Before placement of the chickens the laying house tested negative for ESBL/pAmpC-*E. coli*. Based on the positive environmental samples, negative cloacal swabs during the laying period and negative cecal samples at the end of the experiment, the chickens most likely introduced CMY-2-*E. coli* into the laying pens and after that ceased shedding CMY-2-*E. coli*. Environmental contamination might have persisted after the birds ceased excretion. Others report survival of *E. coli* and ESBL-*E. coli* in faeces and soil for months (Merchant et al., 2012). Although CMY-2-*E. coli* was still present in the environment during egg collection, none of the samples taken from the eggs and offspring were found positive for ESBL/pAmpC-*E. coli*.

MLST ST	pAmpC gene	Other gene	Plasmid	Week	Pen	Sample type
224	CMY-2	TEM-1	IncA/C	21	L2	environment
224	CMY-2	TEM-1	IncA/C	34	L1	cloacal
2485	CMY-2		IncA/C	17	R3	environment
2485	CMY-2		IncA/C	18	R3	environment
2485	CMY-2		IncA/C	19	R3 M	environment
648	CMY-2	TEM-1	IncA/C	16	R2	environment
155	CMY-2		IncA/C	12	R4	cloacal
155	CMY-2		IncA/C	19	R1	environment
156	CMY-2	TEM-1	IncA/C	17	R2	environment
156	CMY-2	TEM-1	IncA/C	19	R2 M	environment
156	CMY-2	TEM-1	IncA/C	19	R4 M	environment
355	CMY-2		IncA/C	16	R3	environment
355	CMY-2		IncA/C	18	R3	cloacal
355	CMY-2		IncA/C	19	R3	environment
new	CMY-2		IncA/C	19	R2	environment
new	CMY-2		IncA/C	44	L1	environment
10	CMY-2	TEM-1	IncA/C	12	R1	cloacal
10	CMY-2		IncA/C	12	R2	cloacal
10	CMY-2	TEM-1	IncA/C	12	R3	cloacal
10	CMY-2	TEM-1	IncA/C	16	R1	cloacal
10	CMY-2	TEM-1	IncA/C	16	R1	environment
10	CMY-2		IncA/C	16	R4	environment
10	CMY-2	TEM-1	IncA/C	17	R1	environment
10	CMY-2		IncA/C	17	R4	environment
10	CMY-2	TEM-1	IncA/C	18	R1	environment
10	CMY-2		IncA/C	18	R2	environment
10	CMY-2	TEM-1	IncA/C	19	R4	environment
10	CMY-2	TEM-1	IncA/C	21	L1	environment
10	CMY-2	TEM-1	IncA/C	24	L1	environment
10	CMY-2		IncA/C	24	L2	environment
10	CMY-2		IncA/C	34	L1	laying nest
10	CMY-2		IncA/C	34	L1	environment
10	CMY-2	TEM-1	IncA/C	34	L2	laying nest
10	CMY-2	TEM-1	IncA/C	34	L2	environment
10	CMY-2	TEM-1	IncA/C	35	L2	cloacal
10	CMY-2		IncA/C	35	L1	laying nest
10	CMY-2		IncA/C	35	L1	environment
10	CMY-2	TEM-1	IncA/C	35	L2	laying nest
10	CMY-2		IncA/C	35	L2	environment
10	CMY-2		IncA/C	44	L2	environment
10	CMY-2	TEM-1	IncA/C	45	L2	environment
10	CMY-2	TEM-1	IncA/C	46	L2	environment
10	CMY-2		IncA/C	47	L2	environment
10	CMY-2	TEM-1	IncA/C	49	L2	environment

Figure 2 Multilocus sequence typing (MLST), gene- and plasmid characteristics of cloacal and environmental samples from females and males (M) in different pens during rearing (R1-R4) and laying (L1, L2) period.

Conclusions

This study showed that in a parent flock at an experimental farm, in absence of antibiotics, prevalence of pAmpC gene bla_{CMY-2} on IncA/C plasmid decreased and is not detected in the offspring. This may not be true for other farms, with different ESBL/pAmpC-plasmids in *E. coli*, and under field conditions. The mechanism behind this should be studied further as this might lead to possible interventions to reduce ESBL/pAmpC prevalence and transmission in the broiler production chain.

Acknowledgements

This work was supported by Public Private Partnership project: 1H4F-Reduction of ESBLs: evaluation of ESBL interventions (TKI-AF-14210). We thank the experimental farm for participation in this study, Daniela Ceccarelli and Kees Veldman for their technical assistance and Apostolos Liakopoulos for his help creating the MLST dendrogram.

CHAPTER 3

Transmission routes of ESBL/pAmpC producing bacteria in the broiler production pyramid, a literature review

Anita Dame-Korevaar, Egil A.J. Fischer, Jeanet van der Goot, Arjan Stegeman, Dik Mevius.

Preventive Veterinary Medicine (2019) 162:136-150.

Abstract

Plasmid mediated Extended Spectrum Beta-Lactamase and AmpC Beta-Lactamase (ESBL/pAmpC) producing bacteria are resistant to beta-lactam antimicrobials and are widespread in humans, the environment and animals. Animals, especially broilers, are an important reservoir of ESBL/pAmpC producing bacteria. To control ESBL/pAmpC prevalence in broilers, transmission within the entire broiler production pyramid should be considered. This study, including 103 articles originating from two electronic databases, searched for evidence for possible routes of transmission of ESBL/pAmpC producing bacteria in the broiler production pyramid. Possible routes of transmission were categorised as 1) vertical between generations, 2) at hatcheries, 3) horizontal on farm, and 4) horizontal between farms and via the environment of farms. This review presents indications for transmission of ESBL/pAmpC producing bacteria for each of these routes. However, the lack of quantitative results in the literature did not allow an estimation of the relative contribution or magnitude of the different routes. Future research should be specifically targeted towards such information as it is crucial to guide reduction strategies for the spread of ESBL/pAmpC producing bacteria in the broiler production chain.

Introduction

Antimicrobial resistance (AMR) is an increasing threat to human health (World Health Organization, 2016). An important group of antimicrobial resistant bacteria is associated with plasmid mediated Extended Spectrum Beta-Lactamase and AmpC Beta-Lactamase (ESBL/pAmpC) producing bacteria. These beta-lactamase producing bacteria hydrolyse beta-lactam antimicrobials, such as penicillins and cephalosporins. Spread of ESBL/pAmpC resistance occurs via clonal and plasmid spread (Nikaido, 2009). ESBL/pAmpC producing bacteria are widely distributed in animals and humans, as reviewed by Ewers et al. (2012); Karanika et al. (2016) as well as in food (Leverstein-van Hall et al., 2011; Cohen Stuart et al., 2012; Kluytmans et al., 2013; Rasmussen et al., 2015; Randall et al., 2017) and in the environment, as reviewed by Huijbers et al. (2015a). In animals, a high prevalence of ESBL/pAmpC producing bacteria is found in broilers and broiler parents (Mesa et al., 2006; Smet et al., 2008; Dierikx et al., 2013a; Dierikx et al., 2013b; Blaak et al., 2015; Huijbers et al., 2016; Dame-Korevaar et al., 2017), and a high level of heterogeneity in ESBL-genes and plasmids has been reported in the poultry production pyramid, as reviewed by Saliu et al. (2017). Carriage of ESBL/pAmpC producing bacteria by humans is associated with living or working on broiler farms in several Dutch studies (Dierikx et al., 2013b; Huijbers et al., 2014; Huijbers et al., 2015b; van Hoek et al., 2016; Dorado-Garcia et al., 2018). Transfer via the consumption of meat is indicated in some studies (Leverstein-van Hall et al., 2011; Kluytmans et al., 2013; Hijazi et al., 2016; Dorado-Garcia et al., 2018). However, in other studies this association was not observed (Leistner et al., 2013; Carmo et al., 2014; de Been et al., 2014).

The decrease in ESBL/pAmpC-*E. coli* prevalence in broilers reported in the Netherlands and in Denmark is most likely related to a reduction of antimicrobial usage (DANMAP, 2015; MARAN, 2018). The use of antimicrobials and the presence of ESBL/pAmpC producing bacteria, and more specific resistance against extended-spectrum cephalosporins (ESC), is associated with the use of third generation cephalosporins for example in hatcheries (Dutil et al., 2010; Baron et al., 2014; Baron et al., 2016; MARAN, 2018). However, even in the absence of antimicrobials ESBL/pAmpC producing bacteria are able to colonise chickens and the plasmids encoding for ESBL/pAmpC production are able to spread within a bacterial and animal population (Le Devendec et al., 2011; Fischer et al., 2014; Huijbers et al., 2016; Ceccarelli et al., 2017; Dame-Korevaar et al., 2017). Next to the use of antimicrobials, other drivers related to the structure of the poultry production pyramid and management on farms might contribute to the occurrence of resistance (Dorado-Garcia et al., 2016).

To control ESBL/pAmpC prevalence in broilers, the pyramidal structure of broiler production is of importance (Figure 1). At the top of the production pyramid Great Grandparent Stock (GGPS) birds are produced by a few breeder companies at pedigree farms. The offspring of these GGPS are hatched at hatcheries and transported to Grandparent Stock (GPS) rearing farms. Around the age of 18 weeks these GPS are moved to GPS multiplier farms. These birds produce offspring

which are the Parent Stock (PS) of the birds raised for meat production at broiler farms. Therefore, controlling ESBL/pAmpC prevalence in broilers requires taking into account possible transmission of ESBL/pAmpC producing bacteria throughout the entire pyramid, as has been described for *Salmonella* (Van Immerseel et al., 2009) and *Campylobacter* (Idris et al., 2006; Katsma et al., 2007) and was suggested for ESBL-*E. coli* (Borjesson et al., 2013b; Dierikx et al., 2013a).

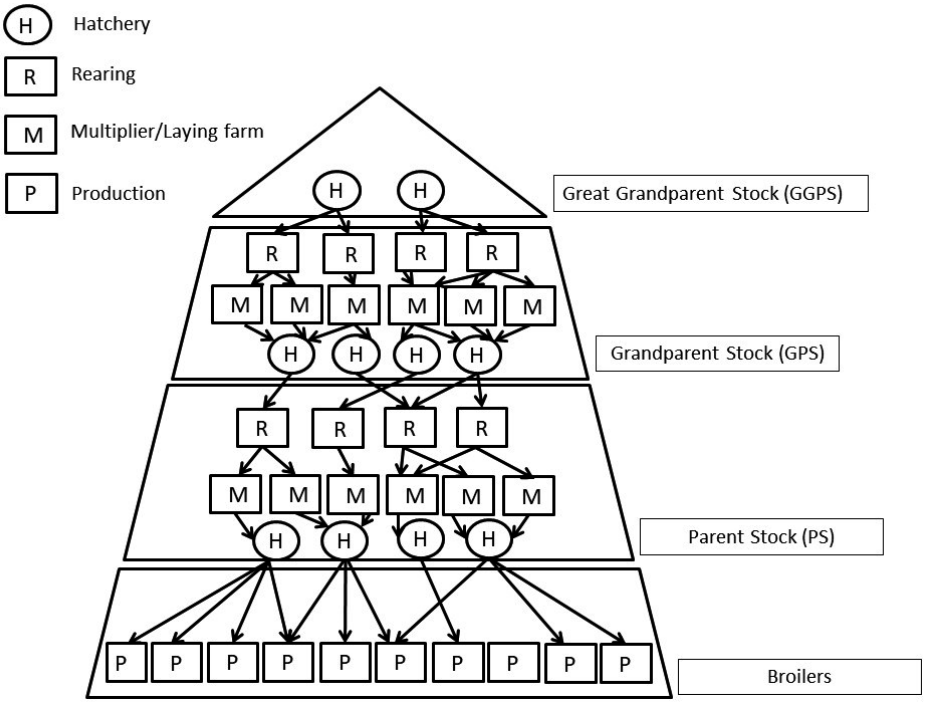

Figure 1 Schematic view of the broiler production pyramid. The broiler production pyramid includes different levels; Great Grandparent Stock (GGPS) birds at the top of the pyramid, Grandparent Stock (GPS), Parent Stock (PS), and broilers at the bottom of the pyramid, with increasing numbers of farms from top to bottom. Levels in the pyramid are linked through hatcheries (H).

The presence of ESBL/pAmpC producing bacteria in the different levels of the broiler production chain has been described in the past decennia. However, an overview of possible transmission routes of ESBL/pAmpC producing bacteria in the broiler production chain is lacking. This review aims to describe possible routes of transmission of ESBL/pAmpC producing bacteria described in the literature, because understanding these routes is a prerequisite for control. It includes four different types of transmission, which correspond to the structure of the poultry production

pyramid: 1) transmission from generation to generation (e.g. parent to offspring), 2) transmission at hatcheries, 3) horizontal on farm transmission and 4) horizontal between farm and environmental transmission.

Methods

Search strategy

A systematic search in PubMed and CAB Abstracts was done, including articles until 26 April 2018. Three search terms were used: [poultry] AND [ESBL/AmpC] AND [spread]. Complete search terms, including synonyms, are given in Appendix 1. No limits were set on time.

Data selection

Duplicates were removed from the dataset before selection of useful articles. Selection was done in three steps. First and second selections were done using an online program (www.covidence.org), on the basis of title and abstract. The first selection was done using the following inclusion criteria: a) the study concerns broilers or layers, turkeys or ducks, including results on chain, farm and animal level, b) the study concerns plasmid mediated resistance. The second selection was done using the following inclusion criteria: the study concerns ESBL/pAmpC producing bacteria in the broiler production pyramid. Relevant studies concerning layers, turkeys or ducks, were included when transmission routes described in these animals were also relevant in the broiler production chain. Studies were selected if isolates were typed as ESBL/pAmpC producing bacteria or if resistance against beta-lactam antimicrobials was reported. Beta-lactam antimicrobials were restricted to extended-spectrum cephalosporins (ESC), although a few articles reporting resistance against first or second generation cephalosporins were included as they contained additional information regarding possible transmission routes.

Third selection was based on full articles, available in pdf or hard copy, in Dutch, English or German language. In this selection a study was included if it suggested or described a certain route or mechanism of spread of ESBL/pAmpC producing bacteria in poultry, or if it mentioned risk factors or interventions on the occurrence of ESBL/pAmpC producing bacteria. Studies containing no primary data were excluded from further analysis, with the exception of some reviews containing information in addition to the selected articles. After the third selection duplicates missed in the first elimination were removed manually. The articles were then studied, the data was extracted from the full articles and included in the dataset. Data was categorised based on: 1) route: vertical transmission from generation to generation, transmission at hatcheries, horizontal on farm transmission or horizontal between farm and environmental transmission, 2) type of the study: observational or experimental, 3) type of results: quantitative or qualitative, 4) typing of the isolates: phenotypic or genotypic and 5) region of origin of the data. In addition, any mentioned

risk factors and interventions were recorded.

Definition of transmission routes

Vertical transmission from generation to generation

Two types of vertical transmission from generation to generation are distinguished: true vertical transmission and apparent vertical transmission. True vertical transmission is transmission via ovarian or uterine infection, as is known for *Salmonella* Enteritidis (Guard-Petter, 2001; Buck et al., 2004). Apparent vertical transmission is transfer from the parent stock to the next generation via the environment, for example via faecal contamination of the egg shell at the parent farm (Wilkinson, 1999). It is often impossible to distinguish apparent and true vertical transmission. Furthermore, the role of the hatchery between parents and offspring is difficult to untangle. In section "Vertical transmission from generation to generation" all studies mentioning the role of the parents are discussed, studies describing the potential role of the hatcheries, as a link between generations, are discussed separately (transmission at hatcheries).

Transmission at hatcheries

Transmission at the hatchery can originate from ESBL/pAmpC producing bacteria present in the environment of the hatchery or can originate from eggs of the supplying parent flocks carrying ESBL/pAmpC producing bacteria, leading to contamination of other eggs or newly hatched birds in the hatching units. In section "Transmission at hatcheries" all studies mentioning the role of the hatchery in transmission of ESBL/pAmpC producing bacteria are discussed.

Horizontal on farm transmission

Horizontal transmission on a farm occurs within and between flocks. Transmission can occur via direct physical or faecal contact between birds and via indirect contact, for example via humans, shared equipment, a contaminated poultry house, or other animals or vectors, such as flies. Here we exclude vertical transmission between parents and offspring, or contamination in a hatchery. Horizontal transmission between flocks at a farm can occur between both parallel and serially housed flocks. In section "Horizontal on farm transmission" articles mentioning horizontal transmission on a farm are discussed.

Horizontal between farm and environmental transmission

Horizontal transmission between farms can occur via indirect contact, for example via humans, other animals, trucks or shared equipment or via the environment, at different levels of the poultry production chain. In section "Horizontal between farm and environmental transmission" articles mentioning horizontal transmission between farms are discussed.

Results

In total 9212 articles were retrieved from PubMed and CAB Abstracts. During the first and second selection rounds 8576 articles were excluded, because they did not concern poultry, plasmid mediated resistance, or both. After the third selection round 103 articles, containing information on possible transmission routes, were included in the database for this review (Figure 2, Table 1). Most articles report results from Europe (56/103), followed by Asia (25/103), North-America (6/103), Latin-America (6/103), Africa (5/103), and Oceania (1/103). The remaining 4 articles report results from more than one continent. Of the 103 articles, 98 articles report primary data. The other 5 articles are reviews (Olsen et al., 2014; Zurek and Ghosh, 2014; Hille et al., 2014; Ljubojevic et al., 2016; Wang et al., 2017) containing valuable additional information regarding possible transmission routes.

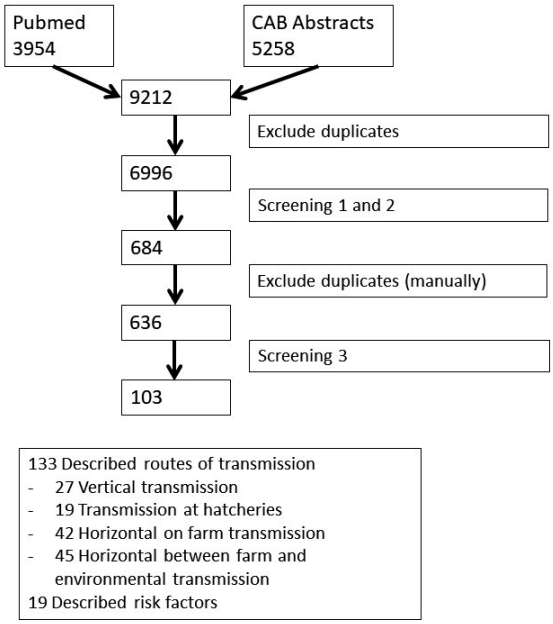

Figure 2 Schematic view of included articles (*n*) per selection step, and the described routes of transmission (*n*=133) and risk factors (*n*=19) in the final included articles.

Transmission routes included in this review are categorised based on the observations and suggestions described by the authors. Some articles describe more than one possible transmission route or a single route in different types of poultry, resulting in a total of 133 descriptions of

possible routes and 19 descriptions of risk factors. Of these 133 descriptions, 27 include vertical transmission from generation to generation, 19 transmission at hatcheries, 42 horizontal on farm transmission, and 45 horizontal between farm and environmental transmission. The vast majority of descriptions (122/133) originate from observational studies, 6 from experimental studies, and the remaining 5 from reviews. Most described or suggested routes are based on qualitative interpretation of data (106/133), whereas only 27/133 of the described routes are based on quantitative data. Studies from different regions suggest different transmission routes. The vertical transmission route and transmission at hatcheries is mostly suggested by descriptions including data from Europe (35/46), whereas only half (23/45) of the descriptions suggesting between farm and environmental transmission include data from Europe. In this review the region of data collection is reported. The routes are discussed in the following paragraph and summarised in Table 1 and Figure 3.

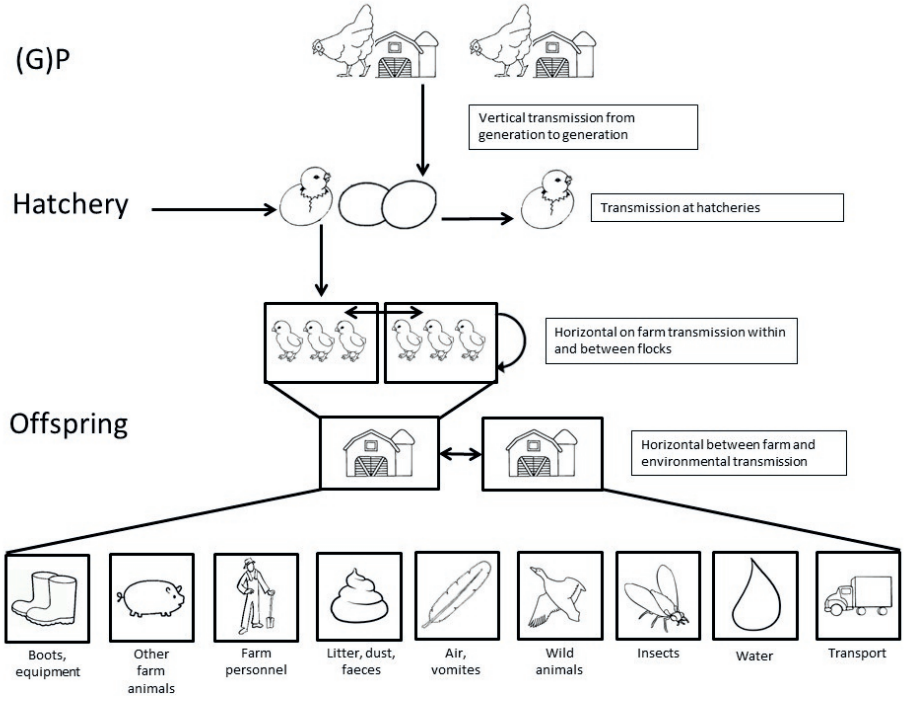

Figure 3 Schematic view of possible transmission routes of ESBL/pAmpC producing bacteria in the broiler production pyramid, as described in paragraph "Routes of transmission", including Great Grandparent, Grandparent and Parent Stock ((G)P), hatcheries and their offspring. At each level farms, flocks or individual birds can be part of the transmission route, as well as the environment.

Table 1 Included articles in the database, describing vertical transmission (*n*=27), transmission at hatcheries (*n*=19), horizontal on farm transmission (*n*=42), horizontal between farm and environmental transmission (*n*=45), or risk factors (*n*=19) categorised per route on region of origin of data, type of study, type of results, typing of isolates.

Route	Region of data collection	*n*	Type study [a]	*n*	Type data	*n*	Typing isolates [b]	*n*	Articles [c]	Quantitative Results [d]
Vertical transmission from generation to generation	EU	21	O	23	qual	20	G	20	Agersø et al., 2014	
	Asia	1					P	0	Borjesson et al., 2013a,b	
	Africa	1							Bortolaia et al., 2010	
	Oceania	2							Carmo et al., 2014	
	>1 continent	2							Cohen Stuart et al., 2012	
									Dierikx et al., 2013a	
									Dolejska et al., 2011	
									Mezhoud et al., 2016	
									Mo et al., 2014	
									Myrenas et al., 2018	
									Nilsson et al., 2014	
									Obeng et al., 2014 (2)	
									Paivarinta et al., 2016	
									Projahn et al., 2017	
									Projahn et al., 2018	
									Yossapol et al., 2017	
									Zurfluh et al., 2014a,b	
					quant	3	G	2	Alvarez Fernandez et al., 2012	Higher frequencies of multi-resistant *E. coli* on eggshells from conventional barns (95%) than from organic (30%) or domestic (20%) farms ($p<0.05$)
							P	1		
									Gay et al., 2018	OR 0 (0.00-0.91) ESBL-*Enterobacteriaceae* occurrence if chicks are produced at the farm
									Mo et al., 2016	OR 6.3 (1.6-25) ESC-resistance if >2 supplying parent flocks
			E	2	qual	2	P	2	Jimenez-Belenguer et al., 2016	
									Roth et al., 2017	
			R	2	qual	2			Hille et al., 2014	
									Olsen et al., 2014	
Transmission at hatcheries	EU	14	O	19	qual	10	G	9	Bortolaia et al., 2010	
	N-America	1					P	1	Baron et al., 2018	
	L-America	1							Dierikx et al., 2013a	
	Asia	2							Osman et al., 2018	
	Africa	1							Ozaki et al., 2017	
									Projahn et al., 2017	
									Projahn et al., 2018	
									Schwaiger et al., 2013	
									Shahada et al., 2013	
									Weill et al., 2004	

Table 1 continued.

Route	Region of data collection	*n*	Type study [a]	*n*	Type data	*n*	Typing isolates [b]	*n*	Articles [c]	Quantitative Results [d]
					quant	9	G P	6 3	Baron et al., 2014 (4)	Layers and broilers: presence of bla_{CMY-2} differs between hatchery of origin (p<0.05); Layers and broilers: *in ovo* treatment with ceftiofur higher prevalence of ESC-resistance (35.1 vs 11.2 for broilers, 46.4 vs 21.9 for layers)
									Boulianne et al., 2016	Higher prevalence ESC- resistance in broiler flocks if treatment with ceftiofur in hatchery (35%) vs no ceftiofur treatment (25%) (p=0.05)
									Braykov et al., 2016	Decreasing prevalence of ESC-resistant *E. coli* with age (p<0.05)
									Chauvin et al., 2013 (2)	OR 0.94 (0.91-0.97) non-susceptible isolates with increasing age (weekly) Similar proportions of ESC-resistant isolates in pullet and layer flocks originating from the same hatcheries (p=0.002)
									Persoons et al., 2011	Occurrence of ESC-resistance differs between hatchery of origin (p<0.01)
Horizontal on farm transmission	EU N-America L-America Asia Africa >1 continent	32 1 1 5 2 1	O	38	qual	28	G P	20 8	Agersø et al., 2014, Blaak et al., 2014 (2) Borjesson et al., 2013a Daehre et al., 2018 Dierikx et al., 2013a Hering et al., 2016 Ho et al., 2015 Huijbers et al., 2016 Laube et al., 2013 (3) Lu et al., 2010 Mattiello et al., 2015 Mezhoud et al., 2015 Nguyen et al., 2016 Oguttu et al., 2008 Persoons et al., 2010 (3) Projahn et al., 2018 Reich et al., 2013 Schwaiger et al., 2013 Smith et al., 2017 Sola-Gines et al., 2015 Stokes et al., 2012 Zurfluh et al., 2014a,b	
					quant	10	G P	8 2	Boulianne et al., 2016	Higher prevalence of ESC-resistance if growing turkey on previous used chicken litter (p=0.02) (7 vs 0% resistant isolates per flock)

Table 1 continued.

Route	Region of data collection	*n*	Type study [a]	*n*	Type data	*n*	Typing isolates [b]	*n*	Articles [c]	Quantitative Results [d]
									Huijbers et al., 2016	R_0 of ESBL/pAmpC in organic broilers 1.70 (0.55-5.25)
									Jones et al., 2013 (5)	OR 0.44 (0.17-1.14) ESC-resistant *E. coli* in fattening turkeys if flocks are compartmentalised OR 73.05 (5.93 – 900.12) ESC-resistant *E. coli* in breeding turkeys if keeping birds in one house OR 0.15 (0.03-0.78) ESC-resistant *E. coli* in breeding turkeys if other domestic animals are absent on farm OR 2.8 (1.36-5.76) ESC-resistant *E. coli* in fattening turkey if staff working with other livestock OR 0.47 (0.22 – 1.02) ESC-resistant *E. coli* in fattening turkey if staff wear gloves
									Mo et al., 2016 (2)	OR 12.7 (4.8-33.5) ESC-resistant *E. coli* in broiler flock if in previous flock ESC-resistance was present OR 0.1 (0.03-0.6) ESC-resistant *E. coli* in broiler flock if disinfecting floor in between production rounds
									Nguyen et al., 2015	OR 4.82 (1.27-18.27) presence ESBL-*E. coli* on chicken farm if fish ponds are present
			E	3	qual	2	G	2	Hiroi et al., 2012 Mo et al., 2017	
					quant	1	G	1	Ceccarelli et al., 2017	Transmission rate (β) of ESBL-*E. coli* in SPF broilers 1.33 (0.600-2.51) per day
			R	1	qual	1			Zurek and Ghosh, 2014	
Horizontal between farm and environmental transmission	EU N-America L-America Asia Africa >1 continent	23 4 4 10 1 3	O	42	qual	38	G P	30 8	Amadi et al., 2015 (2) Antilles et al., 2015 Aw et al., 2015 Blaak et al., 2014 (2) Blaak et al., 2015 (4) Bonnedahl et al., 2015 Borges et al., 2017 Cohen Stuart et al., 2012 Colomer-Lluch et al., 2011 Daehre et al., 2018 Friese et al., 2013 Gao et al., 2014 Hasan et al., 2012 Hasan et al., 2016 Hassan, 2015 Horton et al., 2011	

Table 1 continued.

Route	Region of data collection	*n*	Type study [a]	*n*	Type data	*n*	Typing isolates [b]	*n*	Articles [c]	Quantitative Results [d]
									Jiang et al., 2011 Kim et al., 2005 Laube et al., 2014 (2) Ma et al., 2012 Mathys et al., 2017 Nhung et al., 2015 Oh et al., 2015 Oluduro, 2012 Pohjola et al., 2016 Projahn et al., 2018 Raza et al., 2017 Schaufler et al., 2016 Siemon et al., 2007 Stedt et al., 2015 Vergara et al., 2017 Zurfluh et al., 2014a	
					quant	4	G	4	Chen et al., 2016	Higher proportion of ESBL-*E. coli* in rivers in regions where more chickens are raised (33.8 vs 21.1 %) (p=0.013)
									Jones et al., 2013	OR 2.6 (1.16-5.83) ESC-resistant *E. coli* on fattening turkey farms if neighbour farms with pigs
									Mo et al., 2016	OR 9.3 (1.6-55.1) ESC-resistant *E. coli* if transport personnel enter the room where broilers are raised
									Parker et al., 2016	Wild birds form urban origin associated with the presence of resistant *E. coli* (p<0.01)
			E	1	qual	1	G	1	Duijkeren et al., 2015	
			R	2	qual	2			Ljubojevic et al., 2016	
									Wang et al., 2017	
Risk factors	EU N-America L-America Asia Africa	5 1 2 10 1	O	17	qual	4	G	4	Fitch et al., 2016 Li et al., 2014 Samanta et al., 2015 Xu et al., 2014	
					quant	13	G P	7 6	Braykov et al., 2016	Higher percentage of antimicrobial resistant bacteria in production versus household birds (p<0.01)
									Brower et al., 2017	OR 9.55 (6.14 – 14.85) ESBL-*Enterobacteriaceae* presence in broiler versus layer farms. Prevalence higher in broiler (87%) versus layer farms (42%)

Table 1 continued.

Route	Region of data collection	*n*	Type study [a]	*n*	Type data	*n*	Typing isolates [b]	*n*	Articles [c]	Quantitative Results [d]
									Bui et al., 2018	Prevalence of CTX-M-*E. coli* lower in backyard chickens (13.9%) compared to chickens on large-scale farms (71.1%) (p<0.05)
									Gay et al., 2018	OR 12.72 (1.25–671.77) ESBL-*Enterobacteriaceae* occurrence in broilers in recently built premises (>1999)
									Miranda et al., 2008	Rates multi-resistant *Enterobacteriaceae* lower in organic chicken meat (41.7%) compared to conventional chicken meat (63.3%) (p=0.0197)
									Nguyen et al., 2015	OR 13.02 (1.89-89.61) presence of ESBL-*E. coli* if purchase of day old chicks from other sources than industrial hatchery
									Persoons et al., 2011	Risk of presence of ceftiofur resistant *E. coli*: OR 3.47 (1.05-11.50) if no acidification of the drinking water OR 8.25 (1.39-48.80) if >3 feed changes per cycle OR between 1.02 and 655.89 for different hatcheries of origin OR 9.14 (2.30-36.41) if breed is Ross OR between 5.08-8.04 if litter material other than wood curls OR 5.18 (1.55-17.29) if clean hygienic condition of medicinal treatment reservoir
									Qiao et al., 2017 (2)	Prevalence of ESBL-*Salmonella* in retail chicken varies between Chinese provinces (1.6 – 50%) (p<0.05) and higher percentage of ESBL-*Salmonella* isolates found in autumn (20%) compared to spring (7.9%) (p<0.05)
									Randall et al., 2017	Prevalence of ESBL-*E. coli* in retail chicken meat varies between UK regions (40.6-53.1 vs 75.0-80.6%) (p=0.001)
									Sapkota et al., 2014	Lower percentage of ESC-resistant *Salmonella* in farms that changed from conventional to organic practice compared to conventional poultry houses (p=0.043)
									Wu et al., 2013 (2)	Prevalence of ESBL-*Salmonella* in retail chicken varies between Chinese regions (0-24.64%) (p<0.05) and higher percentage of ESBL-*Salmonella* isolates found in autumn (24.64%) compared to spring (7.44%) and winter (6.12%) (p<0.05)
			E	2	quant	2	G	2	Ceccarelli et al., 2017	Lower median ESBL-*E. coli* excretion (CFU/g faeces) (1.17-2.22 vs 5.68 CFU) (p<0.001) and lower transmission rate (β) (0.669-0.331 vs ∞) (p<0.001) if animals treated with competitive exclusion product prior to ESBL challenge
									Nuotio et al., 2013	Lower cecal ESBL-*E. coli* colonisation (CFU/g cecal content) if broilers are treated with competitive exclusion product (p<0.001)

[a] O=observational, E=experimental, R=review; [b] G=genotypic, P=phenotypic. For reviews (R) typing of isolates is not included. ; [c] *n*, if >1 transmission route reported. ; [d] OR = Odds Ratio (95% CI)

Routes of transmission

Vertical transmission from generation to generation

In a longitudinal study in Norway the odds for ESC-resistance in broilers increased 6-fold (OR 6.3; 95% CI 1.6-25.0) when more than two parent flocks were supplying the broiler flock. This could be explained by the increased probability of at least one of the parent flocks being positive. However, no direct association between the status of the supplying parent flock and the broiler flock was found (Mo et al., 2016). The probability of detecting ESBL-*Enterobacteriaceae* was decreased when chicks were produced at the farm (OR 0; 95% CI 0.00-0.91), suggesting reduced risk of vertical transmission without introduction of new chicks in the farm (Gay et al., 2018).

ESBL/pAmpC-*E. coli* isolates have been observed in different levels of the broiler production pyramid (Dierikx et al., 2013a; Nilsson et al., 2014; Zurfluh et al., 2014a; Zurfluh et al., 2014b; Agerso et al., 2014; Projahn et al., 2018). The finding of genetically similar IncI1 plasmids associated with ESBL-*E. coli* (Zurfluh et al., 2014a; Zurfluh et al., 2014b), or highly related *E. coli* isolates carrying pAmpC gene bla_{CMY-2} (Nilsson et al., 2014) in different levels of the broiler production chain indicates vertical transmission and suggests a common source. Genetically similar or closely related resistant *E. coli* found in broilers and their broiler parents support the likelihood of vertical transmission (Bortolaia et al., 2010; Olsen et al., 2014; Projahn et al., 2018). However, introduction via an earlier event could also have occurred (Projahn et al., 2018), and many other factors, such as the hatchery, might be of influence (Hille et al., 2014).

The possibility of vertical transmission via eggs is shown by the detection of ESC-resistant *Enterobacteriaceae* on crushed decontaminated eggshells and eggshell surface of 7/186 broiler hatching eggs (Mezhoud et al., 2016). Moreover, genetically highly related *E. coli* isolates with ESBL gene $bla_{CTX-M-1}$ were found in a parent flock and on their outer egg shells before decontamination of the eggs (Projahn et al., 2017). Furthermore, in a study on chicken table eggs, multi-resistant *E. coli* was found on eggshells from conventional barns as well as from organic and domestic flocks (Alvarez-Fernandez et al., 2012)

Several observational studies suggest vertical transmission as explanation of the finding of ESC-resistance in birds not treated with antibiotics. The presence of ESBL/pAmpC producing bacteria in organic broiler meat originating from farms without antibiotic usage was explained by the possibility of vertical transmission resulting in introduction of ESBL carrying one-day old broilers into the organic farms (Cohen Stuart et al., 2012). Moreover, in countries like Denmark, Norway, Sweden and Finland, where cephalosporins have never been used in poultry, ESC-resistant *E. coli* were found in different levels of the production pyramid. Use of cephalosporins at hatcheries in the supplying countries was suggested to be the cause of the presence of ESC-resistant *E. coli* in (grand)parent stock, which spread vertically to their offspring via the import of breeding animals and hatching eggs (Borjesson et al., 2013a; Borjesson et al., 2013b; Mo et al., 2014; Carmo et al., 2014; Paivarinta et al., 2016; Myrenas et al., 2018). The finding of *E. coli*, resistant to several

antimicrobials, in one-day old broiler chicks (Jimenez-Belenguer et al., 2016; Roth et al., 2017; Yossapol et al., 2017) and ampicillin resistant *E. coli* in young broilers (Obeng et al., 2014) suggest vertical transmission from breeder birds to offspring. Moreover, ampicillin resistant *E. coli* found in young pullets (Obeng et al., 2014) and clonally related ESBL-*E. coli* isolates in different turkey farms supplied by the same producer, support that vertical transmission between generations can occur in poultry (Dolejska et al., 2011).

Transmission at hatcheries

The occurrence of ESC-resistant *E. coli* in broilers and layers is associated with the supplying hatchery (Persoons et al., 2011; Baron et al., 2014). Moreover, similar proportions of ESC-resistant isolates in pullet and layer flocks originating from the same hatcheries (p=0.002) indicate a relationship between ESC-resistance and the supplying hatchery (Chauvin et al., 2013). The prevalence of ESC-resistant *E. coli* is higher at young ages in broilers and layer hens (p<0.05) (Braykov et al., 2016) and in pullets (OR 0.94; 95% CI 0.91-0.97) (Chauvin et al., 2013), which indicates that colonisation occurs mainly at a young age and suggests that the hatchery has a role in the spread of ESC-resistant *E. coli*.

The relationship between hatchery and ESC resistance could be the result of a selection due to antimicrobial treatment at the hatchery. Treatment in the hatchery with ceftiofur *in ovo* resulted in a higher prevalence of ESC-resistant isolates in broiler and layer flocks compared to non-treated flocks at the age of 0-7 days (35.1% versus 11.2% for broilers and 46.4% versus 21.9% for layers) (Baron et al., 2014). Treatment with ceftiofur in the hatchery led to a higher prevalence of ESC-resistance in Canadian broiler chicken flocks (p=0.05) (Boulianne et al., 2016). However, antimicrobial treatment in the hatchery is not common practice in all countries.

The presence of *E. coli* with AmpC gene bla_{CMY-2} in the hatchery units and in one-day old birds (Dierikx et al., 2013a) and genetically related isolates in the hatchery environment and the fattening flock (Projahn et al., 2018) suggests that colonisation of young broilers can occur at the hatchery. Investigation of ESBL/pAmpC bacteria in a German hatchery showed presence of ESBL/pAmpC-*Enterobacteriaceae* on the surface of eggs from ESBL/pAmpC positive broiler parent flocks before disinfection (5/280) and even after disinfection (1/280). At hatch ESBL/pAmpC-*Enterobacteriaceae* were found in dust, crushed egg shell and environmental samples. Although the proportion of positive samples was low and all hatchlings were negative for ESBL/pAmpC producing bacteria, the existence of resistant *Enterobacteriaceae* in the hatching environment being phylogenetically related with the ESBL/pAmpC-*Enterobacteriaceae* present in the parent flock indicates transmission via the hatchery (Projahn et al., 2017). Broiler flocks from different rounds and different farms but from the same hatchery showed genetically similar ampicillin- or ESC-resistant *E. coli* types, indicating the hatchery as common source (Bortolaia et al., 2010; Shahada et al., 2013; Schwaiger et al., 2013; Ozaki et al., 2017; Baron et al., 2018). Possible transmission from hatchery to farm was also found in a French study, where one single hatchery

was identified as the supplier for six chicken farms on which *Salmonella enterica* with ESBL gene $bla_{CTX\text{-}M\text{-}9}$ was found (Weill et al., 2004).

In another study different resistance genes were detected in hatchlings versus their hatchery environment, indicating another source (Osman et al., 2018). Moreover, the high similarity of plasmids in broiler flocks and in the supplying hatchery, but also to plasmids earlier reported in other regions and animal species makes it difficult to determine the origin of the contamination (Baron et al., 2018).

Horizontal on farm transmission

Transmission within flocks

The basic reproduction ratio (R_0) of ESBL/pAmpC-*E. coli* in an organic broiler flock, thus without the use of antibiotics, was 1.70 (95% CI 0.55-5.25) (Huijbers et al., 2016), indicating that ESBL/pAmpC-*E. coli* are able to persist in the broiler flock without a selective advantage. The probability of being ESBL/pAmpC-*E. coli* positive depends on both the presence of ESBL/pAmpC-*E. coli* positive birds and ESBL/pAmpC-*E. coli* present in the environment of the broiler flock (Huijbers et al., 2016). Also, experimental transmission studies show that ESBL/pAmpC-*E. coli* are able to spread between young specific pathogen free (SPF) birds without selective advantage (β of 1.33 per day (95% CI 0.600-2.51) (Ceccarelli et al., 2017). Housing management may influence the presence of resistant bacteria in a flock: a study on fattening turkeys showed that compartmentalization of flocks decreased the presence of ESC-resistant *E. coli* in fattening turkeys, whereas keeping breeding turkeys in one house, from day-old to depopulation, increased the probability that ESC-resistant *E. coli* was present (OR 73.05; 95% CI 5.93–900.12), although only a few turkey flocks were included in the analysis (Jones et al., 2013).

The finding of similar plasmid-gene combinations within broiler and broiler breeder flocks, although in different *E. coli* types, suggests that within a flock horizontal spread of plasmids encoding ESBL/pAmpC-genes occurs (Zurfluh et al., 2014b). Also reproduction could play a role in spread of ESBL/pAmpC producing bacteria, since ESBL-*E. coli* were found in the reproduction tract of broiler breeder roosters, possibly as a result of faecal contamination (Mezhoud et al., 2015). Genetically related isolates found in chicken samples from the same farm, pen or market can be the result of horizontal transmission of ESC-resistant bacteria between animals (Lu et al., 2010; Ho et al., 2015).

Transmission between subsequent flocks in the same house

In a Norwegian study the presence of ESC-resistant *E. coli* in broiler flocks without antimicrobial usage was associated with the presence of ESC-resistance in the previous flock in the same house (OR 12.7; 95% CI 4.8-33.5) and disinfecting the floor between two production rounds was associated with a decreased presence of ESC-resistant *E. coli* in the subsequent flock (OR 0.1; 95% CI 0.03-0.6) (Mo et al., 2016). Transmission between flocks might occur via litter, dust, or faeces

(Laube et al., 2013). In turkeys, prevalence of ESC-resistant *E. coli* isolates was higher when turkeys were transferred to litter previously used by chickens (p=0.02) (Boulianne et al., 2016). A lab study showed that transfer of plasmids carrying bla_{CMY-2} occurs between bacteria, at temperatures down to 25°C, which might enable the plasmids to spread in the broiler production environment (Mo et al., 2017).

The presence of phylogenetically similar ESBL/pAmpC-*E. coli* in broilers at the end of the previous round, in the broiler house at day 1 before arrival and in the broilers and their environment during the subsequent round (Huijbers et al., 2016; Daehre et al., 2018) and the persistence of resistant bacteria in consecutive rounds (Persoons et al., 2010; Reich et al., 2013) indicate that these bacteria can transmit between subsequent flocks.

Insufficient cleaning might lead to persistence of ESBL/pAmpC producing bacteria in the poultry house environment and play a role in the persistence of ESBL/pAmpC-genes on a farm (Laube et al., 2013; Mattiello et al., 2015). In an experimental study antibiotic treatment of flocks raised in broiler houses that tested ESBL positive in previous rounds did result in ESBL-*E. coli* colonisation, whereas no ESBL-*E. coli* was found in a flock treated with antibiotics and raised in a clean laboratory animal room (Hiroi et al., 2012), indicating that raising ESBL-*E. coli* free broilers in hygienic circumstances can prevent their colonisation with ESBL/pAmpC producing bacteria. However, even after cleaning and disinfection, carry-over at farm level from flock to flock can occur and might result in recirculation at farm level (Oguttu et al., 2008; Schwaiger et al., 2013; Daehre et al., 2018), also depending on the fitness of the strain (Agerso et al., 2014; Huijbers et al., 2016). Colonisation of young birds might be followed by a rapid increase of ESBL/pAmpC prevalence in a broiler flock (Dierikx et al., 2013a). Horizontal transmission between subsequent flocks might contribute, next to vertical transmission, to the high prevalence of ESBL/pAmpC producing bacteria in countries, where no antibiotics have been used (Borjesson et al., 2013a).

Transmission between houses or parallel flocks on the same farm

Transmission between flocks kept in parallel might occur via dust and faeces on equipment, clothes or shoes (Laube et al., 2013) and presence of farm personnel and other farm animals was suggested as possible explanation for transmission (Persoons et al., 2010). Transmission via farm personnel can be reduced by hygiene measures; a study including turkeys showed that staff wearing gloves reduced the risk of occurrence of ESC-resistant *E. coli* in fattening turkeys (OR 0.47; 95% CI 0.22–1.02) (Jones et al., 2013). Farm personnel working with other livestock was positively associated with the presence of ESC-resistant *E. coli* at fattening turkey farms (OR 2.8; 95% CI 1.36-5.76) (Jones et al., 2013). The absence of other domestic farm animals on a turkey farm was associated with decreasing presence of ESC-resistant *E. coli* in breeding turkeys (OR 0.15; 95% CI 0.03-0.78) (Jones et al., 2013), whereas the presence of fish ponds was associated with increasing presence of ESBL-*E. coli* on a chicken farm (OR 4.82; 95% CI 1.27-18.27) (Nguyen et al., 2015). The finding of ESBL/AmpC-*Salmonella* in fish and poultry meat suggests that interaction between

animal species for example via faecal contact may lead to spread of bacteria between different hosts (Nguyen et al., 2016).

Genetically related ESBL-*E. coli* strains from different barns at the same farm suggest transmission between flocks at the same farm (Projahn et al., 2018). Furthermore, investigation at German broiler farms showed that flocks kept in parallel are not independent, as the number of ESC-resistant samples was comparable between flocks on the same farm (Hering et al., 2016). However, these findings can also be explained by a similar source of the birds such as the hatchery or breeding flock.

Transmission via other animal species can occur via a shared environment. The finding of pCT-like plasmids in *E. coli* with ESBL gene $bla_{CTX\text{-}M\text{-}14}$ in cattle, turkeys and humans (Stokes et al., 2012) and *E. coli* with ESBL gene $bla_{CTX\text{-}M\text{-}1}$ on ST3-Inc1 plasmids in chicken, cattle, pig and river water samples (Zurfluh et al., 2014a) suggests that plasmids can be transmitted between food producing animals and the environment.

Several studies have shown the presence of resistant bacteria in insects (Zurek and Ghosh, 2014). ESBL/AmpC-*E. coli* isolates were found in wild birds and flies (Smith et al., 2017). Similar ESBL-*E. coli* genotypes in flies and isolates selected from manure and rinse water at layer and broiler farms (Blaak et al., 2014), and flies captured at a broiler farm at different sampling times carrying the same type of ESBL-*E. coli* (Sola-Gines et al., 2015), show that it is likely that flies can act as a vector between animals facilitating the dissemination of ESBL/pAmpC-*E. coli*.

Horizontal between farm and environmental transmission

Several vehicles might facilitate between farm transmission. Transport personnel entering the farm was positively associated with the presence of ESC-resistant *E. coli* in broiler flocks (OR 9.3; 95% CI 1.6–55.1), suggesting their role in cross contamination between farms (Mo et al., 2016). Another source of between farm transmission might be environmental contamination through animals on neighbouring farms. On fattening turkey farms, the presence of ESC-resistant *E. coli* was positively associated with having neighbouring farms with pigs (OR 2.6; 95% CI 1.16-5.83) (Jones et al., 2013). Cattle grazing on a neighbouring pasture were suggested as possible source of ESC-resistant *Salmonella* found on a free-range poultry farm (Siemon et al., 2007). Also backyard poultry with outdoor access were considered a potential reservoir of pAmpC-*E. coli* (Pohjola et al., 2016).

Farms positive for ESC-resistant bacteria can transmit these bacteria to their environment. Similar profiles of *E. coli* isolates found in samples from barn air and ambient air (50 meter downwind), slurry and ground surfaces outside the barn were found (Laube et al., 2014). Moreover, closely related ESBL/AmpC-*E. coli* strains found in faeces and pasture and soil surrounding the ventilation exhausting air (Daehre et al., 2018), and identical ESBL-*E. coli* isolates found in poultry faeces and environmental samples from the same farm indicate potential transmission from broiler and layer farms to the environment outside the barn (Blaak et al., 2015).

Transmission between farms via transport crates and trucks is suggested by the finding of ESC-resistant *E. coli* on trays used for transport of both conventional and free-range eggs (Aw et al., 2015). ESBL-*E. coli* isolates found on a transportation truck and the litter and faeces of the connected fattening flock clustered together in MLST analysis, indicating that transmission can occur via the transportation system (Projahn et al., 2018).

Fomites, such as feathers, may spread resistant bacteria further from farms to their surroundings (Kim et al., 2005). Furthermore, poultry litter used as fertiliser, containing resistant *E. coli*, can contaminate soil, surface and ground water (Ljubojevic et al., 2016). The finding of ESBL/pAmpC-*E. coli* in broiler faeces, slurry and in the fields fertilised within 6 weeks before the sampling indicates that transmission through poultry litter is a potential route of spread and may contaminate animal farms in the neighbourhood (Friese et al., 2013). A wide variation in levels of ESBL-*E. coli* excretion is found between cattle, pigs and chickens. Therefore, depending on the housing management, for example if the animals have access to the outside, different animal species may vary in their contribution to environmental contamination (Horton et al., 2011).

The finding of similar ESBL-*E. coli* genotypes in rinse water, waste water, surface water and in the manure at layer and broiler farms indicates that transmission of ESBL-*E. coli* via contaminated water may occur (Blaak et al., 2014; Blaak et al., 2015). Important vehicles for this spread include drinking water (Jiang et al., 2011), water troughs (Hassan, 2015) and duck swimming pools contaminated with faeces (Ma et al., 2012). A natural watercourse, such as a river, may also play a role in environmental transmission. Presence of ESBL-*E. coli* in river water was found to be higher in regions with large numbers of chickens being raised compared to regions with lower numbers of chickens being raised (p=0.013) (Chen et al., 2016). Similar isolates from the river and faecal samples indicate that ESBL-*E. coli* can spread, probably via waste water, to the environment (Gao et al., 2014; Zurfluh et al., 2014a) and possibly to other farms.

Resistant bacteria can also be transmitted via wild birds that share the same environment (Hasan et al., 2012; Amadi et al., 2015; Hasan et al., 2016). ESBL-*E. coli* isolates from wild birds, humans, companion animals and the environment showed high similarity, suggesting an exchange of resistant genes between hosts sharing the same environment (Stedt et al., 2015; Schaufler et al., 2016). Although resistant *E. coli* were found in a higher number of wild birds from urban compared to rural origin (p<0.01) (Parker et al., 2016), migration of wild birds utilising both urban and rural areas might result in spread of ESC-resistance. Moreover, wild birds can serve as a reservoir of ESC-resistant bacteria (Borges et al., 2017) and potentially transmit them over long distances via migration (Antilles et al., 2015; Oh et al., 2015; Mathys et al., 2017; Raza et al., 2017; Vergara et al., 2017). However, others have concluded that local dissemination in a shared environment is more important for the presence of resistance genes in wild birds than migration of the birds (Bonnedahl et al., 2015). Besides transmission via wild birds, other wild animals might play a role (Wang et al., 2017), for example rats (Nhung et al., 2015) and bats (Oluduro, 2012) are indicated as sources of resistant *E. coli.* An experiment with 8-week old SPF mice failed, however,

to demonstrate persistence of avian ESBL-*E. coli* strains in mice (van Duijkeren et al., 2015).

Bacteriophages present in the animal environment have been found to carry ESBL genes suggesting, that they may help maintenance and horizontal transfer of antimicrobial resistant genes in the animal environment (Colomer-Lluch et al., 2011).

Transmission of ESC-resistant bacteria from the environment to farms was also suggested as explanation for the presence of resistant bacteria in both free-range and organic chickens that had not received antibiotics (Cohen Stuart et al., 2012; Amadi et al., 2015).

Miscellaneous risk factors

This review focusses primarily on transmission routes in the broiler production pyramid. However, also risk factors and interventions for the presence of ESC-resistant bacteria are mentioned in the selected articles, and are reported in this section.

Organic and backyard farms tended to have a lower occurrence of resistant bacteria compared to conventional farms (Miranda et al., 2008; Samanta et al., 2015; Bui et al., 2017). Farms that changed from conventional to organic practice showed a decrease in ESC-resistant isolates (Sapkota et al., 2014). Commercially kept chickens showed higher percentages of resistance than non-commercially kept birds in Ecuador (p<0.01) (Braykov et al., 2016), and in India the prevalence of ESBL-*Enterobacteriaceae* was higher in broiler farms compared to layer farms (OR 9.55; 95% CI 6.14 – 14.85) (Brower et al., 2017). These differences between production systems were most likely caused by both the level of antibiotic use and by bird density. At farm level in Vietnam, the purchase of one-day old chickens from sources other than industrial hatcheries was associated with the presence of ESBL-*E. coli* (OR 13.02; 95% CI 1.89-89.61) (Nhung et al., 2015). Presence of ESBL-*Enterobacteriaceae* at broiler farms in Reunion was associated with recently built premises, possibly confounded by antibiotic use which might be higher in modern farms (Gay et al., 2018).

Prevalence of ESBL-*E. coli* and ESBL-*Salmonella* in retail chickens varied between regions (Wu et al., 2013; Qiao et al., 2017; Randall et al., 2017) possibly depending on different farm practices (Wu et al., 2013; Xu et al., 2014). Chicken carcasses from supermarkets from different regions in China showed the lowest prevalence in the region with a low human population density and mainly free ranging chickens. The highest prevalence was found in Beijing, where chickens were raised in high density and antimicrobials were used (Xu et al., 2014). Trade of poultry and poultry products might have contributed to the spread of resistant bacteria in Brazil, where genetically related ESBL producing *Salmonella*, carrying variants of bla_{CTX-M}, were found in different regions (Fitch et al., 2016).

Moreover, season might also influence prevalence, with higher percentages of ESBL producing bacteria in retail chicken found in autumn, compared to spring (p<0.05) (Wu et al., 2013; Qiao et al., 2017) and winter (p<0.05) (Wu et al., 2013). Possibly there was a relationship between frequently occurring epidemic poultry diseases in autumn and consequently more antibiotic usage (Wu et al., 2013).

A Belgian study showed that besides antimicrobial use, no acidification of the drinking water, more than three feed changes per cycle, hatchery of origin, breed and litter material are associated with the presence of ESC-resistant *E. coli*. Strikingly, a clean hygienic condition of the reservoir for medicinal treatment increased the risk of presence of ESC-resistant *E. coli* (Persoons et al., 2011). On the other hand, at a farm with no clearance of faeces higher numbers of ESBL-*E. coli* isolates were found compared to farms where faeces was cleared (Li et al., 2014), showing that poor farm hygiene may facilitate the occurrence and transfer of resistant bacteria. Possibly, risk factors as hygiene, drinking water and litter type might influence the microbiome and consequently the ability of ESC-resistant bacteria to colonise the gut (Persoons et al., 2011). Supplying a competitive exclusion product to broilers can reduce colonisation of ESBL-*E. coli* (p<0.001) (Nuotio et al., 2013), and decrease excretion (p<0.001) and transmission (p<0.001) of ESBL-*E. coli* during the first two weeks of life (Ceccarelli et al., 2017).

Discussion and conclusions

Four possible transmission routes of ESC-resistant bacteria in the broiler production pyramid are discussed in this review: 1) vertical transmission from generation to generation (e.g. parent to offspring), 2) transmission at hatcheries, 3) horizontal on farm transmission and 4) horizontal between farm and environmental transmission. Evidence of the existence and information on the magnitude of transmission along these transmission routes is, however, scarce and mainly based on observational studies. Only 27 of 133 described routes have quantified probabilities of transmission along one of the four routes identified. Due to a lack of quantitative data, a meta-analysis could not be performed, neither was it possible to quantify the level of evidence of the four transmission routes. Moreover, the general lack of quantitative results did not allow for a proper assessment of bias. Only a few experimental studies investigated the described routes, therefore hardly any causal evidence is found.

In order to find possible interventions to control ESBL/pAmpC prevalence and spread in the broiler production pyramid, it is important to determine the existence of transmission routes, and to quantify the magnitude of spread along the different transmission routes.

Although no direct relationship between the status of the parent flock and their offspring is reported, the presence of genetically related resistant isolates in different levels of the production chain (Bortolaia et al., 2010; Nilsson et al., 2014; Zurfluh et al., 2014a; Zurfluh et al., 2014b; Olsen et al., 2014; Projahn et al., 2018) and the presence of ESBL/pAmpC producing bacteria on farms and in countries, where no cephalosporins have been used (Cohen Stuart et al., 2012; Borjesson et al., 2013a; Borjesson et al., 2013b; Carmo et al., 2014; Mo et al., 2014; Paivarinta et al., 2016; Myrenas et al., 2018), suggests that transmission between generations may occur. A possible route is via egg shells carrying ESBL/pAmpC producing bacteria (Mezhoud et al., 2016; Projahn

et al., 2017) where the contaminated egg shells can result in colonisation of the offspring. It is, however, difficult to untangle transmission from parent to offspring from contamination in the hatcheries. Even low numbers of contaminated eggs might result in transmission from parent stock to the next generation, and therefore the magnitude of apparent vertical transmission might be overestimated, and contamination due to transmission at the hatchery underestimated. Therefore, we need both quantification of the probability of contamination from parent to offspring, as quantification of transmission among birds, as done in earlier studies (Ceccarelli et al., 2017). No transmission was observed in a field study where eggs from a contaminated parent flock were collected and broilers were hatched aiming to quantify vertical transmission. However, the number of eggs and the ESBL/pAmpC-*E. coli* prevalence in the parent flock were low (Dame-Korevaar et al., 2017). These kinds of studies with larger numbers of eggs are, however, feasible and can be performed to quantify transmission between generations. This can also be studied in experimental studies by hatching offspring of contaminated hens.

None of the included studies quantified the transmission occurring at the hatchery, but the available literature indicates the importance of spread via the hatchery. After introduction of resistant strains originating from parent stock they may spread to the hatchery inventory and further contaminate eggs or newly hatched birds from ESBL/pAmpC-free parent stock. Treatment at the hatchery leads to selection of resistant strains, resulting in an increased probability of colonisation (Baron et al., 2014; Boulianne et al., 2016). Quantification of the role of hatcheries is difficult but can be studied by zooming into specific parts of the route. For example, experimentally determining the transmission from contaminated eggs or the hatchery environment to hatchlings is possible in research facilities.

Strict hygiene measures might reduce the risk of recirculation at farm level, although in several studies even intensive cleaning and disinfection did not result in elimination of resistant strains (Oguttu et al., 2008; Schwaiger et al., 2013; Daehre et al., 2018). This might be caused by the presence of other, unknown sources of ESBL/pAmpC producing bacteria or indirect transmission at poultry farms, enabling transmission between flocks kept in parallel. Transmission between parallel and consecutive flocks on the same farm can be studied with longitudinal observational studies on farms (Huijbers et al., 2016; Dame-Korevaar et al., 2017) as by experimental studies (Ceccarelli et al., 2017). Transmission within and between flocks could be quantified using transmission experiments under controlled circumstances (Dame-Korevaar et al., unpublished data). In these experiments also possible interventions to reduce or prevent transmission could be tested (Ceccarelli et al., 2017).

Contamination of the environment surrounding farms can lead to indirect transmission between farms, but also between houses and subsequent flocks. Several articles report the presence of ESBL/pAmpC producing bacteria in litter, air, dust (Friese et al., 2013; Laube et al., 2013; Blaak et al., 2014; Laube et al., 2014; Sola-Gines et al., 2015; Blaak et al., 2015; Daehre et al., 2018), but also in insects (Zurek and Ghosh, 2014; Smith et al., 2017) and in wildlife (Oluduro, 2012; Nhung et

al., 2015; Stedt et al., 2015; Schaufler et al., 2016; Wang et al., 2017). However, the magnitude of this transmission route has not been quantified. Epidemiological and environmental studies can indicate associations between risk factors and contamination of farms or the environment, but quantification of environmental transmission is extremely difficult, if not impossible, and requires large amounts of longitudinal data. Studies from different regions suggest different transmission routes, transmission via the environment was mainly suggested based on data originating from non-European regions. Possibly this is related to differences in structure or management in poultry production between regions.

Observational studies in which the genetic similarity between isolates is used to study associations or possible transmission routes, often include only a small number or a selection of isolates and a complete view on different resistant genes/plasmids present in the animal or environment is lacking. This limits the insight in possible transmission routes. Also conclusions based on animal products or slaughterhouse samples should be interpreted with caution. Sampling in slaughterhouses or animal products, such as meat and eggs, are likely to be influenced by cross contamination during processing and handling (Cohen Stuart et al., 2012; Mollenkopf et al., 2014). These samples determine the exposure of humans, rather than represent an insight in the contamination of the production pyramid.

This review on different routes of transmission of ESBL/pAmpC producing bacteria in the broiler production pyramid shows scarce to no causal evidence of transmission along these routes and a lack of quantitative data. Therefore the relative contribution or magnitude of transmission via these routes cannot be quantified. This is a major gap in the knowledge on the transmission of ESBL/pAmpC producing bacteria in the poultry production chain and hampers the design of optimal intervention strategies.

Recommendations

There is a need for studies aiming to determine the existence, and quantify the magnitude of transmission via the four hypothesised routes of ESBL/pAmpC producing bacteria in the broiler production pyramid. Accurate detection of ESBL/pAmpC producing bacteria and typing of genes and plasmids using standardised methods is needed, especially because of the high level of heterogeneity in genes, plasmids and strains. This will help to compare occurrences within and between farms and countries and determine the extent to which resistant bacteria are transmitted through the production chain. High-resolution typing techniques, such as whole genome sequencing (WGS), can be used to study transmission routes, however even with these high-resolution typing techniques there is a need for standardised methods to conclude on and show the importance of transmission routes. With information on the contribution of different transmission routes to the occurrence of ESBL/pAmpC producing bacteria in the production pyramid, interventions with the highest impact could be identified.

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Appendix 1

Complete search terms, including synonyms

Search term 'poultry' was defined in Pubmed as: *("Birds"[Mesh]) OR (poultry[tiab]) OR (broiler[tiab]) OR (broilers[tiab]) OR (laying hen[tiab]) OR (laying hens[tiab]) OR (farm[tiab]) OR (farms[tiab]) OR (breeder[tiab]) OR (parent stock[tiab]) OR (flock[tiab]) OR (chicken[tiab])* and in CAB Abstracts as: *exp birds/ or exp poultry/ or exp broilers/ or exp hens/ or exp farms/ or breeder.ti. or breeder.ab. or parent stock.ti. or parent stock.ab. or exp flocks/ or exp fowls/ or chicken.ti. or chicken.ab.*

Search term 'ESBL' was defined in Pubmed as: *("beta-Lactamases"[Mesh]) OR ("AmpC beta-lactamases"[Supplementary Concept]) OR (beta lactamase[tiab]) OR (beta-lactamase[tiab]) OR (beta lactamases[tiab]) OR (ampC[tiab]) OR (ampC beta lactamase[tiab]) OR (beta lactamase ampC[tiab]) OR (esbl[tiab]) OR (extended spectrum beta lactamase[tiab]) OR (resistance[ti]) OR (resistant[ti]) OR (plasmid[tiab]) OR (CMY[tiab]) OR (CTX[tiab]) OR (TEM[tiab]) OR (SHV[tiab]) OR (lactamase[tiab])* and in CAB Abstracts as: *exp beta-lactamase/ or beta lactamase.ti. or beta lactamase.ab. or ampc.ti. or ampc.ab. or esbl.ti. or esbl.ab. or exp extended spectrum beta-lactamase/ or exp drug resistance/ or exp plasmids/ or cmy.ti. or cmy.ab. or tem.ti. or tem.ab. or ctx.ti. or ctx.ab. or shv.ti. or shv.ab. or lactamase.ti. or lactamase.ab. or resistance.ti. or resistant.ti. or plasmid.ti. or plasmid.ab.*

Search term 'spread' was defined in Pubmed as: *("Disease Vectors"[Mesh]) OR (disease vectors[tiab]) OR (insect vectors[tiab]) OR (vectors[tiab]) OR (Route[tiab]) OR (Mechanism[tiab]) OR (mechanisms[tiab]) OR (Pathway[tiab]) OR ("Basic Reproduction Number"[Mesh]) OR (basic reproduction number[tiab]) OR (basic reproductive rate[tiab]) OR (basic reproductive ratio[tiab]) OR (R0[tiab]) OR (epidemic growth rate[tiab]) OR ("Infectious Disease Incubation Period"[Mesh]) OR (incubation period[tiab]) OR (generation time[tiab]) OR (transmission rate[tiab]) OR (transmission ratio[tiab]) OR (infection rate[tiab]) OR ("Disease Transmission, Infectious"[Mesh]) OR (transmission) OR (infection transmission[tiab]) OR [transmission infection[tiab]) OR (infectious disease transmission[tiab]) OR (transmission infectious disease[tiab]) OR (communicable disease[tiab]) OR (disease communicable[tiab]) OR (pathogen transmission[tiab]) OR (horizontal transmission[tiab]) OR (vertical transmission[tiab]) OR (horizontal[tiab]) OR (vertical[tiab]) OR (Spread[tiab]) OR (Introduction) OR (Dynamics[tiab]) OR (Transfer[tiab]) OR ("Disease Outbreaks"[Mesh]) OR (disease outbreak[tiab]) OR (disease outbreaks[tiab]) OR (outbreak[tiab]) OR (epidemic[tiab]) OR ("Endemic Diseases"[Mesh]) OR (endemic disease[tiab]) OR (endemic[tiab]) OR ("Incidence"[Mesh]) OR (incidence[tiab]) OR (occurrence[tiab]) or (prevalence[tiab]) or (dissemination[tiab])* and in CAB Abstracts as: *exp disease vectors/ or vector.ti. or vector.ab. or route.ti. or route.ab. or mechanism.ti. or mechanism.ab. or mechanisms.ti. or mechanisms.ab. or*

pathway.ti. or pathway.ab. or R0.ti. or R0.ab. or reproduction number.ti. or reproduction number.ab. or reproductive rate.ti. or reproductive rate.ab. or reproductive ratio.ti. or reproductive ratio.ab. or epidemic growth.ti. or epidemic growth.ab. or exp prepatent period/ or generation time.ti. or generation time.ab. or transmission.mp. or exp disease transmission/ or exp infectious diseases/ or exp disease distribution/ or vertical.ti. or vertical.ab. or horizontal.ti. or horizontal.ab. or spread.ti. or spread.ab. or introduction.mp. or dynamics.ti. or dynamics.ab. or exp transfer/ or exp outbreaks/ or endemic.ti. or endemic.ab. or exp disease incidence/ or occurrence.ti. or occurrence.ab. or prevalence.ti. or prevalence.ab. or dissemination.ti. or dissemination.ab.

CHAPTER 4

Effect of challenge dose of plasmid-mediated extended-spectrum β-lactamase and AmpC β-lactamase producing *Escherichia coli* on time-until-colonization and level of excretion in young broilers

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Abstract

Plasmid-mediated extended-spectrum β-lactamase and AmpC β-lactamase (ESBL/pAmpC) producing bacteria are present at all levels of the broiler production pyramid. Young birds can be found positive for ESBL/pAmpC-producing *Escherichia coli* shortly after arrival at farm. The aim of this study was to determine the effect of different challenge doses of ESBL/pAmpC-producing *E. coli* on time-until-colonization and the level of excretion in young broilers. One-day-old broilers (specific-pathogen free (SPF) and conventional Ross 308) were housed in isolators and challenged with 0.5 mL ESBL/pAmpC-producing *E. coli* strains of varying doses (10^1-10^5 CFU/mL). Presence and concentration (CFU/gram faeces) of ESBL/pAmpC-producing *E. coli* and total *E. coli* were determined longitudinally from cloacal swabs, and in cecal content 72 hours after challenge. Higher challenge doses resulted in shorter time-until-colonization. However, even the lowest dose (10^1 CFU/mL) resulted in colonization of the broilers which excreted >10^6 CFU/gram faeces 72 hours after inoculation. Conventional broilers were colonized later than SPF broilers, although within 72 hours after challenge all broilers were excreting ESBL/pAmpC-producing *E. coli*. A probabilistic model was used to estimate the probability of colonization by initial inoculation or transmission. The higher the dose the higher the probability of excreting ESBL/pAmpC-producing *E. coli* as a result of inoculation. In conclusion, low initial doses of ESBL/pAmpC-producing *E. coli* can result in rapid colonization of a flock. Interventions should thus be aimed to eliminate ESBL/pAmpC-producing bacteria in the environment of the hatchlings and measures focusing at reducing colonization and transmission of ESBL/pAmpC-producing *E. coli* should be applied shortly after hatching.

Introduction

Plasmid-mediated extended-spectrum β-lactamase and AmpC β-lactamase (ESBL/pAmpC) producing bacteria are resistant to extended-spectrum cephalosporins (ESC), and are present in humans, animals and the environment (Blaak et al., 2015; Dorado-Garcia et al., 2018). Studies in European countries have revealed that the prevalence of ESBL/pAmpC-producing *Escherichia coli* in broilers is high (Saliu et al., 2017; MARAN, 2019) and ESBL/pAmpC-producing *E. coli* can also be present in chicken at higher levels of the broiler production pyramid such as in (grand)parent stocks (Dierikx et al., 2013a). Several field studies have shown that young birds can be positive for ESBL/pAmpC-producing *E. coli* within the first week after arrival at farm (Dierikx et al., 2013a; Huijbers et al., 2016; Dame-Korevaar et al., 2017). Transmission of ESBL/pAmpC-producing bacteria occurs throughout the broiler production pyramid via several routes. At every level of the broiler production pyramid young chickens can become colonized as a result of vertical transmission between generations, at hatcheries, horizontal transmission at the farm, between farms and via the environment (Dame-Korevaar et al., 2019a). However, the concentrations of ESBL/pAmpC-producing bacteria these birds are exposed to are not known. Therefore, it is important to understand what level of exposure leads to colonization, and at what age birds are colonized by ESBL/pAmpC-producing bacteria. This information is needed to apply successful interventions to reduce transmission within the pyramid. Transmission of ESBL/pAmpC-producing bacteria can occur between subsequent flocks (Dame-Korevaar et al., 2019a) and therefore colonization of young chickens by ESBL/pAmpC-producing bacteria can be affected by biosecurity measures between production rounds. Raising broilers in hygienic circumstances (Hiroi et al., 2012) and cleaning and disinfecting the floor between production rounds (Mo et al., 2016) are associated with absence and reduced (Odds Ratio 0.1; 95% Confidence Interval (CI) 0.03-0.60) occurrence of ESC-resistant *E. coli*. However, even after intensive cleaning and disinfection of poultry houses, ESC-resistant *E. coli* can still be found in broiler flocks. Thus, persistence of low numbers of resistant bacteria in the farm or in the hatchery environment might lead to colonization of young broilers (Oguttu et al., 2008; Schwaiger et al., 2013; Projahn et al., 2017; Projahn et al., 2018; Daehre et al., 2018; Dierikx et al., 2018). The use of competitive exclusion products has shown to reduce colonization, excretion and transmission in broilers, but not to prevent it (Nuotio et al., 2013; Ceccarelli et al., 2017). However, in those studies broilers were challenged with a high dose of ESBL-producing *E. coli* (0.5 mL of 10^5 – 10^8 CFU/mL), whereas in the field broilers will be most likely exposed to much lower levels of resistant bacteria (Laube et al., 2013; Laube et al., 2014). To evaluate the effectivity of potential measures, the relation between the exposure dose and colonization in groups of young broilers needs to be understood. This knowledge will help to assess interventions against colonization and transmission of ESBL/pAmpC-producing bacteria at all levels of the poultry production pyramid.

The aim of this study was to determine the relationship between challenge dose of ESBL/

pAmpC-producing *E. coli* on time-until-colonization and the level of excretion. These outcomes were compared for specific-pathogen free (SPF) and conventional broilers, as well as for two different strains of ESBL/pAmpC-producing *E. coli*. To interpret the results of the time-until-colonization we applied a probabilistic model to estimate the probability of colonization after inoculation, and transmission, as a post-hoc analysis after having observed the data.

Materials and Methods

We conducted two experiments (Table S1). Experiment I consisted of three replicate studies with SPF broilers inoculated with five doses ($10^1 – 10^5$). Experiment II consisted of two replicate studies with SPF and conventional broilers inoculated with two doses ($10^1 – 10^2$).

Birds and housing conditions

Before the start of the experiment samples were taken from the parent flocks, incubators, hatchers and isolators to confirm the absence of ESBL/pAmpC-producing *E. coli*. In experiment I (May – July 2016), 80 (replicates 1 and 2) and 120 (replicate 3) 18-days incubated Cobb/Hybro/Ross crossbred eggs from a specified pathogen free (SPF) parent flock (Animal Health Service, Deventer, the Netherlands) were transported to the animal facilities (Utrecht University, Utrecht, the Netherlands). Age of the parent stock varied between replicates from 31 to 62 weeks. All eggs were individually disinfected using a tissue with 3% hydrogen peroxide, then placed in egg trays in the hatcher and hatched after 3 days. At day 0 of the experiment, hatchlings were collected, tagged with an individual number, weighed and randomly divided over different isolators, with a maximum of fifteen (*n*=15) broilers per isolator. The extra chicks were used to prevent differences in group sizes at the moment of inoculation. At day 1, ten (*n*=10) broilers (not sexed, i.e. consisting of males and females) were selected in each isolator for the remainder of the experiment and were inoculated with a specific dose of the ESBL/pAmpC-producing *E. coli* challenge strain. Broilers with signs of reduced health or development, low hatching weight and, if needed, randomly chosen extra broilers were removed from the isolator and euthanized using cervical dislocation.

In experiment II (September – October 2016), in both replicates 50 SPF eggs were transported to the animal facilities and handled according to the same procedures as in experiment I. In addition, at day 0 of the experiment, 50 just hatched conventional broilers (Ross 308) were transported to the animal facilities, individually tagged, weighed and randomly divided over the isolators. The conventional eggs were disinfected with formaldehyde and eggs were treated in the hatcher with 37% formaldehyde solution. No *in ovo* vaccination or antimicrobials were supplied. Age of the parent stock varied between replicates from 45 – 50 (SPF) and 48 – 53 (conventional) weeks.

Broilers were housed in negative pressure HEPA isolators. Water and standard mashed broiler diet without any antibiotics or coccidiostatics, radiated with 9 Gy, were available *ad libitum*. On

the floor of all isolators were paper linings covered with fine wood shavings. Isolator temperature was gradually decreased from 37°C at day of hatch until 32°C at the end of the experiment and a lighting schedule of 23 hours light per day was applied. During experiment I, 12 broilers (3 in replicate 1, 7 in replicate 2, and 2 in replicate 3) died or were euthanized before the end of the experiment. In experiment II no broilers died during the experiment.

***E. coli* challenge**

E. coli strain E39.62, which carries the AmpC gene bla_{CMY-2} on an IncK plasmid, and E38.27, which carries the ESBL gene $bla_{CTX-M-1}$ on an IncI1 plasmid, which are representative of common gene-plasmid combinations in *E. coli* isolates from broilers (MARAN, 2019; Ceccarelli et al., 2019), were isolated from healthy broilers in previous studies (Dierikx et al., 2010). Both isolates are resistant to cefotaxime and were used to challenge the broilers in experiments I and II, respectively. MacConkey agar (product no. 212123; Becton Dickinson) was used to culture the *E. coli* challenge strains. Cefotaxime (1 mg/L) used for selective plating throughout the study was obtained from Sigma-Aldrich (Poole, Dorset, UK).

Serial dilutions of the *E. coli* strains were prepared on the day of challenge from fresh culture on heart infusion agar (HIS) with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany) supplemented with cefotaxime (1 mg/L), after resuspending into saline solution. Bacterial dilutions were measured with the McFarland reader and retrospective colony counting. For experiment I, all ten broilers per isolator were challenged at day 1 by individual oral inoculation with *E. coli* strain E39.62 using a 1 mL syringe without a needle with 0.5 mL of $10^1 – 10^5$ CFU/mL (Table S1) prepared in saline solution (0.85% NaCl). For experiment II, all ten broilers per isolator were challenged at day 1 by individual oral inoculation with 0.5 mL of $10^1 – 10^2$ CFU/mL (Table S1) prepared in saline solution (0.85% NaCl) of *E. coli* strain E38.27.

Cloacal and cecal samples

Individual cloacal samples were collected using sterile dry cotton swabs (MW100, Medical Wire & Equipment, England) from all broilers just before challenge, to confirm the absence of ESBL/pAmpC-producing bacteria. In replicate 1 of experiment I all broilers were sampled with cloacal swabs at t = 3, 6, 9, 12, 15, 20, 24, 28, 32, 48, 52, 56 and 72 hours after challenge. In replicates 2 and 3 of experiment I, an additional sample was taken at t = 1.5 hours. In both replicates of experiment II broilers were sampled at t = 3, 6, 9, 12, 15, 20, 24, 32, 56 and 72 hours after challenge. In all replicates, after the last sampling round at day 4, at 72 hours after challenge, broilers were euthanized within the isolator by cervical dislocation and transported to the *post mortem* room in individually sealed bags. *Post mortem* examination was done within 30 minutes after euthanasia of the broiler. Broilers were weighed, checked for exterior and interior abnormalities, sex was determined, and ceca were collected and stored on ice for further analysis.

Ethics of experimentation

Broilers were observed daily and the presence of clinical signs, abnormal behaviour and mortality was recorded. The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands) under registration number AVD108002015314 and all procedures were done in full compliance with all relevant legislation.

ESBL/pAmpC-producing *E. coli* detection

All cloacal samples, except the ones used for quantification of ESBL/pAmpC-producing *E. coli* and total *E. coli* (next paragraph), were enriched in 3 mL Luria Bertani (LB) broth. After overnight incubation at 37°C, 10 µL were inoculated on MacConkey plates supplemented with 1 mg/L cefotaxime and incubated overnight at 37°C. Cloacal samples were processed and analysed individually. *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CMY-2-*E. coli* (EXP I) or CTX-M-1-*E. coli* (EXP II). Colonies inconclusive after visual assessment were typed using MALDI-TOF MS (Bruker Daltonik, Germany).

ESBL/pAmpC-producing *E. coli* and total *E. coli* quantification

Cloacal swabs obtained at t = 9, 32, 56 and 72 hours after challenge were weighed before and after sampling to determine the amount of faeces collected. The weight of the faecal samples on the cloacal swab specimens ranged from 0.01 to 0.43 gram. Each swab was suspended in 3 mL LB broth. At 72 hours after challenge, content from one of the two ceca was collected *post mortem* and 0.1 – 1.0 gram was used to make a 10% dilution in Phosphate Buffered Saline (PBS). A total of 200 µL of the suspension containing the cloacal faeces or cecal content was used to prepare tenfold dilution series (10^{-1} – 10^{-5}) in saline solution (0.85% NaCl); 10 µL of each dilution were inoculated on MacConkey plates without and with 1 mg/L cefotaxime, which were incubated overnight at 37°C. Concentrations of ESBL/pAmpC-producing *E. coli* and total *E. coli* were determined semi-quantitatively (CFU/gram faeces), based on the highest dilution showing growth of typical *E. coli* colonies and the weight of the faeces on the swabs or the amount of cecal content collected (Ceccarelli et al., 2017). *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CMY-2-*E. coli* (EXP I) or CTX-M-1-*E. coli* (EXP II). Colonies inconclusive after visual assessment were typed using MALDI-TOF MS (Bruker Daltonik, Germany).

The LB broth including the swab was also enriched overnight at 37°C. If no growth of *E. coli* colonies was observed on the MacConkey plates with cefotaxime (except for samples at t = 9 hours of experiment I/replicate 1), 10 µL of the overnight enrichment broth were inoculated on MacConkey plates supplemented with 1 mg/L cefotaxime and incubated overnight at 37°C. If colonies were detected, the concentration was assumed to be below detection level of the dilution series and the concentration designated as such (Statistical analysis).

Statistical analysis

First, results of experiment I and experiment II were analysed separately. Secondly, data of broilers challenged with dose 10^1 or 10^2 CFU/mL from experiment I and experiment II were analysed together to compare different *E. coli* strains with diverse plasmid-gene combinations. All results were analysed using R, version 3.4.3. (RStudio Team, 2016), package "survival" (Cox proportional hazard regression), "lme4" (mixed linear regression model) and "bbmle" (maximum log likelihood).

Time-until-colonization

The time-until-colonization was analysed using Cox proportional hazard regression. Validity of the assumptions of proportional hazards was checked using Schoenfeld residuals and assumptions were met. Colonization of individual broilers was measured by excretion of ESBL/pAmpC-producing *E. coli* and defined as the time point of the first cloacal swab of two consecutive cloacal swabs tested positive for ESBL/pAmpC-producing *E. coli*.

Level of excretion

The effect of challenge dose on the level of excretion was analysed using a mixed linear regression model including variables *time*, *isolator*, *replicate*, *sex* and *body weight at hatch* and the interaction between *time* and *dose level*. *Animal* was included as random effect to account for repeated measurements for the same broiler. *Body weight at hatch* was included as continuous variable, the others as categorical values.

In addition, in experiment II the variable *type of broiler* (conventional or SPF), and in the analysis of experiment I + II the variable *E. coli strain* was included. The best fitting model was obtained by backward selection, choosing the model with the lowest AIC value. Models with a difference in AIC of 2 or less were considered of equal fit and the most parsimonious model (lowest number of parameters) was chosen.

Broilers negative for ESBL/pAmpC-producing *E. coli* in the dilution series but positive after overnight culturing were included in the analysis with excretion level 1 $\log_{10}$ CFU/mL LB, as the minimum detection level of the semi-quantitative method was 2 $\log_{10}$ CFU/mL LB. Results from the dilution series where no ESBL/pAmpC-producing *E. coli* were counted following a dilution with a high amount of CFU ESBL/pAmpC-producing *E. coli* or the other way around, or results based on negative swab weight (or weight = 0 gram) were excluded from the analysis. Moreover, samples negative for ESBL/pAmpC-producing *E. coli* after overnight culturing were excluded since the analysis of excretion levels was based on excreting broilers only. Differences in cecal content ($\log_{10}$ CFU/gram) between the different dose levels and type of broilers were tested using a non-parametric Kruskal-Wallis test.

Probability of inoculation or transmission resulting in colonization

The observed differences in time-until-colonization within isolators showed that colonization of

broilers by ESBL/pAmpC-producing *E. coli* can be the result of inoculation leading to colonization or, in case inoculation did not lead to colonization, the result of transmission from other excreting broilers. Post-hoc analysis was performed assuming that the number of ESBL/pAmpC positive broilers as a result of inoculation or as a result of transmission at a point in time has a binomial distribution with

$$1 - \pi = (1 - \pi_{in})(1 - \pi_{tr})^{nprev}$$

and depends on the number of broilers still at risk just before this time point (van den Broek and Heesterbeek, 2007). The probabilities were estimated by maximizing the likelihood function. In this model π is the probability of colonization by ESBL/pAmpC-producing *E. coli* during a certain time interval, based on π_{in}, which is the probability of colonization because of inoculation, and π_{tr}, which is the probability of colonization by ESBL/pAmpC-producing *E. coli* because of transmission. Probability π_{tr} depends on the total number of broilers being positive at the previous time moment in the same isolator (*nprev*), thus transmission can only occur when at least one broiler in the isolator is excreting ESBL/pAmpC-producing *E. coli*.

The probability of being positive as a result of inoculation (π_{in}) and as a result of transmission (π_{tr}) were estimated using a logistic regression model including time as offset, in which *i* is *dose level*, *j* is *replicate,* and *k* is *type of broiler* (SPF or conventional).

$$\ln\left(\frac{\pi_{in}}{1-\pi_{in}}\right) = \alpha_{in} + \beta_{in_i}X_{in_i} + \beta_{in_j}X_{in_j} + \beta_{in_k}X_{in_k} + \Delta t$$

$$\ln\left(\frac{\pi_{tr}}{1-\pi_{tr}}\right) = \alpha_{tr} + \beta_{tr_i}X_{tr_i} + \beta_{tr_j}X_{tr_j} + \beta_{tr_k}X_{tr_k} + \Delta t$$

For experiment I the model was used without the variable *type of broiler* as only SPF broilers were included, for experiment II the model with all variables was evaluated. The best fitting model was chosen based on the lowest AIC value, if the difference between AIC values was <2, the model with the least variables was selected as the best model.

Results

Experiment I

The effect of dose on time-until-colonization

All broilers were colonized with CMY-2-*E. coli*, varying between 3 (dose 10^5 CFU/mL) and 24 (dose 10^1 CFU/mL) hours after challenge (Table S2a). Time-until-colonization of CMY-2-*E. coli* depended on the challenge dose, with a hazard ratio for colonization of 3.20 (95% CI 1.76 – 5.82) for dose 10^2 to 25.43 (95% CI 11.27 – 57.38) for dose 10^5 (reference dose 10^1, Table 1). Body weight at day 0 and day 4, sex and replicate did not influence the time-until-colonization.

Table 1 Hazard Ratio (HR, 95% CI) of time-until-colonization for experiment I (*n*=120), II (*n*=78) and I+II (*n*=95). In experiment I only SPF broilers were included, challenged with different dose levels of CMY-2-*E. coli*. In experiment II SPF and conventional broilers were included, challenged with different dose levels of CTX-M-1-*E. coli*. Results of experiment I and II were combined in order to compare the different *E. coli* strains.

Experiment	Variable		HR (95% CI)
I	Dose*	10^1 (reference)	1
		10^2	3.20 (1.76 - 5.82)
		10^3	12.21 (6.03 - 24.72)
		10^4	14.03 (6.44 - 30.57)
		10^5	25.43 (11.27 - 57.38)
	Replicate	1 (reference)	1
		2	1.00 (0.38 – 2.69)
		3	1.99 (0.89 – 4.44)
	Body weight day 0 (hatch)		0.98 (0.90 – 1.07)
	Body weight day 4		1.01 (0.99 – 1.03)
	Sex	Male (reference)	1
		Female	0.88 (0.57 – 1.35)
II	Dose	10^1 (reference)	1
		10^2	3.94 (2.31 – 6.74)
	Type of broiler	SPF (reference)	1
		Conventional	0.05 (0.01 – 0.22)
	Replicate	1 (reference)	1
		2	0.94 (0.52 – 1.71)
	Body weight day 0 (hatch)		0.94 (0.87 – 1.02)
	Body weight day 4		0.99 (0.95 – 1.02)
	Sex	Male (reference)	1
		Female	1.01 (0.63 – 1.63)
I+II	Dose	10^1 (reference)	1
		10^2	4.80 (2.30 – 10.02)
	Challenge	CMY-2-*E. coli* (reference)	1
		CTX-M-1-*E. coli*	1.22 (0.51 – 2.94)
	Isolator	1 (reference)	1
		2	1.92 (1.01 – 3.64)
		3	0.89 (0.36 – 2.19)
		4	1.03 (0.29 – 3.71)
	Body weight day 0 (hatch)		0.93 (0.86 – 1.00)
	Body weight day 4		1.01 (0.98 – 1.04)
	Sex	Male (reference)	1
		Female	0.93 (0.58 – 1.50)

*HR of all dose levels were significantly different (p<0.05), except for dose levels 10^3 and 10^4, and 10^4 and 10^5.

The effect of dose on level of excretion

Excretion levels of CMY-2-*E. coli* and total *E. coli* increased during the experiment (Figure 1, Table S3). After inoculation (t = 9), excretion levels of CMY-2-*E. coli* increased with the challenge dose, however from t = 32 hours onwards no trend with increasing dose was observed. A higher body

weight at day of hatch was associated with slightly lower excretion levels of CMY-2-*E. coli*, (-0.07 log_{10} CFU/gram faeces, 95% CI -0.10 – -0.04) (Table S3). All broilers showed cecal content levels of CMY-2-*E. coli* between 7.8 – 8.3 log_{10} CFU/gram, with slightly lower levels for dose 10^3 – 10^5 compared to dose 10^1 (p=0.02) (Table S2).

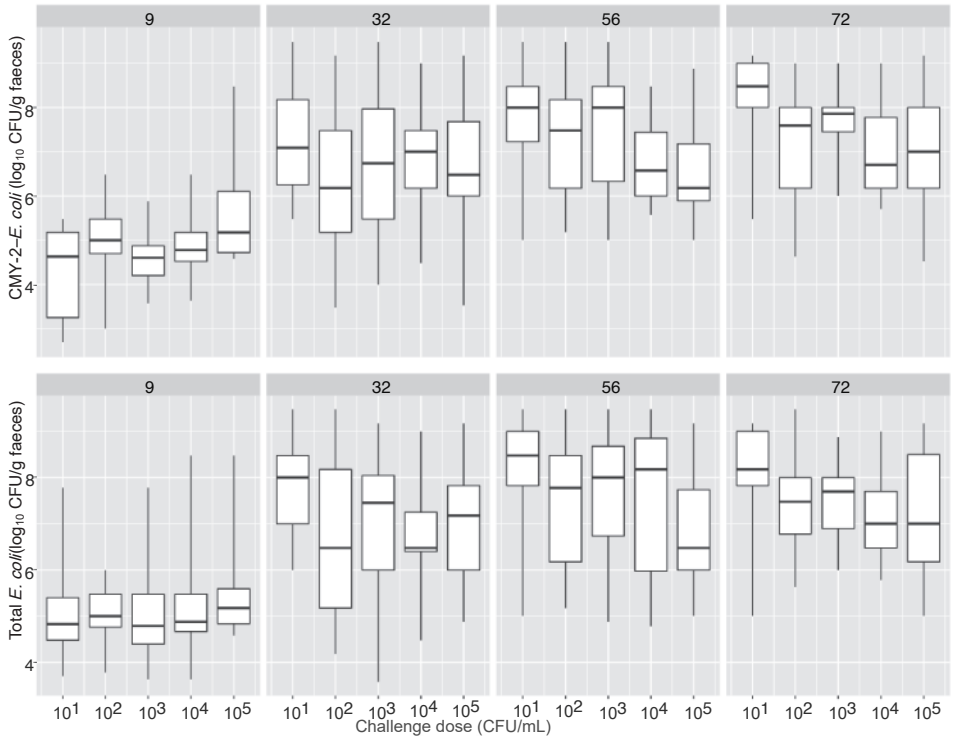

Figure 1 Experiment I: Excretion levels (log_{10} CFU/g faeces) of CMY-2-*E. coli* and total *E. coli* per challenge dose (10^1, 10^2, 10^3, 10^4, 10^5) at 9, 32, 56 and 72 hours after inoculation, including broilers with excretion levels above detection limit. The heavy line indicates the median, the box plot extends from the lower to upper quartile, the whiskers indicate the total range of observations.

Inoculation and transmission

Colonization of an individual broiler by ESBL/pAmpC-producing *E. coli* in this study was either the result of inoculation or the result of transmission from colonized broilers within the same isolator. Different statistical models to estimate the probability of being positive for CMY-2-*E. coli* as a result of inoculation or transmission were evaluated. In the best fitting model, the probability of being positive as a result of inoculation depended on the dose, while the chance of being positive as a result of transmission was equal for all doses. This model was preferred to a model in which

both inoculation and transmission depended on the dose, because of wide confidence intervals around the estimates. The estimated probability for a susceptible broiler to become colonized by CMY-2-*E. coli* as a result of inoculation (Table 2) was 0.03 per hour when challenged with dose 10^1, 0.14 for dose 10^2, 0.34 for dose 10^3, 0.44 for dose 10^4 and 0.83 for dose 10^5. The probability of a susceptible broiler to become colonized as a result of transmission was 0.04, multiplied by the number of excreting broilers in the same isolator per hour (Table 2).

Table 2 Estimates of probability of becoming colonized per susceptible broiler per hour (95% CI), because of inoculation (π_{in}) or transmission (π_{tr}) for experiment I (n=159) and experiment II (n=120), per challenge dose ($10^1 – 10^5$) and type of broiler (SPF and conventional), analysed with a model using the maximum likelihood of a binomial distribution with π_{in} and π_{tr}.

Experiment	Variable	Type of broiler	Dose level	Probability positive per broiler per hour (95% CI)
I	π_{in}	SPF	10^1	0.03 (0.01 – 0.07)
			10^2	0.14 (0.02 – 0.58)
			10^3	0.34 (0.05 – 0.81)
			10^4	0.44 (0.07 – 0.88)
			10^5	0.83 (0.23 – 0.99)
	π_{tr}	SPF	10^1-10^5	0.04 (0.02 – 0.06)
II	π_{in}	SPF	10^1	0.05 (0.02 – 0.10)
			10^2	0.11 (0.02 – 0.40)
		Conventional	10^1	0.01 (0.001 – 0.04)
			10^2	0.01 (0.001 – 0.18)
	π_{tr}	SPF	10^1-10^2	0.05 (0.02 – 0.08)
		Conventional	10^1-10^2	0.02 (0.005 – 0.09)

Experiment II

The effect of dose on time-until-colonization

All broilers were colonized with CTX-M-1-*E. coli*, varying between 56 (dose 10^2 CFU/mL) to 72 (dose 10^1 CFU/mL) hours after challenge (Table S2b). Time-until-colonization depended on the CTX-M-1-*E. coli* dose, with a hazard ratio of 3.94 (95% CI 2.31 – 6.74) for dose 10^2 (reference dose 10^1, Table 1). For conventional broilers, the hazard rate of colonization by CTX-M-1-*E. coli* was lower than for SPF broilers with a hazard ratio of 0.05 (95% CI 0.01 – 0.22). Body weight at day 0 and day 4, sex and replicate did not influence the time-until-colonization.

The effect of dose on level of excretion

Conventional broilers excreted lower levels of CTX-M-1-*E. coli* compared to SPF broilers (Figure 2, Table S3). For both conventional and SPF broilers excretion levels increased during the experiment and were higher for broilers receiving challenge dose 10^2, however this difference was only minor (0.66 $\log_{10}$ CFU/gram faeces, 95% CI 0.23 – 1.12). Levels in cecal content were also lower in

conventional broilers compared to SPF broilers (7.05 versus 8.01 $\log_{10}$ CFU/gram cecal content, p=0.03). *E. coli* was detected in conventional broilers before the moment of inoculation. At t = 9 hours after inoculation conventional broilers excreted higher levels of total *E. coli* compared to SPF broilers (Figure 2). Results of total *E. coli* in isolator 5 of replicate 1 were excluded from the analysis because of a contamination with *Citrobacter freundii* in the isolator, making visual assessment of *E. coli* growth on MacConkey plates not possible.

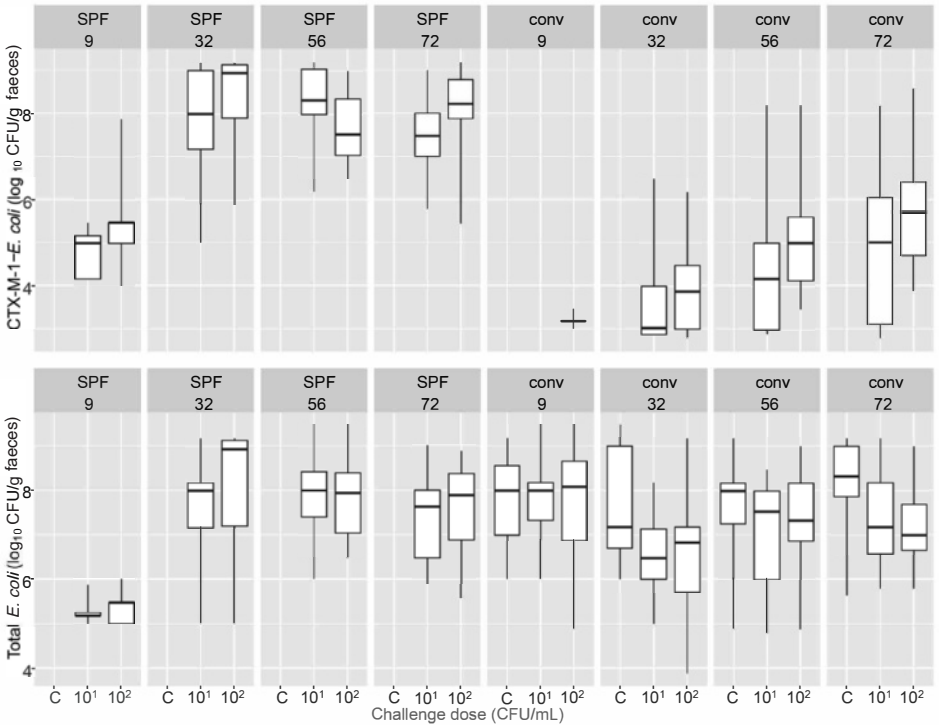

Figure 2 Experiment II: Excretion levels ($\log_{10}$ CFU/g faeces) of CTX-M-1-*E. coli* and total *E. coli* per challenge dose (control (C), 10^1 and 10^2) at 9, 32, 56 and 72 hours after inoculation, for SPF and conventional (conv) broilers, including broilers with excretion levels above detection limit. The heavy line indicates the median, the box plot extends from the lower to upper quartile, the whiskers indicate the total range of observations.

Inoculation and transmission

Similar to Experiment I, different statistical models were fitted to estimate the probability of being CTX-M-1-*E. coli* positive as a result of inoculation or as the result of transmission from other inoculated broilers within the same isolator. The best fitting model included a probability of being positive because of inoculation, depending on challenge dose (10^1 or 10^2) and type of

broiler (SPF or conventional), and transmission. Transmission depended on type of broiler but was independent of challenge dose. The estimates of the probability of colonization as a result of inoculation were 0.05 (dose 10^1) and 0.11 (dose 10^2) per hour for a susceptible SPF broiler, and 0.01 per hour (doses 10^1 and 10^2) for a susceptible conventional broiler (Table 2). The estimates of the probability of colonization as a result of transmission were independent of dose level and were 0.05 for a susceptible SPF broiler and 0.02 for a susceptible conventional broiler, both multiplied by the number of excreting broilers in the same isolator per hour.

Experiment I + II

The hazard rate of being colonized did not differ between the two ESBL/pAmpC-producing *E. coli* strains used in experiment I and II for SPF broilers receiving challenge doses 10^1 or 10^2 (Table 1). However, the hazard rate of colonization increased together with the challenge dose with a hazard ratio of 4.80 (95% CI 2.30 – 10.02) for dose 10^2 versus dose 10^1. Excretion levels of ESBL/pAmpC-producing *E. coli* in both cloacal and cecal samples of SPF broilers did not differ between the two strains (data not shown). Total *E. coli* excretion was slightly lower in SPF broilers challenged with CTX-M-1-*E. coli* (-0.76 $\log_{10}$ CFU/gram faeces, 95% CI -1.25 – -0.27).

Discussion

These experiments have shown that the probability of colonization by ESBL/pAmpC-producing *E. coli* of a single young broiler per hour upon inoculation increases with the challenge dose. Moreover, the time-until-colonization decreases with increasing challenge dose. Eventually, all broilers within the flock became colonized either due to colonization or because of transmission, even after challenge with a dose as low as 10^1 CFU/mL. Furthermore, 72 hours after inoculation the level of excretion was not different between the challenge doses. Our results were reproduced for two different *E. coli* strains carrying a different gene-plasmid combination and for both SPF and conventional broilers. Conventional broilers showed a delay in colonization compared to SPF broilers, which might be due to competition by resident *E. coli*, which was detected in conventional broilers before the moment of inoculation. Distinguishing between the rate of colonization by ESBL/pAmpC-producing *E. coli* after challenge as a result of inoculation or transmission shows that presence of only a few ESBL/pAmpC-producing bacteria within a poultry house or hatchery can lead to colonization of some of the broilers, and that subsequently between-broiler transmission will result in a high prevalence of colonized broilers in the flock. This process likely includes, at least in conventional broilers, transmission (via conjugation) of the plasmid present in the inoculum *E. coli* to other *E. coli* strains. In our experimental design, we intentionally decided to follow the resistance phenotype – provided by the ESBL/pAmpC gene encoding plasmid - independently on the *E. coli* strain, which we did not aim to fully characterize. This approach was chosen to reflect the

dynamics of ESBL/pAmpC-producing *E. coli* transmission in nature, i.e. the chicken gut (Huijbers et al., 2016; van Hoek et al., 2018), where it is known that horizontal gene transfer occurs and is an integral part of resistance spread in broilers. Yet, to not overlook this biological aspect completely, the use of RAPD PCR as a quick tool to define strain variability was used and the finding of only one RAPD profile in the SPF birds but different profiles in the conventional birds (data not shown) indicated that plasmid transfer to different *E. coli* strains occurred in the conventional birds.

Our method to estimate the probability of colonization by inoculation and transmission may have overestimated the probability of colonization upon inoculation, because a main assumption was that the probability that a bird starts to excrete due to colonization after inoculation remains constant over time during the entire experiment. In reality, the probability that a bird starts excreting as a result of colonization after inoculation, will decline in time, because the initial inoculated bacteria will colonize the bird, or the inoculated bacteria will pass through the gastro-intestinal tract without colonization during the first hours after inoculation. On the other hand, our model did take into account that the probability of a susceptible broiler being colonized because of transmission, will increase in time due to an increase in the number of already colonized broilers excreting ESBL/pAmpC-producing *E. coli*. The model included the number of excreting broilers and did not take into account the build-up of bacteria, being excreted by the broilers, in the environment, which might have led to underestimation of the infectious pressure. Even though we might have underestimated transmission compared to the probability of colonization by inoculation, the relevance of transmission in dose-effect experiments performed in small groups of broilers is demonstrated, which is in line with the results in earlier experiments with *Campylobacter jejuni* (Line et al., 2008).

Importantly our study provides evidence that the probability of colonization as a result of transmission is independent of the initial dose of ESBL/pAmpC-producing *E. coli* introduced in a flock. Excretion levels higher than the initial challenge doses at 9 hours post inoculation, similar excretion levels between the challenge doses quickly after inoculation (32 hours post inoculation) in experiment I and only slightly different excretion levels in experiment II do support this idea. Excretion levels of ESBL/pAmpC-producing *E. coli* at the end of the experiment were higher in SPF broilers than in conventional broilers, and were comparable to earlier reported excretion levels in SPF broilers during the first week after challenge (Ceccarelli et al., 2017).

Finding excretion levels similar for all dose levels in experiment I and only slightly different in experiment II indicates that excretion levels do not depend on the initial inoculum, but on processes within the bird. Several bird characteristics might influence excretion levels. In experiment I, broilers with lower hatching weights were excreting slightly higher levels of ESBL/pAmpC-producing *E. coli*. Possibly these hatchlings had limited microbial diversity or abundance, which increased the susceptibility to ESBL/pAmpC-producing *E. coli* colonization, as was suggested earlier for *C. jejuni* (Han et al., 2017). Moreover, other host characteristics such as differences in genetics (reviewed by Kers et al., 2018) between SPF and conventional broilers might affect the

microbiome, possibly also in relation to growth performance, and subsequently their response to *E. coli* challenges. In experiment II, conventional broilers showed a delayed time-until-colonization and reduced excretion levels compared to SPF broilers, possibly explained by the microbiota, which included *E. coli*, present before inoculation. The initially present *E. coli* did not carry ESBL/pAmpC, shown by the absence of ESBL/pAmpC-producing *E. coli* in samples at start of the experiment and in the control groups during the experiment. The presence of initial *E. coli* might have reduced susceptibility to the challenge *E. coli*, as previously observed with the supply of competitive exclusion cultures leading to a reduction in colonization (Hofacre et al., 2002; Nuotio et al., 2013; Ceccarelli et al., 2017). The stage of microbiota development and thus the age of broilers might affect the susceptibility to colonization (Jurburg et al., 2019), such as ESBL/pAmpC-producing *E. coli*. Our result show that small amounts of ESBL/AmpC-producing *E. coli* are able to colonize young birds in the early stages of microbiota development.

Inoculation with two different *E. coli* strains with other plasmid and ESBL/pAmpC-gene combinations did not result in differences in time-until-colonization and in excretion levels of ESBL/pAmpC-producing *E. coli* in SPF broilers. Both ESBL/pAmpC-producing *E. coli* strains were obtained from a poultry monitoring program (Dierikx et al., 2010) and were able to colonize and transmit, even in absence of antimicrobials, as observed in earlier studies (Le Devendec et al., 2011; Fischer et al., 2014; Huijbers et al., 2016; Dame-Korevaar et al., 2017; Ceccarelli et al., 2017). The successful colonization of one of the strains in conventional broilers and both strains in SPF broilers suggest that strains well adapted to broilers are suitable for animal models to study interventions to reduce ESBL/pAmpC-producing bacteria in poultry.

Conclusions

The presence of small amounts of bacteria in a hatchery or poultry house could result in colonization of young birds followed by high levels of excretion and transmission within the flock. Interventions, such as hygiene measures, should aim towards eliminating ESBL/pAmpC-producing *E. coli* in the environment of the hatchling, i.e. the hatchery, transport vehicles and the broiler farm. Furthermore, within 72 hours of challenge all broilers excreted ESBL/pAmpC-producing *E. coli*, therefore measures focusing at reducing colonization and transmission of ESBL/pAmpC-producing *E. coli,* such as administration of competitive exclusion cultures, should be applied within a very short time frame after hatching.

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Supplementary material

Table S1 Design of experiment I and II, including date, age of the parent stock, dose level and type of challenge, type of animal, number of broilers at start and end of experiment, and sex of the animals, per replicate.

Replicate	Date	Age parent stock	Dose level (CFU/mL)	Challenge[a]	Animal[b]	*n* broilers at day 0	*n* broilers inoculated	*n* broilers at day 4	Sex[c]
Experiment I									
1	13 – 20 May 2016	62 weeks	Control	PBS	SPF	14	10, 1 died during inoculation	8	5 M; 3 F; 2 NA
			10^2	CMY-2	SPF	14	10	10	10 M; 0 F
			10^3	CMY-2	SPF	14	10	10	8 M; 2 F
			10^4	CMY-2	SPF	14	10	9	6 M; 4 F
			10^5	CMY-2	SPF	14	10	10	6 M; 4 F
2	3 – 10 June 2016	31 weeks	Control	PBS	SPF	14	10	9	8 M; 2 F
			10^1	CMY-2	SPF	14	10	8	7 M; 3 F
			10^2	CMY-2	SPF	14	10	10	8 M; 2 F
			10^3	CMY-2	SPF	13	10	7	7 M; 3 F
			10^4	CMY-2	SPF	13	10	9	7 M; 3 F
3	24 June – 1 July 2016	34 weeks	Control	PBS	SPF	14	10	10	7 M; 3 F
			10^1	CMY-2	SPF	13	10	10	7 M; 2 F; 1 NA
			10^1 (other dilution series)	CMY-2	SPF	14	10	10	7 M; 3 F
			10^2	CMY-2	SPF	13	10	10	7 M; 3 F
			10^3	CMY-2	SPF	15	10	9	5 M; 5 F
			10^5	CMY-2	SPF	13	10	9	4 M; 6 F
Experiment II									
1	11 – 18 Sept 2016	SPF: 45 weeks Conv: 48 weeks	Control	PBS	SPF	15	10	10	6 M; 4 F
			Control	PBS	Conv	15	10	10	4 M; 6 F
			10^1	CTX-M-1	SPF	14	10	10	5 M; 5 F
			10^1	CTX-M-1	Conv	15	10	10	6 M; 4 F
			10^2	CTX-M-1	SPF	14	10	10	3 M; 5 F; 2 NA
			10^2	CTX-M-1	Conv	15	10	10	7 M; 3 F
2	14 – 21 Oct 2016	SPF: 50 weeks Conv: 53 weeks	Control	PBS	SPF	10	10	10	8 M; 2 F
			Control	PBS	Conv	15	10	10	4 M; 6 F
			10^1	CTX-M-1	SPF	10	10	10	7 M; 3 F
			10^1	CTX-M-1	Conv	15	10	10	5 M; 5 F
			10^2	CTX-M-1	SPF	10	10	10	4 M; 6 F
			10^2	CTX-M-1	Conv	15	10	10	6 M; 4 F

[a] *E. coli* strain E39.62 with bla_{CMY-2} on IncK, *E. coli* strain E38.27 with $bla_{CTX-M-1}$ on IncI1

[b] Specific Pathogen Free (SPF), Animal Health Service Deventer, Conventional (Conv) broilers (Ross 308) originated from poultry facility with hatchery

[c] Number of Males (M), Females (F) and not assessed (NA) broilers

Table S2a Detection (+/-) and quantification (log_{10} CFU/g faeces) of CMY-2-*E. coli* in broilers in experiment I, determined at *n* hours post inoculation and in the cecal content (log_{10} CFU/g cecal content).

Replicate	Iso	Dose	Bird ID	Hours post inoculation														
				0	1.5	3	6	9	12	15	20	24	28	32	48	52	56	72	Ceca
1	1	0	0001	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	1	0	0011	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	1	0	0021	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	1	0	0026	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	1	0	0031	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	1	0	0036	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	1	0	0041	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	1	0	0046	-		-	-	-	-	-	†								
1	1	0	0051	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	2	10^2	0002	-		-	+	5.18	+	+	+	+	+	7.48	+	+	7.52	+	7.98
1	2	10^2	0007	-		+	+	4.70	+	+	+	+	+	4.18	+	+	8.18	+	8.00
1	2	10^2	0012	-		+	+	+	+	+	+	+	+	6.18	+	+	6.52	6.78	7.95
1	2	10^2	0017	-		-	-	-	-	+	+	+	+	+	+	+	6.48	+	7.93
1	2	10^2	0022	-		-	+	5.00	+	+	+	+	+	+	+	+	5.18	+	7.96
1	2	10^2	0037	-		-	+	5.00	+	+	+	+	+	3.48	+	+	6.00	4.88	7.95
1	2	10^2	0042	-		+	+	5.00	+	+	+	+	+	3.48	+	+	5.48	4.63	7.95
1	2	10^2	0047	-		-	-	-	+	+	+	+	+	8.18	+	+	6.00	6.88	7.96
1	2	10^2	0057	-		-	+	5.48	+	+	+	+	+	4.18	+	+	6.78	7.70	7.95
1	2	10^2	0062	-		-	-	-	+	+	+	+	+	+	+	+	8.00	7.88	8.00
1	3	10^3	0008	-		+	+	4.06	+	+	+	+	+	8.00	+	+	8.00	8.00	7.94
1	3	10^3	0013	-		+	+	4.57	+	+	+	+	+	5.88	+	+	8.48	8.00	7.97
1	3	10^3	0023	-		+	+	-	+	+	+	+	+	7.00	+	+	8.00	8.00	7.96
1	3	10^3	0028	-		+	+	4.63	+	+	+	+	+	6.00	+	+	5.00	8.18	7.96
1	3	10^3	0033	-		-	+	4.63	+	+	+	+	+	6.00	+	+	6.18	7.18	7.95
1	3	10^3	0043	-		+	+	3.57	+	+	+	+	+	4.88	+	+	8.88	7.88	7.95
1	3	10^3	0048	-		+	+	4.36	+	+	+	+	+	5.00	+	+	6.00	7.48	7.95
1	3	10^3	0053	-		-	+	4.27	+	+	+	+	+	5.18	+	+	6.00	7.00	7.95
1	3	10^3	0058	-		+	+	4.63	+	+	+	+	+	5.48	+	+	6.48	7.18	7.96
1	3	10^3	0063	-		+	+	4.70	+	+	+	+	+	6.48	+	+	5.88	6.00	8.03
1	4	10^4	0004	-		+	+	4.70	+	+	+	+	+	6.48	+	+	5.78	5.78	7.95
1	4	10^4	0009	-		+	+	+	+	+	+	+	+	6.48	+	+	7.18	7.00	8.07
1	4	10^4	0014	-		+	+	4.70	+	+	+	+	+	6.18	+	+	6.00	7.00	7.98
1	4	10^4	0024	-		+	+	4.78	+	+	+	+	+	7.18	+	+	5.78	6.70	7.96
1	4	10^4	0029	-		+	+	5.78	+	+	+	+	+	5.48	+	+	6.57	6.70	7.94
1	4	10^4	0034	-		+	+	4.63	+	+	+	+	+	5.48	+	+	7.00	5.88	7.96
1	4	10^4	0039	-		+	+	4.30	+	+	+	+	+	9.00	+	+	5.57	6.18	7.96
1	4	10^4	0049	-		+	+	3.63	+	+	+	+	+	7.18	+	+	8.48	7.78	7.96
1	4	10^4	0054	-		+	+	4.52	+	†									
1	4	10^4	0064	-		+	+	+	+	+	+	+	+	7.48	+	+	7.18	8.00	7.97
1	5	10^5	0005	-		+	+	4.57	+	+	+	+	+	7.48	+	+	8.48	9.18	7.96
1	5	10^5	0010	-		+	+	4.63	+	+	+	+	+	6.18	+	+	6.00	6.18	7.93
1	5	10^5	0015	-		+	+	4.78	+	+	+	+	+	6.48	+	+	5.63	8.00	8.30
1	5	10^5	0020	-		+	+	4.57	+	+	+	+	+	6.00	+	+	5.78	6.48	7.97
1	5	10^5	0025	-		+	+	5.88	+	+	+	+	+	7.48	+	+	6.88	7.00	7.84
1	5	10^5	0030	-		+	+	4.70	+	+	+	+	+	5.88	+	+	5.00	7.88	7.97
1	5	10^5	0035	-		+	+	4.63	+	+	+	+	+	7.48	+	+	5.48	6.18	7.95

Table S2a continued.

Replicate	Iso	Dose	Bird ID	Hours post inoculation														
				0	1.5	3	6	9	12	15	20	24	28	32	48	52	56	72	Ceca
1	5	10^5	0040	-		+	+	5.00	+	+	+	+	+	6.00	+	+	6.00	6.00	7.85
1	5	10^5	0055	-		+	+	+	+	+	+	+	+	8.48	+	+	6.00	6.00	7.97
1	5	10^5	0065	-		+	+	5.88	+	+	+	+	+	6.00	+	+	5.00	5.18	8.26
2	1	0	0071	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0076	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0081	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0086	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0090	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0119	-	-	-	-	-	†										
2	1	0	0124	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0134	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	0	0139	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
2	2	10^1	0072	-	-	-	-	4.78	+	+	+	+	+	8.00	+	+	8.78	8.00	8.10
2	2	10^1	0082	-	-	-	-	-	+	+	+	+	+	8.18	+	+	+	8.00	8.02
2	2	10^1	0087	-	-	-	+	-	+	+	+	+	+	6.48	+	+	9.18	9.00	8.01
2	2	10^1	0091	-	-	-	+	-	+	+	+	+	+	6.18	+	+	9.48	6.00	7.98
2	2	10^1	0098	-	-	-	-	-	-	-	+	+	+	+	+	+	6.00	6.00	7.98
2	2	10^1	0103	-	-	-	-	-	-	-	+	+	+	6.88	+	+	8.48	7.78	8.05
2	2	10^1	0108	-	-	-	-	4.48	-	-	+	+	+	8.48	+	+	7.88	8.00	8.16
2	2	10^1	0115	-	-	-	-	2.70	-	-	-	+	†						
2	2	10^1	0125	-	-	-	-	2.78	+	+	+	+	+	9.18	+	+	6.00	8.88	7.98
2	2	10^1	0130	-	-	-	+	2.78	+	+	+	+	+	+	+	+	†		
2	3	10^2	0073	-	-	-	-	4.78	+	+	+	+	+	9.18	+	+	9.48	8.48	7.97
2	3	10^2	0078	-	-	+	+	4.88	+	+	+	+	+	9.18	+	+	8.63	9.00	7.99
2	3	10^2	0088	-	-	+	+	+	+	+	+	+	+	7.48	+	+	7.48	5.57	7.99
2	3	10^2	0092	-	-	+	+	+	+	+	+	+	+	6.18	+	+	6.18	8.00	7.98
2	3	10^2	0099	-	-	+	+	5.00	+	+	+	+	+	5.88	+	+	6.18	6.48	7.99
2	3	10^2	0110	-	-	-	+	5.88	+	+	+	+	+	6.48	+	+	5.48	8.18	7.96
2	3	10^2	0116	-	-	-	+	5.00	+	+	+	+	+	7.18	+	+	8.18	7.88	7.96
2	3	10^2	0121	-	-	-	+	5.48	+	+	+	+	+	7.48	+	+	+	8.00	7.98
2	3	10^2	0126	-	-	+	+	5.48	+	+	+	+	+	+	+	+	8.18	9.00	7.97
2	3	10^2	0136	-	-	+	+	3.00	+	+	+	+	+	8.18	+	+	7.78	9.00	7.95
2	4	10^3	0074	-	-	-	+	5.78	+	+	+	+	+	6.78	†				
2	4	10^3	0079	-	-	+	+	4.01	+	+	+	+	+	6.44	+	+	7.18	7.44	7.98
2	4	10^3	0084	-	+	-	+	4.12	+	+	+	+	+	7.70	+	+	8.00	7.90	7.96
2	4	10^3	0093	-	-	-	+	5.88	+	-	+	+	+	7.88	+	+	6.00	7.70	7.98
2	4	10^3	0095	-	-	-	+	4.36	+	+	+	+	+	7.70	+	†			
2	4	10^3	0100	-	-	-	+	4.78	+	+	+	+	+	6.70	†				
2	4	10^3	0105	-	-	+	+	3.63	+	+	+	+	+	5.27	+	+	8.00	7.70	7.99
2	4	10^3	0122	-	-	-	+	4.78	+	+	+	+	+	7.78	+	+	8.48	7.84	7.96
2	4	10^3	0127	-	-	+	+	4.88	+	-	+	+	+	7.70	+	+	8.48	7.78	7.95
2	4	10^3	0137	-	-	+	+	4.88	+	+	+	+	+	4.57	+	+	6.48	7.78	7.96
2	5	10^4	0075	-	-	+	+	4.88	+	+	+	+	+	7.48	+	+	8.48	9.00	7.96
2	5	10^4	0080	-	-	+	+	4.00	+	+	+	+	+	7.88	+	+	6.48	6.48	7.96
2	5	10^4	0085	-	+	+	+	6.00	+	+	+	+	+	+	+	+	7.70	8.00	7.94
2	5	10^4	0096	-	-	+	+	6.48	†										

Table S2a continued.

Replicate	Iso	Dose	Bird ID	Hours post inoculation														
				0	1.5	3	6	9	12	15	20	24	28	32	48	52	56	72	Ceca
2	5	10^4	0101	-	+	+	-	5.00	+	+	+	+	+	4.48	+	+	+	7.52	7.96
2	5	10^4	0106	-	-	+	+	3.70	+	+	+	+	+	+	+	+	8.40	+	7.98
2	5	10^4	0113	-	-	+	+	5.18	+	+	+	+	+	+	+	+	+	8.33	7.95
2	5	10^4	0118	-	+	+	+	+	+	+	+	+	+	+	+	+	+	6.30	7.96
2	5	10^4	0128	-	+	-	-	5.18	+	+	+	+	+	+	+	+	6.00	5.88	7.98
2	5	10^4	0138	-	+	-	-	6.18	+	+	+	+	+	7.00	+	+	6.18	5.70	8.01
3	1	0	0107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0113	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0125	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0143	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0149	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0155	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0161	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	0	0179	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	2	10^1	0102	-	-	+	+	5.48	+	+	+	+	+	9.48	+	+	8.18	9.00	7.98
3	2	10^1	0108	-	-	-	-	-	+	+	+	+	+	7.00	+	+	7.48	9.00	8.05
3	2	10^1	0114	-	-	-	-	-	-	+	+	+	+	6.88	+	+	7.88	8.48	7.94
3	2	10^1	0126	-	-	-	+	5.48	+	+	+	+	+	6.18	+	+	8.00	8.18	8.00
3	2	10^1	0132	-	-	-	-	-	+	+	+	+	+	7.18	+	+	8.18	5.48	7.94
3	2	10^1	0138	-	-	-	+	5.48	+	+	+	+	+	7.00	+	+	8.70	8.78	8.03
3	2	10^1	0150	-	-	-	-	-	+	+	+	+	+	9.48	+	+	7.70	8.88	7.97
3	2	10^1	0156	-	-	-	-	5.18	+	+	+	+	+	6.18	+	+	8.18	8.48	8.04
3	2	10^1	0162	-	-	-	-	-	+	+	+	+	+	6.00	+	+	5.88	9.18	7.95
3	2	10^1	0180	-	-	-	+	3.48	+	+	+	+	+	8.00	+	+	8.78	7.88	7.99
3	3	10^1	0103	-	-	-	-	-	+	+	+	+	+	7.48	+	+	+	8.78	7.96
3	3	10^1	0109	-	-	-	-	3.18	+	+	+	+	+	6.48	+	+	+	9.00	7.96
3	3	10^1	0115	-	-	-	-	-	+	+	+	+	+	8.48	+	+	8.48	8.18	7.98
3	3	10^1	0121	-	-	-	+	5.18	+	+	+	+	+	6.00	+	+	6.48	9.00	+
3	3	10^1	0127	-	-	-	-	-	-	+	+	+	+	7.88	+	+	6.00	8.18	7.96
3	3	10^1	0139	-	-	-	+	+	+	+	+	+	+	8.18	+	+	8.00	8.18	7.98
3	3	10^1	0145	-	-	-	-	-	+	+	+	+	+	6.48	+	+	5.00	9.00	7.98
3	3	10^1	0151	-	-	-	-	-	-	+	+	+	+	5.48	+	+	7.48	9.18	7.99
3	3	10^1	0163	-	-	-	+	3.48	+	+	+	+	+	9.00	+	+	7.48	9.18	8.08
3	3	10^1	0181	-	-	+	+	5.18	+	+	+	+	+	6.18	+	+	8.18	9.00	7.96
3	4	10^2	0104	-	-	+	+	+	+	+	+	+	+	5.18	+	+	9.48	7.48	8.01
3	4	10^2	0116	-	-	-	+	4.78	+	+	+	+	+	5.18	+	+	9.18	8.00	7.97
3	4	10^2	0122	-	-	+	+	6.48	+	+	+	+	+	7.88	+	+	8.88	6.18	7.98
3	4	10^2	0128	-	-	-	-	-	+	+	+	+	+	5.18	+	+	6.00	6.00	+
3	4	10^2	0134	-	-	+	-	3.48	+	+	+	+	+	4.57	+	+	6.48	6.00	8.00
3	4	10^2	0140	-	-	-	-	-	+	+	+	+	+	6.00	+	+	8.00	8.00	8.00
3	4	10^2	0146	-	-	-	+	3.48	+	+	+	+	+	5.18	+	+	6.18	6.18	7.99
3	4	10^2	0158	-	-	-	+	4.18	+	+	+	+	+	5.18	+	+	5.48	6.78	8.01
3	4	10^2	0164	-	-	+	+	6.48	+	+	+	+	+	8.18	+	+	7.48	6.00	7.98
3	4	10^2	0182	-	-	-	+	4.18	+	+	+	+	+	7.48	+	+	8.00	8.18	7.99
3	5	10^3	0105	-	-	+	+	4.48	+	+	+	+	+	5.48	+	+	8.48	8.48	7.98
3	5	10^3	0123	-	-	+	+	+	+	+	+	+	+	8.00	+	+	8.00	6.88	8.01

Table S2a continued.

Replicate	Iso	Dose	Bird ID	Hours post inoculation														
				0	1.5	3	6	9	12	15	20	24	28	32	48	52	56	72	Ceca
3	5	10^3	0129	-	-	+	+	+	+	+	+	+	+	8.18	+	+	9.18	9.00	7.98
3	5	10^3	0141	-	-	+	+	4.18	+	+	+	+	+	3.99	+	+	9.48	7.88	7.99
3	5	10^3	0147	-	+	+	+	5.48	+	+	+	+	+	8.18	+	+	7.00	8.88	8.00
3	5	10^3	0159	-	+	+	+	+	+	+	+	+	+	8.48	+	+	9.00	8.00	7.98
3	5	10^3	0170	-	+	+	+	4.48	+	+	+	+	+	8.00	+	+	6.00	8.78	7.95
3	5	10^3	0171	-	-	+	+	5.48	+	+	+	+	+	9.48	+	+	8.18	6.88	7.93
3	5	10^3	0177	-	-	+	+	5.48	+	+	+	+	+	5.18	+	+	7.88	†	
3	5	10^3	0183	-	-	+	+	5.18	+	+	+	+	+	8.18	+	+	7.88	9.00	7.97
3	7	10^5	0106	-	-	+	+	7.18	+	+	+	+	+	6.18	+	+	8.18	8.18	7.98
3	7	10^5	0112	-	-	+	+	8.48	+	+	+	+	+	4.88	+	+	6.00	8.00	7.94
3	7	10^5	0118	-	+	+	+	5.00	+	+	+	+	+	6.78	+	+	8.88	6.18	7.94
3	7	10^5	0124	-	+	+	+	5.48	+	+	+	+	+	8.18	+	+	8.00	9.18	7.99
3	7	10^5	0136	-	+	+	+	+	+	+	+	+	+	6.00	+	+	6.48	8.00	+
3	7	10^5	0148	-	+	+	+	6.18	+	+	†								
3	7	10^5	0154	-	+	+	+	5.18	+	+	+	+	+	9.18	+	+	6.18	8.00	8.12
3	7	10^5	0166	-	+	+	+	6.18	+	+	+	+	+	8.48	+	+	7.48	9.00	7.96
3	7	10^5	0172	-	+	+	+	6.48	+	+	+	+	+	7.88	+	+	6.18	6.63	7.96
3	7	10^5	0178	-	+	+	+	5.18	+	+	+	+	+	3.52	+	+	6.48	4.52	7.96

+ in quantification series are broilers excreting CMY-2-*E. coli* (i.e. growth of *E. coli* on MacConkey + cefotaxime), but excretion values were below detection limit or missing.

† chick died

Table S2b Detection (+/-) and quantification (log_{10} CFU/g faeces) of CTX-M-1-*E. coli* in broilers (SPF and conventional) in experiment II, determined at *n* hours post inoculation and in the cecal content (log_{10} CFU/g cecal content).

Replicate	Iso	Broiler	Dose	Bird ID	Hours post inoculation											
					0	3	6	9	12	15	20	24	32	56	72	Ceca
4	1	SPF	0	0001	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0004	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0007	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0010	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0019	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0025	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0028	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0034	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0037	-	-	-	-	-	-	-	-	-	-	-	-
4	1	SPF	0	0043	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0053	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0056	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0062	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0071	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0077	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0080	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0083	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0086	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0089	-	-	-	-	-	-	-	-	-	-	-	-
4	2	Conv	0	0092	-	-	-	-	-	-	-	-	-	-	-	-
4	3	SPF	10^1	0002	-	-	-	-	+	+	+	+	+	6.18	7.00	8.03
4	3	SPF	10^1	0005	-	-	+	5.00	+	+	+	+	8.18	8.18	8.88	7.99
4	3	SPF	10^1	0008	-	-	-	-	+	+	+	+	9.18	7.48	9.00	8.02
4	3	SPF	10^1	0011	-	+	-	5.18	+	+	+	+	7.48	9.18	7.63	7.97
4	3	SPF	10^1	0014	-	-	-	-	+	+	+	+	7.18	9.18	7.00	7.98
4	3	SPF	10^1	0023	-	-	-	-	-	+	+	+	+	8.00	6.88	7.98
4	3	SPF	10^1	0026	-	-	-	-	-	+	+	+	7.18	9.00	8.00	8.01
4	3	SPF	10^1	0029	-	-	-	-	-	+	+	+	5.00	8.48	7.18	7.91
4	3	SPF	10^1	0032	-	-	+	4.18	+	+	+	+	9.18	9.18	9.00	7.99
4	3	SPF	10^1	0035	-	-	+	4.88	+	+	+	+	8.88	8.18	7.88	7.97
4	4	Conv	10^1	0051	-	-	-	-	-	-	-	-	2.88	3.88	6.00	7.98
4	4	Conv	10^1	0057	-	-	-	-	-	-	-	+	-	3.00	5.18	7.98
4	4	Conv	10^1	0063	-	-	-	-	-	-	-	-	-	5.00	8.18	8.11
4	4	Conv	10^1	0069	-	-	-	-	-	-	-	+	3.18	6.18	5.00	7.99
4	4	Conv	10^1	0072	-	-	-	-	-	-	-	+	-	4.48	6.18	8.00
4	4	Conv	10^1	0075	-	-	-	-	-	-	+	-	6.48	8.18	8.18	7.87
4	4	Conv	10^1	0078	-	-	-	-	-	-	+	-	+	4.18	5.00	8.15
4	4	Conv	10^1	0087	-	-	-	-	-	-	-	-	-	4.18	5.18	8.07
4	4	Conv	10^1	0090	-	-	-	-	-	-	+	-	2.88	5.48	4.88	7.98
4	4	Conv	10^1	0093	-	-	-	-	-	-	-	+	-	3.00	4.78	8.04
4	5	SPF	10^2	0003	-	+	-	-	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0006	-	+	+	-	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0009	-	-	-	+	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0012	-	-	-	-	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0015	-	-	-	-	+	+	+	+	+	+	+	+

Table S2b continued.

Replicate	Iso	Broiler	Dose	Bird ID	Hours post inoculation											
					0	3	6	9	12	15	20	24	32	56	72	Ceca
4	5	SPF	10^2	0018	-	-	+	-	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0021	-	+	-	+	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0024	-	-	-	+	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0027	-	+	-	+	+	+	+	+	+	+	+	+
4	5	SPF	10^2	0042	-	-	-	+	+	+	+	+	+	+	+	+
4	6	Conv	10^2	0052	-	-	-	-	-	-	-	-	-	3.88	8.00	7.97
4	6	Conv	10^2	0055	-	-	-	3.00	+	+	+	+	4.88	6.00	4.70	7.99
4	6	Conv	10^2	0061	-	-	-	3.18	-	-	+	-	-	3.88	4.63	8.00
4	6	Conv	10^2	0067	-	-	-	-	-	-	+	+	2.78	4.48	8.57	8.04
4	6	Conv	10^2	0070	-	-	-	-	-	-	+	-	3.88	4.18	5.70	8.15
4	6	Conv	10^2	0076	-	-	-	3.18	+	+	-	+	3.88	4.88	+	8.07
4	6	Conv	10^2	0079	-	+	-	-	-	+	+	+	5.18	5.18	6.00	7.94
4	6	Conv	10^2	0085	-	+	+	-	+	-	-	+	2.88	5.18	6.18	7.97
4	6	Conv	10^2	0088	-	+	-	3.18	-	+	+	-	3.78	5.00	7.78	8.11
4	6	Conv	10^2	0094	-	-	-	-	-	-	-	+	3.00	6.00	4.70	8.00
5	1	SPF	0	0001	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0004	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0007	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0010	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0013	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0016	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0019	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0022	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0025	-	-	-	-	-	-	-	-	-	-	-	-
5	1	SPF	0	0028	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0031	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0034	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0040	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0043	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0049	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0055	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0058	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0061	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0064	-	-	-	-	-	-	-	-	-	-	-	-
5	2	Conv	0	0067	-	-	-	-	-	-	-	-	-	-	-	-
5	3	SPF	10^1	0002	-	-	-	-	+	+	+	+	9.18	9.00	+	7.96
5	3	SPF	10^1	0005	-	-	+	4.18	+	+	+	+	9.00	8.00	+	7.98
5	3	SPF	10^1	0008	-	-	-	-	-	+	+	+	8.18	9.18	7.48	7.99
5	3	SPF	10^1	0011	-	-	-	-	-	+	+	+	9.18	8.48	+	7.94
5	3	SPF	10^1	0014	-	-	+	4.18	+	+	+	+	7.18	7.78	6.88	8.01
5	3	SPF	10^1	0017	-	-	+	5.18	+	+	+	+	+	8.00	7.70	8.04
5	3	SPF	10^1	0020	-	+	+	5.18	+	+	+	+	5.88	6.18	5.78	7.98
5	3	SPF	10^1	0023	-	+	+	5.48	+	+	+	+	8.00	8.48	8.78	7.97
5	3	SPF	10^1	0026	-	-	-	-	-	+	+	+	7.88	8.00	7.48	8.02
5	3	SPF	10^1	0029	-	-	-	-	+	+	+	+	5.18	9.18	7.48	7.97
5	4	Conv	10^1	0035	-	-	-	-	-	-	-	-	-	2.88	6.78	7.10
5	4	Conv	10^1	0038	-	-	-	-	-	-	-	-	-	-	3.18	4.00
5	4	Conv	10^1	0041	-	-	-	-	-	-	-	-	-	3.00	3.18	3.97
5	4	Conv	10^1	0044	-	-	-	-	-	-	-	-	-	-	2.88	5.03

Table S2b continued.

Replicate	Iso	Broiler	Dose	Bird ID	Hours post inoculation											
					0	3	6	9	12	15	20	24	32	56	72	Ceca
5	4	Conv	10^1	0053	-	-	-	-	-	-	-	-	-	-	2.88	3.96
5	4	Conv	10^1	0059	-	-	-	-	-	-	-	-	-	3.48	5.00	6.85
5	4	Conv	10^1	0062	-	-	+	-	-	-	-	-	-	-	7.00	4.92
5	4	Conv	10^1	0065	-	-	-	-	-	-	-	-	-	-	2.78	3.00
5	4	Conv	10^1	0068	-	-	+	-	-	-	-	-	-	-	2.88	4.01
5	4	Conv	10^1	0074	-	-	-	-	-	-	-	-	-	-	2.88	5.02
5	5	SPF	10^2	0003	-	-	+	5.48	+	+	+	+	7.88	8.00	8.88	8.02
5	5	SPF	10^2	0006	-	+	+	4.00	+	+	+	+	8.00	8.48	8.78	8.07
5	5	SPF	10^2	0009	-	-	+	5.48	+	+	+	+	9.00	7.00	5.44	8.40
5	5	SPF	10^2	0012	-	+	+	-	+	+	+	+	9.18	8.48	7.88	8.07
5	5	SPF	10^2	0015	-	-	+	5.18	+	+	+	+	5.88	7.88	9.18	7.99
5	5	SPF	10^2	0018	-	+	+	6.00	+	+	+	+	6.00	6.48	7.88	8.13
5	5	SPF	10^2	0021	-	-	+	7.88	+	+	+	+	9.00	7.00	8.00	7.97
5	5	SPF	10^2	0024	-	+	-	5.48	+	+	+	+	9.18	7.18	6.57	8.02
5	5	SPF	10^2	0027	-	-	+	5.00	+	+	+	+	8.88	7.18	8.44	7.89
5	5	SPF	10^2	0030	-	+	+	5.00	+	+	+	+	9.18	9.00	8.78	7.94
5	6	Conv	10^2	0033	-	-	-	-	-	-	-	+	3.00	6.88	6.63	7.98
5	6	Conv	10^2	0036	-	-	-	-	+	-	+	+	5.18	4.00	5.70	7.94
5	6	Conv	10^2	0039	-	-	-	-	-	-	+	+	6.18	7.18	4.78	7.95
5	6	Conv	10^2	0045	-	-	+	-	-	-	-	-	-	4.48	4.88	6.94
5	6	Conv	10^2	0048	-	+	-	-	-	-	+	+	4.18	5.00	5.48	6.96
5	6	Conv	10^2	0057	-	-	-	-	-	-	+	+	3.48	3.48	3.88	8.00
5	6	Conv	10^2	0063	-	-	+	-	-	-	-	-	2.78	3.48	4.00	7.96
5	6	Conv	10^2	0066	-	-	-	3.48	-	+	+	+	4.18	8.18	6.70	7.95
5	6	Conv	10^2	0069	-	+	+	-	+	+	+	+	4.48	5.18	5.78	5.97
5	6	Conv	10^2	0075	-	-	-	3.18	-	-	-	+	2.88	5.48	3.88	5.98

+ in quantification series are broilers excreting CTX-M-1-*E. coli* (i.e. growth of *E. coli* on MacConkey + cefotaxime), but excretion values were below detection limit or missing.

† chick died

Table S3 Parameter estimates for excretion levels (log_{10} CFU/g faeces, 95% CI) of CMY-2-*E. coli* in experiment I (*n*=129) and CTX-M-1-*E. coli* in experiment II (*n*=70), including broilers with excretion levels above detection limit, using a mixed linear regression model.

Experiment	Variable		Estimate (95% CI)
I	Time (T hours post inoculation)	T=9, Dose 10^1 (intercept*)	6.94 (5.67 – 8.21)
		T=32	3.12 (2.44 – 3.81)
		T=56	3.45 (2.76 – 4.15)
		T=72	4.06 (3.38 – 4.74)
	Body weight day 0 (hatch)		-0.07 (-0.10 – -0.04)
	Interaction Time (T) : Dose	T=9 * Dose 10^2	0.87 (0.12 – 1.61)
		T=32 * Dose 10^2	-0.83 (-1.43 – -0.22)
		T=56 * Dose 10^2	-0.23 (-0.83 – 0.38)
		T=72 * Dose 10^2	-0.91 (-1.51 – -0.31)
		T=9 * Dose 10^3	0.66 (-0.06 – 1.38)
		T=32 * Dose 10^3	-0.42 (-1.01 – 0.16)
		T=56 * Dose 10^3	0.06 (-0.55 – 0.67)
		T=72 * Dose 10^3	-0.25 (-0.85 – 0.35)
		T=9 * Dose 10^4	0.90 (0.12 – 1.68)
		T=32 * Dose 10^4	-0.23 (-0.98 – 0.51)
		T=56 * Dose 10^4	-0.53 (-1.25 – 0.19)
		T=72 * Dose 10^4	-1.07 (-1.75 – -0.40)
		T=9 * Dose 10^5	1.66 (0.89 – 2.44)
		T=32 * Dose 10^5	-0.22 (-0.90 – 0.44)
		T=56 * Dose 10^5	-0.79 (-1.47 – -0.11)
		T=72 * Dose 10^5	-0.78 (-1.44 – -0.12)
II	Time (T hours post inoculation)	T=9, Dose 10^1, SPF, Replicate 1 (intercept*)	5.28 (4.65 – 5.90)
		T=32	2.77 (2.13 – 3.42)
		T=56	2.94 (2.30 – 3.57)
		T=72	2.64 (1.99 – 3.28)
	Dose	Dose 10^2	0.66 (0.23 – 1.12)
	Replicate	Replicate 2	-0.55 (-1.00 – -0.12)
	Interaction Time (T) : Type of broiler	T=9 * Conventional	-2.60 (-3.70 – -1.49)
		T=32 * Conventional	-4.57 (-5.28 – -3.85)
		T=56 * Conventional	-3.63 (-4.26 – -3.02)
		T=72 * Conventional	-2.68 (-3.27 – -2.09)

*the intercept for experiment I gives the estimate of excretion level of CMY-2-*E. coli* at 9 hours post inoculation, for dose 10^1, body weight at day 0 = 0 gram. The intercept for experiment II gives the estimate of excretion level of CTX-M-1-*E. coli* at 9 hours post inoculation, for SPF broilers receiving dose 10^1, in replicate 1.

CHAPTER 5

Early life supply of competitive exclusion products reduces colonization of Extended Spectrum Beta-Lactamase producing *Escherichia coli* in broilers

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Abstract

Broilers are an important reservoir of ESBL/pAmpC-producing bacteria. In previous studies a single supply of a competitive exclusion (CE) product before challenge with a high dose of ESBL/pAmpC-producing *E. coli* led to reduced colonization, excretion and transmission, but could not prevent colonization. The hypothesized mechanism is competition; therefore in this study the effect of a prolonged supply of CE products on colonization, excretion and transmission of ESBL-producing *E. coli* after challenge with a low dose at day 0 or day 5 was investigated. Day-old broilers (Ross 308) (*n*=220) were housed in isolators. Two CE products, containing unselected fermented intestinal bacteria (CEP) or a selection of pre- and probiotics (SYN), were supplied in drinking water from day 0 to 14. At day 0 or day 5 broilers were challenged with 0.5 mL with 10^1 or 10^2 CFU/mL *E. coli* encoding the beta–lactamase gene $bla_{CTX\text{-}M\text{-}1}$ on an IncI plasmid (CTX-M-1-*E. coli*). Presence and concentration of CTX-M-1-*E. coli* was determined using cloacal swabs (day 0-14, 16, 19, 21) and cecal content (day 21). Cox proportional hazard model and a mixed linear regression model were used to determine the effect of the intervention on colonization and excretion ($\log_{10}$ CFU/gram). When challenged at day of hatch, no effect of CEP was observed. When challenged at day 5, both CEP and SYN led to a prevention of colonization with CTX-M-1-*E. coli* in some isolators. In the remaining isolators, we observed reduced time until colonization (HR between 3.71 $\times 10^{-3}$ and 3.11), excretion (up to -1.60 $\log_{10}$ CFU/gram) and cecal content (up to -2.80 $\log_{10}$ CFU/gram) and a 1.5 to 3-fold reduction in transmission rate. Colonization after a low dose challenge with ESBL-producing *E. coli* can be prevented by CE products. However, if at least one bird is colonized it spreads through the whole flock. Prolonged supply of CE products provided shortly after hatch, may be applicable as an intervention to reduce prevalence of ESBL/pAmpC-producing bacteria in the broiler production chain.

Introduction

Plasmid mediated Extended Spectrum Beta-Lactamase and AmpC Beta-Lactamase (ESBL/pAmpC) producing bacteria are resistant to extended spectrum cephalosporins (ESC). ESBL/pAmpC-producing *Escherichia coli* are present in the environment, humans and animals (Blaak et al., 2015). Although prevalence has decreased in recent years in different animal sectors (Dorado-Garcia et al., 2016; MARAN, 2019; Hesp et al., 2019), broilers are still an important reservoir of ESBL/pAmpC-producing bacteria and high prevalence in poultry flocks and poultry products are reported from several European countries (Saliu et al., 2017). The broiler production chain has a pyramidal structure with a few purebred pedigree farms at the top and many broiler farms at the bottom, with multiplier and crossbreeding steps in between. ESBL/pAmpC-producing *E. coli* have been found in all levels of the production chain (Dierikx et al., 2013a; Apostolakos et al., 2019). Transmission occurs via several routes, vertically between different levels of the chain, horizontally within and between farms, and via the (farm) environment (Dame-Korevaar et al., 2019a). Consequently, introduction of ESBL/pAmpC-producing *E. coli* in a broiler flock can occur at different moments, e.g. in the hatchery, during transport or shortly after arrival at the farm, or during the fattening phase.

To reduce the prevalence of ESBL/pAmpC-producing *E. coli* in the broiler production chain, interventions targeted at different transmission routes are needed. Examples include reducing exposure of the flock to bacteria from the farm environment using hygiene barriers, or from the previous flock by cleaning and disinfection between production rounds. However, these interventions are not always sufficient in preventing colonization (Daehre et al., 2018). In addition, other types of interventions can be used to attempt to prevent colonization of resistant *E. coli*, such as supplying products via feed or water, like feed additives (Roth et al., 2017). Interventions applicable simultaneously at different levels of the production chain will most likely help control the spread of ESBL/pAmpC-producing *E. coli* in broilers, and consequently in meat products, as measures taken at the top of the pyramid can affect the presence of ESBL/pAmpC-producing *E. coli* at lower levels of the pyramid as well. Furthermore, the rapid colonization of young broilers, even after exposure to a low dose of ESBL/pAmpC-producing *E. coli* (Dame-Korevaar et al., 2019b) shows that interventions should be taken as soon as possible after hatching. Delayed colonization observed in conventional broilers which carried initial *E. coli*, compared to SPF broilers not carrying *E. coli* upon hatch (Dame-Korevaar et al., 2019b), suggests that the gut microbiome plays an important role in susceptibility to colonization of ESBL/pAmpC-producing *E. coli*, and that this susceptibility may vary between development phases (Jurburg et al., 2019). Therefore, influencing the gut microbiome at an early age could potentially be a high impact intervention, applicable at different levels of the broiler pyramid. This can be done using the concept of competitive exclusion.

Competitive exclusion (CE) is based on early establishment of natural intestinal bacteria, to protect the bird from colonization with certain other bacteria (Nurmi et al., 1992). Different

types of competitive exclusion products, containing non-pathogenic bacterial cultures of single or mixed strains (Callaway et al., 2008), are available for poultry. The bacterial strains in these products can be defined, or consist of unselected intestinal bacteria from adult specific pathogen-free (SPF) chickens (e.g. Aviguard®). Also, some products contain a selection of pre- and probiotics, so called synbiotics. These CE products reduce colonization of foodborne pathogens, such as *Salmonella* (Nakamura et al., 2002; Ferreira et al., 2003; Luoma et al., 2017; Markazi et al., 2018). The administration of a CE product to day-old broilers before challenge resulted in decreased intestinal and cecal colonization with resistant pathogenic *E. coli* (Hofacre et al., 2002). Other studies showed that, in absence of antibiotics, a single oral supply of a CE product led to reduced cecal content (CFU/gram) (Nuotio et al., 2013; Methner et al., 2019), excretion and transmission (Ceccarelli et al., 2017) upon challenge with a high dose (10^5 to 10^8 CFU/mL) of ESBL/pAmpC-producing *E. coli*, but could not prevent colonization in the gut. However, under field circumstances the first colonized birds have likely been exposed to much lower numbers of ESBL/pAmpC-producing *E. coli* (Laube et al., 2013; Blaak et al., 2015), especially in a properly cleaned and disinfected poultry house. Exposure to a lower dose of ESBL/pAmpC-producing *E. coli* will reduce the risk of colonization (Dame-Korevaar et al., 2019b), and the bacteria present in the CE products will most likely result in further reduction of this risk. In addition, a longer supply of a CE product might be more effective by supplying more of the competitive bacteria.

In this study, we investigated the effect of prolonged supply of CE products in drinking water on time until colonization, excretion and transmission of ESBL-producing *E. coli* after challenge with a low dose. In three transmission experiments with contact birds and orally inoculated seeder birds the effect of two types of CE products (unselected fermented intestinal bacteria from SPF chickens (CEP) and a synbiotic selection of pre- and probiotics (SYN)) was investigated. Two scenarios of ESBL-producing *E. coli* introduction were studied: exposure of broilers to a low dose of ESBL-producing *E. coli* at day of hatch (experiment I) and during the first week of life (experiments II and III).

Material and methods

Three consecutive experiments were conducted (Table 1). In experiment I, broilers were challenged at day of hatch (day 0) with 0.5 mL with 10^1 or 10^2 CFU/mL CTX-M-1-*E. coli* and intervention groups received a competitive exclusion product in the drinking water, derived from unselected fermented intestinal bacteria from SPF birds (CEP). In experiments II and III, broilers were challenged at day 5 with 0.5 mL with 10^1 or 10^2 CFU/mL CTX-M-1-*E. coli* and intervention groups received either CEP or a competitive exclusion product based on synbiotics containing a selection of pre- and probiotics (SYN).

Table 1 Date, age parent stock (weeks), day (0 or 5) and dose (non-inoculated, or 0.5 mL of 10^1 or 10^2 CFU/mL) of challenge, and intervention (None (-), CEP or SYN) for experiments I, II and III.

Experiment	I		II		III	
Date	12 April – 3 May 2017		24 May – 14 June 2017		23 Oct – 13 Nov 2017	
Parent flock [a]	A, 54 weeks		A, 60 weeks		B, 57 weeks	
Day of challenge [b]	Day 0		Day 5		Day 5	
Isolator	**Challenge**	**Intervention**	**Challenge (CFU/mL)**	**Intervention**	**Challenge (CFU/mL)**	**Intervention**
1	Non-inoculated (-)	-	Non-inoculated (saline solution)	-	Non-inoculated (saline solution)	-
2	10^1	None (-)	10^1	None (-)	10^2	None (-)
3	10^1	CEP	10^1	CEP	10^2	CEP
4	10^1	CEP	10^1	CEP	10^2	CEP
5	10^2	None (-)	10^2	None (-)	10^2	SYN
6	10^2	CEP	10^2	CEP	10^2	SYN
7	10^2	CEP	10^2	CEP	10^2	SYN
8					10^2	SYN

[a] In all three experiments the eggs were disinfected with formaldehyde before incubation and in the hatcher before hatching.

[b] Challenge with *E. coli* E38.27 with $bla_{CTX\text{-}M\text{-}1}$ on IncI1 plasmid.

Ethics of experimentation

Broilers were observed daily and the presence of clinical signs, abnormal behaviour and mortality were recorded. The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands) under registration number AVD108002015314 and all procedures were done in full compliance with all legislation.

Birds, housing and management

In all three experiments, 100 conventional broilers (Ross 308) were transported on the morning of the day of hatch (referred to as day 0 of age and day 0 of the experiment) to the animal facilities (Utrecht University, Utrecht, the Netherlands); they were individually tagged and weighed and randomly divided over the isolators (Table 1). In experiment I, part of the broilers (*n*=43, randomly selected) were placed temporally in two other isolators, 35 of these broilers were selected for the remainder of the experiment and inoculated with CTX-M-1-*E. coli* (see challenge). One hour after inoculation, the inoculated (referred to as *seeder*) broilers were moved using transport boxes and added to the non-inoculated (referred to as *contact*) broilers in isolators 2 to 7 (5 seeder, 5 contact broilers per isolator). In experiments II and III, upon arrival at day 0 all broilers were randomly distributed over isolators 1 to 7 (experiment II) or 1 to 8 (experiment III) (max. 15 broilers per isolator). At day 5, just before the moment of inoculation, 10 broilers per isolator were selected for the remainder of the experiment, and randomly assigned to contact (*n*=5) or seeder (*n*=5) birds. The seeder broilers were inoculated. The surplus broilers not assigned as contacts or seeders in

experiments I, II and III, including all broilers with signs of reduced health or development or low hatching weight, were euthanized using cervical dislocation and removed from the isolator. Before the start of each of the three experiments samples were taken from the parent flock, incubators, hatchers and research facilities to confirm the absence of ESBL/pAmpC-producing *E. coli*.

Broilers were housed in negative pressure HEPA isolators, on paper linings with fine wood shavings. Standard broiler diet without any antibiotics or coccidiostats, radiated with 9 Gy, was available *ad libitum*. Feed and water were available from day 0, 4:00 p.m. The intervention was supplied in the drinking water (next paragraph). A few broilers died or were euthanized before the end of the experiment due to causes unrelated to the experiment (8 in experiment I, 2 in experiment II, and 1 in experiment III).

Intervention: competitive exclusion product

Composition

In this study two CE products were used: 1) competitive exclusion product (CEP) containing unselected, fermented intestinal bacteria, derived from SPF chickens and manufactured by fermentation (Aviguard®, MSD Animal Health Nederland, Boxmeer, the Netherlands) (experiments I, II and III); and 2) a selection of a prebiotic compound and probiotic bacterial strains (SYN): fructo-oligosaccharides (FOS) and *Enterococcus faecium*, *Bifidobacterium animalis*, *Lactobacillus salivarius* (Poultrystar® sol, Biomin Holding GmbH, Getzersdorf, Austria) (experiment III).

Supply

The competitive exclusion products were supplied from day of hatch (day 0), 4:00 p.m., until day 14, 4:00 p.m., twice a day, in the drinking water. Solutions with CEP or SYN in water were prepared in predilution directly before application with a dose according to recommendations of the manufacturer, i.e. 0.125 gram CEP versus 0.2 gram SYN per 10 broilers, and was added to the drinkers within the isolator. The amount of drinking water was restricted between day 0 and 14, based on the expected water consumption of 10 broilers in an isolator to ensure that all supplied CEP or SYN product would be consumed. Control groups received drinking water according to the same schedule, but without any intervention added.

Cloacal and cecal samples

Samples were taken using sterile dry cotton swabs (MW100, Medical Wire & Equipment, England, during day 0-3, and Copan 155C, Copan Diagnostics, USA, from day 4 onwards). All birds were sampled just before inoculation to confirm absence of ESBL/pAmpC-producing bacteria (and additionally at day 1 and 3 in experiments II and III), and from the moment of inoculation until day 7 twice a day (8:00 a.m., 4:00 p.m.), daily between day 8 to 14, and on days 16, 19, 21 (8:00 a.m.). At day 21, after the last sampling, *post mortem* examination was done within at maximum 30 minutes after euthanasia on each broiler. Broilers were weighed, sex was determined, broilers

were checked for exterior and interior abnormalities and ceca were collected and stored on ice for further analysis.

E. coli challenge

Broilers were challenged with *E. coli* strain E38.27, which carries the ESBL gene $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 plasmid, selected from healthy broilers and resistant to cefotaxime (Dierikx et al., 2010) using a 1 mL syringe without a needle with 0.5 mL of 10^1 or 10^2 CFU/mL. From 1 hour after inoculation onwards, 5 contact birds were exposed to 5 seeder birds, either by moving the inoculated seeder birds to the isolators containing the contact birds (experiment I) or by removing the temporal barrier between the inoculated seeder birds and the contact birds within the isolator (experiments II and III). The unchallenged control birds were not inoculated (experiment I) or received 0.5 mL physiological saline solution (experiments II and III).

ESBL-producing _E. coli_ detection

All cloacal samples except the ones used for quantification of ESBL-producing *E. coli* and total *E. coli* (next paragraph) were enriched in 3 mL Luria Bertani (LB) broth. After overnight incubation at 37°C, 10 µL broth was inoculated on MacConkey plates supplemented with 1 mg/L cefotaxime and incubated overnight at 37°C. *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CTX-M-1-*E. coli*. If visual assessment was inconclusive for the presence of *E. coli*, colonies were selected for further analysis using MALDI-TOF MS (Bruker Daltonik, Germany).

ESBL-producing _E. coli_ and total _E. coli_ quantification

Cloacal swabs obtained at 8:00 a.m. were weighed before and after sampling to determine the amount of faeces collected. The weight of the faecal material on the cloacal swab ranged from 0.01 – 0.43 gram. At day 21, content from one of two ceca was collected. Samples were processed as previously described (Dame-Korevaar et al., 2019b). Concentrations of ESBL-producing *E. coli* and total *E. coli* were determined semi-quantitatively (CFU/gram faeces), based on the highest consecutive dilution showing growth of typical *E. coli* colonies (Jett et al., 1997) and the weight of the faeces on the swabs or the amount of cecal content collected, as previously described (Ceccarelli et al., 2017). *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CTX-M-1-*E. coli*. If visual assessment was inconclusive for the presence of *E. coli*, colonies were selected for further analysis using MALDI-TOF MS (Bruker Daltonik, Germany).

Statistical analysis

Statistical analyses were performed in R, version 3.4.3 (RStudio Team, 2016), using packages "survival" (Cox proportional hazard regression) and "lme4" (mixed linear regression model).

Time until colonization

Individual broilers were considered colonized when two consecutive cloacal swabs tested positive for ESBL/pAmpC-producing *E. coli*. Time until colonization, using the first positive cloacal swab, was analysed using Cox proportional hazard regression. Validity of the assumptions of proportional hazards was checked using Schoenfeld residuals, and these assumptions were met.

Excretion

Broilers negative for ESBL/pAmpC-producing *E. coli* in the dilution series but positive after overnight culturing were included in the analysis with excretion concentration 1 $\log_{10}$ CFU/mL LB, as the minimum detection level of the semi-quantitative method was 2 $\log_{10}$ CFU/mL LB. Results based on negative swab weight (or weight = 0 gram) were excluded from the analysis. Moreover, samples negative for ESBL/pAmpC-producing *E. coli* after overnight culturing were excluded since the analysis was based on excreting broilers only. The effect of challenge dose and the intervention on the ESBL-producing *E. coli* and total *E. coli* excretion ($\log_{10}$ CFU/gram) was analysed using a mixed linear regression model including variables *time, intervention, dose, contact/seeder bird, weight at hatch, weight at day 21* and the interaction between *time* and *intervention*. Variable *sex* was only included for experiments I and II, as in experiment III only female birds were delivered by the hatchery. Random intercept was included, per *bird*, to adjust for clustered data in repeated measurements for the same bird. *Weight at hatch* and *weight at day 21* were included as continuous variables, the others as categorical variables. The best fitting model was obtained by backward selection, choosing the model with the lowest AIC value. Models with a difference in AIC of 2 or less were considered of equal fit and the most parsimonious model (lowest number of parameters) was chosen. Differences in ESBL-producing *E. coli* and total *E. coli* in cecal content ($\log_{10}$ CFU/gram) between the control and intervention groups were tested using a linear regression model including variables *intervention*, *dose*, *contact/seeder bird*, *weight at hatch*, *weight at day 21* and *sex*. The best fitting model was obtained by backward selection, choosing the model with the lowest AIC value. Models with a difference in AIC of 2 or less were considered of equal fit and the most parsimonious model (lowest number of parameters) was chosen.

Transmission

The transmission coefficient (β) was estimated using the data of experiments II and III based on the stochastic SI model (Velthuis et al., 2007; Dekker et al., 2013), in which the number of new cases is determined by transmission from infectious (I)-birds to susceptible (S) birds for a total population of (N) birds. The expected number of new cases (C) in time interval Δt is calculated by $E(C) = S(1\text{-}e^{-foi \times \Delta t})$. The force of infection (*foi*) was determined using different models. In model 1, direct transmission with mass action was assumed ($foi = \beta_{direct} \times I/N$), in which the force of infection was determined by the proportion of infectious birds (I-birds). In model 2, the cumulative time of excretion determined the force of infection ($foi = \beta_{time} \times \Sigma excrhours$), in

which Σexcrhours is the cumulative sum of hours that all infectious birds were excreting up to the beginning of the interval. In model 3, the cumulative excretion determined the force of infection ($foi = \beta_{concentration} \times$ Σexcrconcentration), in which Σexcrconcentration is the cumulative sum of excretion ($\log_{10}$ CFU/gram faeces) of all infectious birds. For all 3 models different assumptions regarding the input data of I-birds were compared; assuming that I-birds start to excrete at the moment of the first positive cloaca swab (basic model) or half an interval previous to the first positive cloaca swab (alternative model) (Table 4).

Performance

Differences in performance (growth between day of hatch and day 21) between the control and intervention groups were tested using a linear regression model including variables *intervention*, *dose*, *contact/seeder bird* and *sex*. The best fitting model was obtained by backward selection, choosing the model with the lowest AIC value. Models with a difference in AIC of 2 or less were considered of equal fit and the most parsimonious model (lowest number of parameters) was chosen.

Results

Time until colonization

Experiment I: CTX-M-1-E. coli challenge with 10^1 or 10^2 CFU/mL at day of hatch

All broilers were colonized with CTX-M-1-*E. coli* within 24 hours after inoculation (Table S1). There was no difference in the hazard of colonization between control broilers and CEP broilers, neither between broilers challenged with dose 10^1 or 10^2. However, isolators 2, 6 and 7 had a higher hazard of colonization than isolators 3, 4 and 5 (Table 2). Other variables, being seeder or contact bird, weight at day of hatch, weight at day 21 and sex did not influence the time until colonization.

Experiment II: CTX-M-1-E. coli challenge with 10^1 or 10^2 CFU/mL at day 5

Broilers challenged with 10^1 CFU/mL CTX-M-1-*E. coli* in both the control and the CEP groups were not colonized throughout the entire experiment. All broilers challenged with 10^2 CFU/mL CTX-M-1-*E. coli* were colonized within 48 (control) or within 144 (CEP) hours after inoculation (Figure 1 and Table 3). CEP broilers had a lower hazard of colonization (HR isolator 6: 0.08, 95% CI 0.02 – 0.42 and isolator 7: 3.71×10^{-3}, 95% CI 2.71×10^{-4} – 0.05) than the control isolator (Table 2). Being seeder or contact bird, weight at day of hatch, weight at day 21, sex, and the total *E. coli* excretion ($\log_{10}$ CFU/gram faeces) just before inoculation (day 5) did not influence the time until colonization.

Table 2 Hazard Ratio (HR, 95% CI) of time until colonization for experiments I (*n*=53), II, dose 10^2 (*n*=29) and III (*n*=40). Broilers were challenged with CTX-M-1-*E. coli* at day 0 in experiment I, and at day 5 in experiments II and III.

Experiment	Variable		HR (95% CI)
I	Isolator	2 (reference)	1
		3 (10^1 – CEP)	0.25 (0.09 – 0.72)
		4 (10^1 – CEP)	0.24 (0.08 – 0.68)
		5 (10^2 – control)	0.27 (0.09 – 0.76)
		6 (10^2 – CEP)	0.74 (0.25 – 2.20)
		7 (10^2 – CEP)	0.89 (0.29 – 2.71)
	Seeder/contact bird	Seeder (reference)	1
		Contact	0.67 (0.34 – 1.30)
	Body weight day 0 (hatch)		0.99 (0.91 – 1.09)
	Body weight day 21		1.00 (1.00 – 1.01)
	Sex	Male (reference)	1
		Female	0.91 (0.43 – 1.91)
II	Isolator	5 (10^2 - control)	1
		6 (10^2 - CEP)	0.08 (0.02 – 0.42)
		7 (10^2 - CEP)	3.71×10^{-3} (2.71×10^{-4} – 0.05)
	Seeder/contact bird	Seeder (reference)	1
		Contact	1.09 (0.40 – 2.98)
	Body weight day 0 (hatch)		0.93 (0.800 – 1.09)
	Body weight day 21		1.00 (0.99 – 1.00)
	Sex	Male (reference)	1
		Female	0.64 (0.25 – 1.60)
	Total *E. coli* (CFU/g faeces) day 5		1.06 (0.64 – 1.74)
III	Isolator	2 (10^2 - control)	1
		6 (10^2 - SYN)	0.07 (0.01 – 0.39)
		7 (10^2 - SYN)	1.29 (0.39 – 4.28)
		8 (10^2 - SYN)	3.11 (0.97 – 10.05)
	Seeder/contact bird	Seeder (reference)	1
		Contact	0.43 (0.20 – 0.94)
	Body weight day 0 (hatch)		1.03 (0.94 – 1.13)
	Body weight day 21		1.00 (0.99 – 1.00)
	Total *E. coli* (CFU/g faeces) day 5		1.18 (0.80 – 1.74)

Experiment III: CTX-M-1-E. coli challenge with 10^2 CFU/mL at day 5

Broilers treated with CEP were not colonized with CTX-M-1-*E. coli* during the experiment, whereas the broilers in two control isolators were colonized within 56 hours after inoculation. The broilers in one of the SYN isolators (isolator 5) were not colonized, the broilers in the other three SYN isolators were all colonized within 336 hours after inoculation (Figure 1 and Table 3). Although one of the SYN isolators showed a lower hazard than the control isolator (isolator 6, HR 0.07, 95% CI 0.01 – 0.39), for the broilers in the other isolators there was no effect of SYN on time until colonization (HR isolator 7: 1.29, 95% CI 0.39 – 4.28 and HR isolator 8: 3.11, 95% CI 0.97 – 10.05)

(Table 2). Weight at day of hatch and weight at day 21 and total *E. coli* excretion just before inoculation (day 5) did not influence time until colonization. However, contact birds had a lower hazard of colonization (HR 0.43, 95% CI 0.20 – 0.94) than seeder birds. The variable sex was not analysed, as only female broilers were included in experiment III.

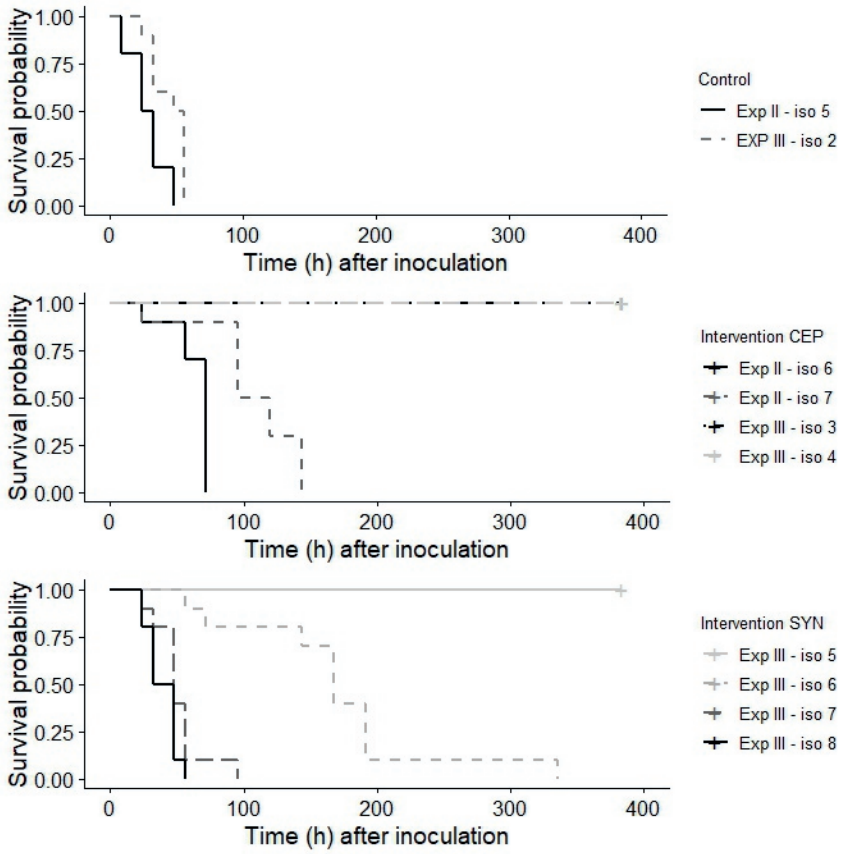

Figure 1 Survival curve of time until colonization of CTX-M-1-*E. coli* for experiments II and III, after challenge at day 5 with dose 10^2 CFU/mL.

Excretion

Experiment I: excretion of CTX-M-1-E. coli and total E. coli

The effect of the CEP product on both total *E. coli* and CTX-M-1-*E. coli* excretion differed per time point. Female birds excreted slightly higher concentrations of CTX-M-1-*E. coli* (0.23, 95% CI 0.03 – 0.43 $\log_{10}$ CFU/gram faeces) than male birds, and broilers challenged with either 10^1 or 10^2

Table 3 Detection (+/-) and quantification (log_{10} CFU/g faeces) of CTX-M-1-*E. coli* in broilers in experiments II (dose 10^2 CFU/mL) and III, determined at *n* hours post inoculation (*p.i.*) at day 5-7 (8:00 a.m. and 4:00 p.m.), 8 – 14, 16, 19, 21 (8:00 a.m.).

Exp	Iso	Dose	Intervention	Bird ID	Seeder/ contact	D5 8:00	D5 4:00	D6 8:00	D6 4:00	D7 8:00	D7 4:00	D8 8:00	D9 8:00	D10 8:00	D11 8:00	D12 8:00	D13 8:00	D14 8:00	D16 8:00	D19 8:00	D21 8:00
					hours p.i.	*0*	*8*	*24*	*32*	*48*	*56*	*72*	*96*	*120*	*144*	*168*	*192*	*216*	*264*	*336*	*384*
II	5	10^2	None	205	Seeder	-	+	3.63	+	5.48	+	5.78	3.57	6.52	6.63	7.57	5.44	6.40	3.88	5.57	5.25
	5	10^2	None	212	Seeder	-	-	2.57	+	4.63	+	5.57	4.52	4.48	4.63	2.57	4.57	5.36	4.52	4.63	4.70
	5	10^2	None	275	Seeder	-	-	3.52	+	4.63	+	2.57	5.78	3.63	3.52	4.57	4.30	5.05	3.52	6.78	4.30
	5	10^2	None	289	Seeder	-	-	2.63	+	4.52	+	3.48	3.44	3.78	2.48	3.00	2.44	2.52	4.70	4.78	6.36
	5	10^2	None	296	Seeder	+	-	-	+	3.63	+	3.63	5.40	6.52	3.57	4.57	5.52	3.48	6.63	4.70	5.22
	5	10^2	None	226	Contact	-	-	-	+	2.48	+	3.57	5.88	5.70	4.63	7.88	4.36	4.27	4.57	4.48	5.12
	5	10^2	None	233	Contact	-	-	-	-	2.33	+	2.52	2.40	2.78	2.52	5.52	3.40	4.70	4.63	6.57	6.13
	5	10^2	None	240	Contact	-	-	-	+	2.70	+	3.52	2.70	2.48	2.70	3.27	2.63	2.57	3.57	4.57	4.27
	5	10^2	None	261	Contact	-	-	2.63	-	2.44	+	2.33	2.48	3.52	3.44	2.27	2.63	3.44	6.57	5.44	4.20
	5	10^2	None	282	Contact	-	+	2.63	+	4.30	+	5.57	7.44	6.48	4.70	4.78	5.10	3.63	4.78	4.44	7.52
	6	10^2	CEP	220	Seeder	-	-	-	-	-	-	2.88	2.48	4.57	3.70	5.57	4.78	2.52	4.63	4.57	3.63
	6	10^2	CEP	234	Seeder	-	-	2.27	+	2.40	+	3.36	4.40	5.52	5.78	3.78	3.30	3.40	5.52	4.48	5.40
	6	10^2	CEP	241	Seeder	-	-	-	-	2.44	-	2.48	2.40	3.70	4.63	5.63	4.48	2.57	2.63	2.52	3.40
	6	10^2	CEP	269	Seeder	-	-	-	-	-	+	2.44	2.57	2.63	2.52	3.63	2.70	3.44	2.48	2.57	2.48
	6	10^2	CEP	290	Seeder	-	-	-	-	-	-	2.44	2.36	2.57	2.48	3.52	2.52	2.63	2.48	3.63	3.33
	6	10^2	CEP	206	Contact	-	-	-	-	-	-	2.63	3.52	4.57	4.00	5.70	2.52	†			
	6	10^2	CEP	227	Contact	-	-	-	-	-	-	2.52	2.52	2.57	3.88	3.63	3.48	4.52	3.57	4.52	4.27
	6	10^2	CEP	248	Contact	-	-	-	-	-	-	2.70	2.57	2.48	3.70	5.63	4.78	6.57	3.57	4.48	5.70
	6	10^2	CEP	255	Contact	-	-	-	-	-	-	2.63	2.40	3.40	3.63	2.57	3.95	3.22	3.57	3.48	3.18
	6	10^2	CEP	276	Contact	-	+	-	-	-	+	2.52	2.52	3.48	4.52	5.44	2.70	3.78	5.52	5.48	5.57
	7	10^2	CEP	207	Seeder	-	-	2.78	+	3.63	+	2.57	2.52	3.57	4.63	3.70	3.63	5.88	4.78	4.57	4.33
	7	10^2	CEP	242	Seeder	-	+	-	-	-	-	-	-	4.00	2.78	3.78	4.63	3.63	5.00	4.36	3.44
	7	10^2	CEP	277	Seeder	-	-	-	-	-	-	-	-	-	2.78	2.70	2.57	3.63	3.57	3.48	4.30
	7	10^2	CEP	291	Seeder	-	-	-	-	-	-	-	-	-	2.57	2.78	2.40	2.40	3.63	3.63	4.33
	7	10^2	CEP	298	Seeder	-	-	-	-	-	-	-	-	-	2.63	5.57	4.88	4.63	5.70	3.63	4.44
	7	10^2	CEP	214	Contact	-	-	-	-	-	-	-	3.44	3.63	4.63	4.63	4.36	3.57	3.48	4.57	4.27
	7	10^2	CEP	235	Contact	-	-	-	-	-	-	-	2.48	2.78	2.88	2.88	2.48	3.57	3.52	4.48	4.44
	7	10^2	CEP	256	Contact	-	-	-	-	-	-	-	2.48	2.63	2.52	3.63	3.52	2.30	4.52	4.44	5.25

Table 3 continued.

Exp	Iso	Dose	Intervention	Bird ID	Seeder/ contact	D5 8:00	D5 4:00	D6 8:00	D6 4:00	D7 8:00	D7 4:00	D8 8:00	D9 8:00	D10 8:00	D11 8:00	D12 8:00	D13 8:00	D14 8:00	D16 8:00	D19 8:00	D21 8:00
					hours p.i.	*0*	*8*	*24*	*32*	*48*	*56*	*72*	*96*	*120*	*144*	*168*	*192*	*216*	*264*	*336*	*384*
	7	10^2	CEP	263	Contact	-	-	-	-	-	-	-	2.57	2.52	2.70	4.33	3.30	3.36	3.70	2.44	2.40
	7	10^2	CEP	284	Contact	-	-	-	-	-	-	-	-	2.63	3.48	3.57	5.57	2.70	3.78	3.88	3.25
III	2	10^2	None	309	Seeder	-	-	-	+	2.30	+	2.52	2.52	4.40	4.48	3.36	4.27	4.25	3.57	3.22	5.27
	2	10^2	None	333	Seeder	-	-	-	+	3.25	+	4.57	4.48	3.57	5.44	5.25	5.40	7.25	5.48	6.03	5.06
	2	10^2	None	341	Seeder	-	-	3.70	+	4.22	+	5.48	2.48	5.57	4.33	4.27	4.36	5.57	4.52	5.18	6.05
	2	10^2	None	349	Seeder	-	-	-	+	2.70	+	3.57	3.48	5.48	4.15	6.25	5.15	3.36	4.48	5.18	5.33
	2	10^2	None	373	Seeder	-	-	-	-	-	+	2.36	4.52	4.15	5.48	5.30	3.57	5.20	5.52	5.25	5.33
	2	10^2	None	317	Contact	-	-	-	-	-	+	3.57	4.40	4.70	7.30	4.33	5.52	4.63	4.63	5.12	6.06
	2	10^2	None	325	Contact	-	-	-	-	2.13	+	2.52	3.44	5.44	6.13	5.36	5.27	5.25	4.57	4.27	7.20
	2	10^2	None	357	Contact	-	-	-	-	-	+	3.48	4.57	4.57	5.33	6.18	7.20	6.33	4.88	5.36	6.33
	2	10^2	None	365	Contact	-	-	-	-	-	+	4.40	3.52	4.52	7.40	4.30	6.00	6.22	4.57	4.25	5.84
	2	10^2	None	381	Contact	-	-	-	-	-	+	2.57	4.52	4.70	4.22	5.18	4.57	4.27	5.63	4.44	5.40
	6	10^2	SYN	305	Seeder	-	-	-	-	-	-	2.33	2.70	2.40	2.13	5.15	5.33	6.40	3.52	4.36	5.48
	6	10^2	SYN	329	Seeder	-	-	-	-	-	-	-	-	-	-	-	2.48	2.27	2.57	3.06	5.40
	6	10^2	SYN	337	Seeder	-	-	-	-	-	-	-	-	-	-	-	-	2.48	-	2.33	4.48
	6	10^2	SYN	353	Seeder	-	-	-	-	-	-	-	-	-	-	2.01	2.12	2.36	2.52	4.20	4.57
	6	10^2	SYN	361	Seeder	-	-	-	-	-	+	2.36	-	2.52	-	2.57	2.25	2.13	2.78	4.15	5.36
	6	10^2	SYN	313	Contact	-	-	-	-	-	-	-	-	-	1.85	2.15	2.40	2.15	2.70	2.15	3.36
	6	10^2	SYN	321	Contact	-	-	-	-	-	-	-	2.52	-	-	-	3.00	2.06	4.00	4.13	3.48
	6	10^2	SYN	369	Contact	-	-	-	-	-	-	-	-	-	-	-	2.36	3.00	3.57	3.13	3.48
	6	10^2	SYN	377	Contact	-	-	-	-	-	-	-	-	-	-	2.57	2.40	3.18	2.63	2.48	4.30
	6	10^2	SYN	385	Contact	-	-	-	-	-	-	-	-	-	-	2.48	2.33	4.57	4.70	3.33	4.63
	7	10^2	SYN	314	Seeder	-	-	2.01	+	2.40	+	4.20	2.36	3.25	4.06	4.30	3.70	4.12	+*	4.25	3.33
	7	10^2	SYN	338	Seeder	-	-	-	+	4.48	+	6.40	4.44	4.44	4.52	3.57	4.63	3.70	3.63	5.12	4.20
	7	10^2	SYN	354	Seeder	-	-	-	-	-	+	-	3.57	2.30	5.18	4.12	4.36	3.44	3.63	6.27	4.52
	7	10^2	SYN	370	Seeder	-	-	-	-	-	+	3.27	4.57	3.48	3.27	3.12	3.36	3.40	-	6.44	3.44
	7	10^2	SYN	394	Seeder	-	-	-	-	2.57	+	3.48	3.63	4.20	4.13	4.33	4.25	5.88	4.88	3.03	4.25
	7	10^2	SYN	322	Contact	-	-	-	-	3.63	+	3.48	3.57	4.30	4.30	5.52	5.30	4.52	4.78	5.20	5.52
	7	10^2	SYN	330	Contact	-	-	-	-	3.44	+	2.27	3.57	4.44	3.70	4.20	5.44	5.25	4.48	5.36	4.30

Table 3 continued.

Exp	Iso	Dose	Intervention	Bird ID	Seeder/ contact	D5 8:00	D5 4:00	D6 8:00	D6 4:00	D7 8:00	D7 4:00	D8 8:00	D9 8:00	D10 8:00	D11 8:00	D12 8:00	D13 8:00	D14 8:00	D16 8:00	D19 8:00	D21 8:00
					hours p.i.	*0*	*8*	*24*	*32*	*48*	*56*	*72*	*96*	*120*	*144*	*168*	*192*	*216*	*264*	*336*	*384*
	7	10^2	SYN	362	Contact	-	-	-	-	2.33	+	2.48	2.78	5.05	5.22	3.33	6.27	4.70	3.70	3.90	4.15
	7	10^2	SYN	378	Contact	-	-	-	-	-	+	3.27	3.70	2.95	4.33	5.57	5.22	6.44	5.70	6.36	6.70
	7	10^2	SYN	386	Contact	-	-	-	-	-	+	3.27	3.44	+*	5.18	4.05	4.30	4.44	3.70	4.12	5.44
	8	10^2	SYN	315	Seeder	-	-	2.52	+	4.57	+	3.33	3.52	5.48	2.91	3.85	3.40	3.18	2.44	3.00	2.78
	8	10^2	SYN	347	Seeder	-	-	2.57	+	2.52	+	3.44	4.25	4.57	3.33	+*	3.52	3.52	2.40	2.44	4.57
	8	10^2	SYN	355	Seeder	-	-	-	+	5.36	+	5.44	6.12	4.27	3.22	4.30	3.10	4.12	2.52	3.40	3.15
	8	10^2	SYN	363	Seeder	-	-	-	+	4.36	+	3.48	6.40	4.88	3.70	4.63	4.48	3.63	2.78	2.44	3.52
	8	10^2	SYN	379	Seeder	-	-	-	+	4.25	+	3.70	4.10	3.63	4.12	3.33	4.18	4.48	2.63	2.27	3.25
	8	10^2	SYN	307	Contact	-	-	-	-	-	+	4.00	2.15	3.40	6.27	3.33	3.08	3.44	2.70	+*	3.40
	8	10^2	SYN	323	Contact	-	-	-	-	2.48	+	2.30	2.99	3.48	3.48	4.30	4.27	3.57	3.88	3.52	4.36
	8	10^2	SYN	331	Contact	-	-	-	-	2.25	+	4.63	4.44	2.63	4.22	3.33	4.40	4.48	-	3.20	4.52
	8	10^2	SYN	387	Contact	-	-	-	-	2.40	+	2.78	3.63	4.44	4.27	3.36	3.27	2.52	2.70	3.13	5.57
	8	10^2	SYN	395	Contact	-	-	-	-	2.18	+	3.48	4.36	4.40	4.36	3.40	4.20	4.44	3.52	3.06	3.70

* + in quantification series are broilers excreting CTX-M-1-*E. coli* (i.e. growth of *E. coli* on MacConkey + cefotaxime), but excretion values were missing.

† chick died

CFU/mL CTX-M-*E. coli* excreted slightly lower concentrations of total *E. coli* than non-inoculated broilers (-0.81, 95% CI -1.14 – -0.48 versus -0.85, 95% CI -1.19 – -0.51 $\log_{10}$ CFU/gram faeces) (Table S2). Concentrations of CTX-M-1-*E. coli* in cecal content were lower in CEP broilers than control broilers (-0.71, 95% CI -1.06 – -0.37 $\log_{10}$ CFU/gram cecal content) and higher in broilers receiving dose 10^2 than dose 10^1 (0.46, 95% CI 0.14 – 0.79 $\log_{10}$ CFU/gram cecal content). Total *E. coli* concentrations in cecal content were slightly lower in CEP broilers than control broilers (-0.36, 95% CI -0.63 – -0.08 $\log_{10}$ CFU/gram cecal content, Table S3).

Experiment II: excretion of CTX-M-1-E. coli and total E. coli

Broilers challenged with 10^1 CFU/mL CTX-M-1-*E. coli* did not excrete CTX-M-1-*E. coli* during the experiment. CEP broilers challenged with 10^2 CFU/mL excreted lower concentrations of CTX-M-1-*E. coli* (-0.89, 95% CI -1.33 – -0.45 $\log_{10}$ CFU/gram faeces) than control broilers. Female birds excreted slightly higher concentrations of CTX-M-1-*E. coli* (0.48, 95% CI 0.04 – 0.92 $\log_{10}$ CFU/gram faeces) than male birds. CEP broilers excreted lower or equal concentrations of *E. coli* than control broilers, except at day 1, but without a clear pattern (Table S2). Mean concentrations of total *E. coli* and CTX-M-1-*E. coli* in cecal content were lower in CEP broilers than control broilers (-0.51, 95% CI -0.79 – -0.22, versus -2.80, 95% CI -3.47 – -2.14 $\log_{10}$ CFU/gram cecal content, Table S3).

Experiment III: excretion of CTX-M-1-E. coli and total E. coli

CEP broilers did not excrete CTX-M-1-*E. coli*. SYN broilers excreted lower concentrations of CTX-M-1-*E. coli* than control broilers from day 10 onwards. Total *E. coli* excretion concentrations in CEP and SYN broilers were lower than or equal to the control broilers, except at day 1, however the excretion per day was highly variable without a clear pattern (Table S2). The concentrations of CTX-M-1-*E. coli* in cecal content of SYN broilers were lower (-1.13, 95% CI -1.94 – -0.33 $\log_{10}$/gram cecal content) than in control broilers. Total *E. coli* concentrations were lower in CEP broilers than in control broilers (-1.50, 95% CI -1.76 – -1.24 $\log_{10}$/gram cecal content, Table S3).

Transmission

The transmission coefficients (β_{direct} , β_{time} and $\beta_{concentration}$) were estimated using the data of experiments II and III. These could not be estimated from experiment I because most broilers (seeder and contact) in the control and CEP isolators were colonized already at the first sampling moment (16 hours) after inoculation. Also, estimation of the transmission coefficients in the CEP groups in experiment III was not possible, because the inoculation did not lead to colonization in the CEP groups.

Transmission coefficients (β_{direct} , β_{time} and $\beta_{concentration}$) estimated using the assumptions in the alternative model (assuming that I-birds start to excrete half an interval previous to the first positive cloaca swab, having slightly lower AIC values than the basic model), were lower in both intervention groups than in the control groups, based on model 2 (β_{time}: CEP: 0.19 day^{-2}, 95%

CI 0.04 – 0.87, SYN: 0.09 day^{-2}, 95% CI 0.02 – 0.40, control: 0.27 day^{-2}, 95% CI 0.13 – 0.49) and model 3 ($\beta_{concentration}$: CEP: 0.12 (CFU × day)$^{-1}$, 95% CI 0.15 – 0.56, SYN: 0.14 (CFU × day)$^{-1}$, 95% CI 0.02 – 0.63, control: 0.31 (CFU × day)$^{-1}$, 95% CI 0.10 – 0.57). The transmission coefficients (β_{direct}, day^{-1}) estimated based on model 1 were not different (β_{direct}: CEP 2.57 day^{-1}, 95% CI 0.51 – 11.47, SYN: 1.58 day^{-1}, 95% CI 0.35 – 6.57, control: 2.19 day^{-1}, 95% CI 1.09 – 3.91) (Table 4). The unit of β in model 2 is day^{-2} and can be interpreted as the number of new colonized broilers caused by a positive broiler per day for each day this broiler has been excreting CTX-M-1-*E. coli*. The unit of β in model 3 is (CFU × day)$^{-1}$ and can be interpreted as the number of new colonized broilers caused by a positive broiler per day, for each excreted unit of $\log_{10}$ CTX-M-1-*E. coli* per gram of faeces. In addition, a second alternative model was tested including the assumption that I-birds not colonized at 32 hours after inoculation were S-birds. However, this assumption did not improve the fit of the model (data not shown).

Table 4 Transmission coefficients (β, 95% CI) for experiments II and III, using an SI-model, for the basic model (assuming I-birds start to excrete at the moment of the first positive cloaca swab) and the alternative model (assuming I-birds start to excrete half an interval previous to the first positive cloaca swab).

Transmission coefficient (β, 95% CI)						
	Basic model			**Alternative model**		
	Model 1 (day^{-1})	Model 2 (day^{-2})	Model 3 (CFU × day)$^{-1}$	Model 1 (day^{-1})	Model 2 (day^{-2})	Model 3 (CFU × day)$^{-1}$
Control	2.93 (1.38 – 5.40)	0.40 (0.19 – 0.76)	0.31 (0.10 – 0.57)	2.19 (1.09 – 3.91)	0.27 (0.13 – 0.49)	0.31 (0.10 – 0.57)
CEP	4.08 (0.76 – 19.43)	0.30 (0.05 – 1.48)	0.12 (0.15 – 0.56)	2.57 (0.51 – 11.47)	0.19 (0.04 – 0.87)	0.12 (0.15 – 0.56)
SYN	2.22 (0.46 – 9.96)	0.12 (0.02 – 0.57)	0.14 (0.02 – 0.63)	1.58 (0.35 – 6.57)	0.09 (0.02 – 0.40)	0.14 (0.02 – 0.63)
AIC	82.5	87.8	102.0	78.7	86.8	102.0

Expected number of cases (C) is in model 1: $E(C) = S(1 - e^{-\beta time \times \frac{I}{N} \times \Delta t \sum excrconcentration})$,
model 2: $E(C) = S(1 - e^{-\beta time \times \sum excrconcentration \times \Delta t})$,
model 3: $E(C) = S(1 - e^{-\beta concentration \times \sum excrconcentration \times \Delta t})$. In model 3 cumulative excretion (CFU/g faeces) is independent of the number of I-birds and is therefore independent of the assumption regarding the start of excretion.

Performance

There was no effect of CEP on growth (experiments I, II, and III). In experiment III, SYN broilers had higher growth (from day of hatch until day 21) than control broilers (1021.1, 95% CI 914.1 – 1128.0 gram versus 914.8, 95% CI 866.5 – 963.1 gram). However, this effect was mainly explained by the higher growth of broilers in one of the SYN isolators (isolator 7, mean growth 1070.0, 95% CI 884.9 – 1228.1 gram).

Discussion

The supply of CE products to broilers during the first two weeks of life resulted in a lower time until colonization and excretion of CTX-M-1-*E. coli* and even in prevention of colonization of broilers challenged with a low dose of CTX-M-1-*E. coli* at day 5. Moreover, transmission rates of CTX-M-1-*E. coli* were lower in the broilers receiving one of the CE products (CEP or SYN) than in the control broilers. In contrast, the supply of CEP when challenged at day of hatch did not affect colonization. Our results show that a prolonged supply of CE products can be a useful intervention to prevent or reduce colonization of ESBL/pAmpC-producing *E. coli* in a broiler flock, when exposure occurs after supply of CE products. These results are in line with earlier studies showing a reduction in transmission, colonization and excretion of *Salmonella* (Nakamura et al., 2002; Ferreira et al., 2003; Luoma et al., 2017; Markazi et al., 2018), pathogenic *E. coli* (Hofacre et al., 2002) and ESBL/pAmpC-producing *E. coli* (Nuotio et al., 2013; Ceccarelli et al., 2017; Methner et al., 2019), when providing CE products before challenge. Moreover, in our study we were able to prevent colonization of CTX-M-1-*E. coli*, possibly as a result of the prolonged supply of CE products, whereas in earlier studies a single supply of CE products did not result in prevention of colonization of a group of birds (Hofacre et al., 2002; Nuotio et al., 2013; Ceccarelli et al., 2017; Methner et al., 2019). In contrast to our study, in the studies of Nuotio et al. (2013) and Ceccarelli et al. (2017) broilers were exposed to high concentrations of ESBL-producing *E. coli*, whereas we used a low dose aiming to mimic the initial stages of colonization of a flock in the field. A prolonged supply of CE product followed by exposure to lower concentrations of EBSL-producing *E. coli* might give more potential for the bacteria in the CE products, and less potential for the ESBL/pAmpC-producing *E. coli*, to colonize.

Challenge with dose 10^1 at day 5 in experiment II did not result in colonization of CTX-M-1-*E. coli* in control and intervention groups, although the results of experiment I and earlier studies showed that with this low dose young broilers could become colonized (Dame-Korevaar et al., 2019b). However, in this earlier study broilers were challenged at day 1, whereas we challenged at day 5, simulating exposure to ESBL/pAmpC-producing *E. coli* during the first week at the farm. This age effect suggests that susceptibility to colonization is reduced with age (Chauvin et al., 2013; Braykov et al., 2016). Although we did not analyse microbiota composition in this study, it is likely that the gut microbiome composition might have played a role, as the different successive stages in microbiome development (Jurburg et al., 2019) may also result in different stages of susceptibility to colonization with certain bacteria. Analysis of the microbiome would require experiments with intensive sampling of intestinal content for comparisons of the changes in microbiota composition in intervention- and control groups, to facilitate understanding of the underlying mechanisms behind the differences in observed time until colonization. However, due to the different factors influencing microbiota composition (Kers et al., 2018) many broilers would need to be tested to avoid spurious correlations.

The difference in hazard rate of colonization between CEP and SYN groups compared to the control groups might be caused by the composition of the products. Both products are aimed at establishing competitive exclusion, but CEP contains natural, live, fermented intestinal microflora from SPF chickens, whereas SYN contains a prebiotic compound (FOS) and probiotic bacterial strains (*Enterococcus faecium*, *Bifidobacterium animalis* and *Lactobacillus salivarius*). In our study the total concentrations of *E. coli* at day 5, just before inoculation, did not influence time until colonization. Therefore, the protective effects of the CE products might not be in the competition between the different *E. coli* strains (initially present, inoculated and in the supplied intervention), but between other (combinations of) supplied bacteria. Likely, the two CE products have affected the gut microbiota composition in different ways, but to what extent and how this may have affected colonization of ESBL/pAmpC-*E. coli* in the intestinal tract cannot be elucidated with the data available from these experiments.

Some of the observed differences between isolators can also be a result of the so-called "cage effect"; animals housed together tend to show less variation in microbiota composition than a random group of animals, as described for mice (Laukens et al., 2016), which might result in differences in susceptibility to colonization between groups. Furthermore, other host and environmental factors can affect the microbiota composition and can influence experimental outcomes, as reviewed by Kers et al. (2018). Although we cannot exclude such effects completely, the experimental design was aimed to keep the impact of potential confounding factors to a minimum. All broilers originated from the same flock, were handled in the same way and the isolators were intensively cleaned and disinfected before the start of the experiment.

The supply of CE products did not affect the time until colonization when provided at the same time as the ESBL-producing *E. coli* challenge (day of hatch, experiment I). This is in line with earlier studies (Ceccarelli et al., 2017), showing that the effect of competitive exclusion depends on the time of supply (Varmuzova et al., 2016) and indicates that the CE products need time to be established in the gut, before they can protect broilers from colonization with low dose of ESBL-producing *E. coli* that may be present at the farm, for example due to insufficient cleaning and disinfection, via parallel housed flocks, or from the environment (Dame-Korevaar et al., 2019a).

The prevention of colonization (experiments II and III), and the fast colonization of one seeder bird in both isolators in experiment II followed by colonization of the remaining seeder birds and the contact birds, suggest that the effect of CE upon low dose exposure mainly lies in the prevention of colonization, rather than substantially affecting transmission. Nevertheless, transmission rates were lower in the intervention groups than in the control groups, according to model 2 and 3. We did not find this reduction when assuming direct transmission. Model 1 did have the lowest AIC value, but from biological reasoning environmental transmission should be a better model. ESBL/pAmpC-producing *E. coli* can survive in the environment for months (Merchant et al., 2012; Friese et al., 2013), therefore the presence of ESBL/pAmpC-producing *E. coli* in the litter will facilitate transmission via the faecal-oral route, as described for *Eimeria acervulina* (Velkers et al., 2012).

Thus, the accumulation of *E. coli* in the environment should be taken into account, as is done in model 2, with force of infection based on excretion time of infectious broilers. We suggest to use this model for generalization to larger populations, as it best describes the biological mechanisms of transmission of ESBL/pAmpC-producing *E. coli.* Model 3, with force of infection based on excretion concentrations, did not improve the fit of the model. However, in both models including the environment CE products reduce the transmission coefficients.

The colonization of ESBL-producing *E. coli* in the broilers' intestinal tract as observed in our experiments likely is a result of both vertical and horizontal (via conjugation) transfer of the plasmids present in the inoculum *E. coli* to other *E. coli* strains. This is reflecting the transmission dynamics of ESBL/pAmpC-producing *E. coli* in field situations (Huijbers et al., 2016; van Hoek et al., 2018), where horizontal gene transfer occurs naturally and is part of the transmission process.

In conclusion, CE products can prevent and reduce initial colonization, but if only one bird is successfully colonized and starts to excrete ESBL/pAmpC-producing *E. coli*, the subsequent spread through the flock is inevitable. Therefore, additional interventions are needed to reduce transmission. CE products need time to get established in the gut, therefore should be applied as soon as possible after hatch, before broilers are exposed to ESBL/pAmpC-producing *E. coli*. It is recommended to further study the mechanisms behind the dynamical processes in the gut responsible for the competitive exclusion effects, and to determine the best timing and type of bacterial composition manipulations to optimize these interventions strategies for practical use.

Acknowledgements

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Table S1 Detection (+/-) and quantification (log_{10} CFU/g faeces) of CTX-M-1-*E. coli* in broilers in experiment I, determined at *n* hours post inoculation (*p.i.*) at day 1-7 (8:00 a.m. (M) and 4:00 p.m. (A)), 8 – 14, 16, 19, 21 (8:00 a.m. (M)).

Iso	Dose	IV[a]	Bird ID	S/C[b]	D0	D1 M	D1 A	D2 M	D2 A	D3 M	D3 A	D4 M	D4 A	D5 M	D5 A	D6 M	D6 A	D7 M	D7 A	D8 M	D9 M	D10 M	D11 M	D12 M	D13 M	D14 M	D16 M	D19 M	D21 M
			hours p.i.		*0*	*16*	*24*	*40*	*48*	*64*	*72*	*88*	*96*	*112*	*120*	*136*	*144*	*160*	*168*	*184*	*208*	*232*	*256*	*280*	*304*	*328*	*376*	*448*	*496*
2	10^1	None	107	S	-	4.88	+	4.78	+	4.60	+	4.27	+	4.56	+	4.18	+	4.23	+	4.40	4.11	4.18	4.00	4.40	4.08	4.12	4.14	4.48	3.81
2	10^1	None	124	S	-	4.60	+	4.78	+	4.78	+	4.26	+	4.08	+	4.33	+	4.18	+	4.65	4.78	4.22	4.48	4.40	4.26	4.41	4.54	4.40	3.97
2	10^1	None	142	S	-	4.00	+	5.08	+	4.56	+	4.33	+	4.56	+	4.21	+	4.12	+	4.48	4.48	4.00	4.48	4.08	4.26	4.02	3.94	4.27	4.26
2	10^1	None	146	S	-	4.95	+	4.78	+	4.56	+	4.48	+	4.18	+	4.48	+	4.18	+	4.48	4.11	4.18	4.40	4.23	3.91	4.48	4.11	4.56	4.18
2	10^1	None	163	S	-	4.48	+	4.88	+	4.48	+	4.56	+	4.22	+	4.27	+	3.97	+	4.57	4.23	4.33	4.48	4.48	4.38	4.30	4.48	4.70	3.97
2	10^1	None	115	C	-	4.65	+	4.70	+	4.48	+	4.12	+	4.14	+	4.56	+	4.33	+	4.30	4.38	4.38	4.60	4.48	4.38	4.18	4.26	4.65	3.92
2	10^1	None	141	C	-	4.18	+	5.26	+	4.40	+	4.35	+	4.32	+	4.48	+	4.21	+	4.60	4.56	4.40	4.88	5.18	4.30	†			
2	10^1	None	154	C	-	4.95	+	5.26	+	4.72	+	4.35	+	4.41	+	4.56	+	4.54	+	4.65	4.60	4.38	4.30	4.48	4.18	4.27	4.28	4.23	4.32
2	10^1	None	178	C	-	4.78	+	4.95	+	4.60	+	4.56	+	4.48	+	4.41	+	4.04	+	4.70	4.60	4.38	4.18	4.70	4.08	4.41	4.21	4.30	3.97
2	10^1	None	191	C	-	4.18	+	+	+	4.95	+	4.48	+	4.48	+	4.40	+	4.48	+	4.48	4.33	4.40	4.38	4.18	4.18	4.35	4.21	4.48	4.18
3	10^1	CEP	116	S	-	-	+	4.57	+	4.57	+	4.21	+	3.88	+	4.30	+	4.30	+	4.05	4.00	3.63	4.26	4.30	4.05	3.93	4.12	3.95	3.57
3	10^1	CEP	129	S	-	-	+	4.78	+	4.85	+	4.18	+	4.26	+	4.48	+	4.30	+	4.38	4.30	4.48	4.23	4.30	3.70	4.35	4.38	3.93	3.97
3	10^1	CEP	155	S	-	+	+	4.78	+	4.70	+	4.40	+	4.13	+	4.88	+	4.27	+	4.57	4.18	4.48	4.05	4.00	3.93	3.78	3.93	4.30	3.70
3	10^1	CEP	168	S	-	5.18	+	4.48	+	4.48	+	4.27	+	4.23	+	+	+	4.26	+	4.35	4.30	4.27	4.05	3.93	4.12	4.22	4.22	4.18	3.93
3	10^1	CEP	192	S	-	-	+	5.08	+	4.48	+	4.18	+	4.27	+	4.57	+	4.26	+	4.30	4.18	4.33	4.08	4.18	4.27	4.18	4.12	4.00	3.55
3	10^1	CEP	118	C	-	4.18	+	4.95	+	4.48	+	4.40	+	4.04	+	4.30	+	4.18	+	4.60	4.18	4.18	4.00	4.40	4.48	3.93	4.11	4.00	3.81
3	10^1	CEP	144	C	-	-	+	4.95	+	4.48	+	4.21	+	4.11	+	4.23	+	4.12	+	4.30	4.48	3.84	4.08	4.00	4.30	3.95	4.30	3.82	3.93
3	10^1	CEP	157	C	-	-	+	4.78	+	4.65	+	4.62	+	4.54	+	4.78	+	4.40	+	4.30	4.30	4.30	4.18	4.18	3.93	3.48	4.12	4.00	3.40
3	10^1	CEP	181	C	-	4.78	+	4.78	+	4.65	+	4.60	+	4.06	+	4.60	+	4.48	+	4.35	4.60	4.30	4.48	4.38	4.38	4.40	4.18	4.38	3.88
3	10^1	CEP	194	C	-	-	+	4.88	+	5.26	+	4.54	+	4.48	+	4.33	+	4.35	+	4.38	4.95	4.30	4.18	3.93	4.65	4.11	4.48	3.88	4.18
4	10^1	CEP	120	S	-	-	+	4.48	+	4.60	+	4.33	+	4.70	+	4.88	+	†											
4	10^1	CEP	137	S	-	4.48	+	4.78	+	5.02	+	4.21	+	4.40	+	4.41	+	4.40	+	4.00	4.70	4.30	4.38	4.26	4.00	4.13	4.13	4.18	3.70
4	10^1	CEP	159	S	-	-	+	4.78	+	4.48	+	4.30	+	4.30	+	4.48	+	4.65	+	4.18	4.48	4.26	4.18	4.48	4.60	4.30	4.30	4.33	3.88
4	10^1	CEP	172	S	-	4.30	+	4.60	+	4.65	+	4.48	+	4.33	+	4.48	+	3.95	+	4.08	4.18	4.11	4.00	4.04	4.35	4.05	4.05	4.18	3.88
4	10^1	CEP	196	S	-	4.78	+	4.88	+	4.57	+	4.48	+	4.18	+	4.33	+	4.30	+	4.48	4.48	4.11	4.35	4.30	4.11	4.38	4.22	4.26	3.88
4	10^1	CEP	145	C	-	-	+	4.78	+	4.88	+	4.18	+	4.12	+	3.88	+	4.12	+	4.11	4.13	4.04	4.18	3.93	4.04	4.22	4.33	4.12	3.97
4	10^1	CEP	158	C	-	-	+	4.70	+	4.48	+	4.48	+	4.35	+	4.57	+	4.26	+	4.30	4.30	4.12	4.40	4.30	4.48	4.18	4.18	4.18	4.00
4	10^1	CEP	171	C	-	-	+	4.70	+	4.60	+	4.48	+	4.48	+	4.00	+	4.48	+	4.40	4.38	4.23	4.78	4.40	4.30	4.12	4.18	4.30	4.22

Table S1 continued.

Iso	Dose	IV[a]	Bird ID	S/C[b]	D0	D1 M	D1 A	D2 M	D2 A	D3 M	D3 A	D4 M	D4 A	D5 M	D5 A	D6 M	D6 A	D7 M	D7 A	D8 M	D9 M	D10 M	D11 M	D12 M	D13 M	D14 M	D16 M	D19 M	D21 M
			hours p.i.		*0*	*16*	*24*	*40*	*48*	*64*	*72*	*88*	*96*	*112*	*120*	*136*	*144*	*160*	*168*	*184*	*208*	*232*	*256*	*280*	*304*	*328*	*376*	*448*	*496*
4	10^1	CEP	182	C	-	-	+	4.60	+	4.48	+	4.23	+	4.05	+	4.26	+	4.48	+	4.05	4.05	4.18	4.26	4.26	4.48	4.30	4.23	4.48	3.93
4	10^1	CEP	195	C	-	-	+	4.48	+	4.60	+	4.33	+	4.18	+	4.23	+	4.30	+	4.30	4.48	3.95	4.35	4.18	4.30	4.27	4.08	4.33	3.88
5	10^2	None	104	S	-	-	+	4.78	+	4.70	+	4.70	+	4.33	+	4.48	+	4.70	+	4.38	4.48	4.27	4.35	4.33	4.30	4.00	4.48	4.48	4.26
5	10^2	None	125	S	-	4.18	+	5.02	+	4.78	+	4.21	+	4.30	+	4.48	+	4.40	+	4.23	3.95	4.60	4.48	†					
5	10^2	None	147	S	-	4.78	+	4.95	+	4.78	+	4.18	+	4.48	+	4.30	+	4.11	+	4.30	4.11	3.82	4.05	4.18	4.00	4.35	4.05	4.04	3.81
5	10^2	None	169	S	-	4.78	+	5.26	+	4.78	+	4.57	+	4.48	+	4.30	+	4.48	+	4.57	3.88	3.74	4.30	4.23	4.00	4.30	4.57	4.38	4.00
5	10^2	None	188	S	-	4.18	+	4.95	+	4.95	+	4.21	+	4.22	+	4.23	+	4.70	+	4.22	4.00	3.76	4.06	4.38	4.24	4.04	3.97	4.11	4.04
5	10^2	None	109	C	-	-	+	4.57	+	4.88	+	4.04	+	4.23	+	4.78	+	4.65	+	4.70	4.40	4.48	4.11	4.18	4.18	4.12	4.18	4.33	†
5	10^2	None	122	C	-	4.08	+	4.88	+	5.26	+	4.41	+	4.18	+	4.26	+	4.00	+	4.26	3.95	4.38	4.33	4.30	4.35	3.91	4.21	4.40	4.03
5	10^2	None	161	C	-	-	+	5.18	+	4.70	+	4.48	+	4.33	+	4.48	+	4.30	+	4.23	4.11	4.30	4.30	3.72	4.30	4.18	4.48	4.18	4.08
5	10^2	None	185	C	-	-	+	4.78	+	4.33	+	4.11	+	4.05	+	4.23	+	4.04	+	4.12	4.26	4.11	4.88	4.11	4.38	4.23	4.40	4.23	3.90
5	10^2	None	198	C	-	-	+	4.78	+	4.48	+	4.12	+	4.18	+	4.11	+	3.81	+	4.08	4.48	4.95	4.78	4.33	4.35	4.00	4.35	4.23	3.93
6	10^2	CEP	112	S	-	+	+	4.48	+	4.60	+	4.22	+	4.30	+	4.40	+	3.88	+	4.35	4.35	4.18	4.05	4.48	3.97	4.18	4.56	4.11	3.97
6	10^2	CEP	134	S	-	4.30	+	4.88	+	+	+	4.38	+	4.48	+	4.33	+	4.08	+	4.23	4.08	4.05	4.48	4.08	3.90	4.23	4.48	†	
6	10^2	CEP	156	S	-	4.88	+	5.18	+	5.32	+	4.48	+	4.48	+	4.40	+	4.95	+	4.35	4.35	4.78	5.08	4.30	4.18	4.08	4.35	†	
6	10^2	CEP	173	S	-	5.26	+	4.78	+	4.57	+	4.41	+	4.40	+	4.40	+	4.48	+	4.08	3.93	4.12	4.11	4.23	4.10	4.18	4.12	4.23	4.06
6	10^2	CEP	193	S	-	4.48	+	4.88	+	4.57	+	4.12	+	4.41	+	4.48	+	4.35	+	4.26	4.38	4.18	4.18	4.33	4.38	4.11	4.18	4.12	3.85
6	10^2	CEP	123	C	-	4.65	+	4.70	+	4.48	+	4.72	+	4.40	+	4.48	+	4.38	+	4.30	4.30	4.23	4.26	4.18	4.60	4.26	4.22	4.30	3.97
6	10^2	CEP	136	C	-	4.60	+	5.18	+	4.78	+	4.72	+	4.57	+	4.38	+	3.82	+	†									
6	10^2	CEP	162	C	-	+	+	4.95	+	4.57	+	4.42	+	4.60	+	4.40	+	4.88	+	4.35	4.48	4.18	4.38	4.40	4.33	4.05	4.18	4.11	3.93
6	10^2	CEP	175	C	-	-	+	4.78	+	4.60	+	4.11	+	4.30	+	4.56	+	4.70	+	4.26	4.30	4.18	4.30	4.65	4.23	4.38	3.95	3.93	3.84
6	10^2	CEP	199	C	-	4.78	+	+	+	4.95	+	4.27	+	4.37	+	4.48	+	4.57	+	4.26	4.48	4.18	4.26	4.30	4.30	4.30	4.03	4.05	4.06
7	10^2	CEP	117	S	-	4.95	+	+	+	4.70	+	4.41	+	4.41	+	4.33	+	4.38	+	4.35	4.00	4.23	4.23	4.30	4.40	3.82	4.04	4.08	3.88
7	10^2	CEP	143	S	-	4.78	+	4.70	+	4.78	+	4.13	+	4.11	+	4.35	+	4.22	+	4.30	4.30	4.22	4.05	4.18	4.18	3.78	4.00	4.18	3.90
7	10^2	CEP	164	S	-	4.78	+	4.95	+	4.65	+	4.48	+	4.40	+	4.35	+	+	+	4.57	4.12	4.18	4.48	4.18	4.48	3.90	4.22	4.30	3.78
7	10^2	CEP	180	S	-	4.38	+	4.57	+	4.40	+	4.18	+	4.30	+	4.26	+	4.40	+	4.38	4.11	4.00	4.05	4.48	4.88	3.78	4.30	3.95	3.75
7	10^2	CEP	197	S	-	4.48	+	4.78	+	4.65	+	4.57	+	4.48	+	4.23	+	4.48	+	4.48	4.48	4.26	4.35	3.93	4.48	4.08	4.40	4.11	4.05
7	10^2	CEP	113	C	-	4.48	+	4.95	+	4.60	+	4.57	+	4.48	+	4.18	+	4.30	+	4.18	4.33	4.22	4.40	4.78	3.66	3.60	4.18	3.88	3.48

Table S1 continued.

Iso	Dose	IV[a]	Bird ID	S/C[b]	D0	D1 M	D1 A	D2 M	D2 A	D3 M	D3 A	D4 M	D4 A	D5 M	D5 A	D6 M	D6 A	D7 M	D7 A	D8 M	D9 M	D10 M	D11 M	D12 M	D13 M	D14 M	D16 M	D19 M	D21 M
			hours p.i.		*0*	*16*	*24*	*40*	*48*	*64*	*72*	*88*	*96*	*112*	*120*	*136*	*144*	*160*	*168*	*184*	*208*	*232*	*256*	*280*	*304*	*328*	*376*	*448*	*496*
7	10^2	CEP	126	C	-	-	+	4.85	+	4.78	+	4.35	+	4.33	+	4.40	+	4.18	+	4.38	4.23	4.13	4.26	4.35	4.65	3.88	4.08	3.91	3.57
7	10^2	CEP	139	C	-	3.70	+	4.85	+	5.26	+	4.48	+	4.22	+	4.65	+	4.23	+	4.27	4.05	4.18	4.26	4.30	4.26	3.97	3.91	4.05	4.10
7	10^2	CEP	176	C	-	4.00	+	4.95	+	4.78	+	4.42	+	4.57	+	4.56	+	4.30	+	4.18	4.08	4.00	4.26	4.18	4.23	3.90	3.84	4.08	3.81
7	10^2	CEP	189	C	-	3.88	+	+	+	4.65	+	4.48	+	4.30	+	4.48	+	4.18	+	4.35	4.33	4.00	4.18	3.95	3.93	3.25	4.08	4.12	3.81

[a] Intervention

[b] Seeder-/Contact bird

† chick died

Table S2 Estimates for excretion (log_{10} CFU/g faeces, 95% CI) of CTX-M-1-*E. coli* and total *E. coli* in experiments I (*n*= 53, 62), II (*n*=29, 68) and III (*n*=42, 79), using a mixed linear regression model.

Experiment	Variable		CTX-M-1-*E. coli* (95% CI)	Total *E. coli* (95% CI)
I	Time	Day 1, male, non-inoculated, control group (intercept)	5.07 (4.50 – 5.63)	6.68 (6.17 – 7.19)
		Day 2	2.40 (1.67 – 3.13)	1.29 (0.72 – 1.86)
		Day 3	1.86 (1.14 – 2.58)	0.90 (0.35 – 1.46)
		Day 4	1.43 (0.71 – 2.15)	1.03 (0.47 – 1.58)
		Day 5	1.34 (0.62 – 2.06)	1.13 (0.58 – 1.69)
		Day 6	1.20 (0.48 – 1.92)	0.66 (0.10 – 1.22)
		Day 7	0.79 (0.07 – 1.51)	0.70 (0.14 – 1.26)
		Day 8	1.05 (0.33 – 1.77)	0.79 (0.24 – 1.35)
		Day 9	0.35 (-0.37 – 1.07)	0.44 (-0.11 – 1.00)
		Day 10	0.36 (-0.36 – 1.08)	0.52 (-0.03 – 1.08)
		Day 11	0.92 (0.20 – 1.63)	0.61 (0.06 – 1.17)
		Day 12	0.83 (0.11 – 1.54)	0.71 (0.15 – 1.27)
		Day 13	0.13 (-0.59 – 0.85)	0.74 (0.18 – 1.30)
		Day 14	0.80 (0.08 – 1.52)	0.61 (0.05 – 1.17)
		Day 16	2.04 (1.33 – 2.76)	1.36 (0.80 – 1.91)
		Day 19	1.05 (0.33 – 1.77)	0.75 (0.19– 1.31)
		Day 21	0.78 (0.07 – 1.50)	0.46 (-0.09 – 1.02)
	Sex	Female	0.23 (0.03 – 0.43)	
	Dose (CFU/mL)	10^1		-0.81 (-1.14 – -0.48)
		10^2		-0.85 (-1.19 – -0.51)
	Time * Intervention	Day 1 * CEP	0.29 (-0.43 – 1.01)	-0.12 (-0.75 – 0.51)
		Day 2 * CEP	-0.28 (-0.90 – 0.33)	0.32 (-0.19 – 0.83)
		Day 3 * CEP	-0.10 (-0.70 – 0.49)	0.39 (-0.10 – 0.87)
		Day 4 * CEP	0.43 (-0.16 – 1.03)	0.20 (-0.28 – 0.69)
		Day 5 * CEP	-0.22 (-0.81 – 0.38)	-0.37 (-0.86 –0.11)
		Day 6 * CEP	0.06 (-0.54 – 0.66)	0.36 (-0.13 – 0.85)
		Day 7 * CEP	-0.12 (-0.72 – 0.48)	-0.53 (-1.02 – -0.04)
		Day 8 * CEP	-0.96 (-1.56 – -0.37)	-0.56 (-1.05 – -0.07)
		Day 9 * CEP	-0.28 (-0.88 – 0.31)	-0.21 (-0.69 – 0.28)
		Day 10 * CEP	-0.38 (-0.98 – 0.21)	-0.52 (-1.00 – -0.03)
		Day 11 * CEP	-1.02 (-1.61 – -0.42)	-0.60 (-1.09 – -0.12)
		Day 12 * CEP	-0.64 (-1.24 – -0.05)	-0.55 (-1.04 – -0.07)
		Day 13 * CEP	0.07 (-0.52 – 0.67)	-0.54 (-1.02 – -0.05)
		Day 14 * CEP	-0.99 (-1.58 – -0.39)	-0.32 (-0.81 – 0.17)
		Day 16 * CEP	-1.39 (-1.98 – -0.79)	-0.70 (-1.19 – -0.21)
		Day 19 * CEP	-1.22 (-1.82 – -0.63)	-0.45 (-0.93 – 0.04)
		Day 21 * CEP	-0.94 (-1.53 – -0.34)	-0.39 (-0.88 – 0.10)
II	Time	Day 1, control group (intercept *E. coli*)		5.22 (4.85 – 5.60)
		Day 3		1.72 (1.25 – 2.20)
		Day 5		2.55 (2.07 – 3.02)
		Day 6, male, control group (intercept CTX-M-1-*E. coli*)	2.53 (1.75 – 3.31)	1.95 (1.48 – 2.42)
		Day 7	0.78 (-0.04 – 1.61)	1.79 (1.32 – 2.27)
		Day 8	0.81 (0.03 – 1.58)	2.16 (1.69 – 2.63)
		Day 9	1.05 (0.30 – 1.81)	1.92 (1.44 – 2.39)

Table S2 continued.

Experiment	Variable		CTX-M-1-*E. coli* (95% CI)	Total *E. coli* (95% CI)
		Day 10	1.49 (0.73 – 2.24)	1.59 (1.12 – 2.06)
		Day 11	1.36 (0.62 – 2.11)	1.16 (0.69 – 1.64)
		Day 12	1.98 (1.23 – 2.72)	1.80 (1.32 – 2.27)
		Day 13	1.54 (0.80 – 2.28)	1.71 (1.23 – 2.18)
		Day 14	1.52 (0.77 – 2.26)	1.13 (0.66 – 1.61)
		Day 16	1.98 (1.23 – 2.72)	1.26 (0.79 – 1.73)
		Day 19	2.12 (1.37 – 2.86)	1.54 (1.06 – 2.01)
		Day 21	2.23 (1.49 – 2.98)	1.80 (1.33 – 2.27)
	Intervention	CEP	-0.89 (-1.33 – -0.45)	
	Time * Intervention	Day 1 * CEP		1.30 (0.82 – 1.77)
		Day 3 * CEP		-0.29 (-0.72 – 0.15)
		Day 5 * CEP		-0.84 (-1.28 – -0.40)
		Day 6 * CEP		-0.43 (-0.87 – 0.01)
		Day 7 * CEP		-0.69 (-1.13 – -0.25)
		Day 8 * CEP		-0.60 (-1.04 – -0.16)
		Day 9 * CEP		-0.83 (-1.27 – -0.40)
		Day 10 * CEP		-0.67 (-1.11 – -0.23)
		Day 11 * CEP		0.06 (-0.38 – 0.50)
		Day 12 * CEP		-0.70 (-1.14 – -0.26)
		Day 13 * CEP		-0.82 (-1.26 – -0.38)
		Day 14 * CEP		-0.15 (-0.59 – 0.29)
		Day 16 * CEP		-0.04 (-0.48 – 0.40)
		Day 19 * CEP		-0.48 (-0.92 – -0.04)
		Day 21 * CEP		-0.56 (-1.00 – -0.12)
	Sex	Female	0.48 (0.04 – 0.92)	
III	Time	Day 1, control group (intercept *E. coli*)		5.58 (5.14 – 6.01)
		Day 3		1.10 (0.53 – 1.67)
		Day 5		1.05 (0.48 – 1.63)
		Day 6, control group (intercept CTX-M-1-*E. coli*)	3.64 (1.82 – 5.47)	0.87 (0.30 – 1.44)
		Day 7	-0.62 (-2.58 – 1.33)	0.32 (-0.25 – 0.89)
		Day 8	-0.14 (-2.02 – 1.75)	0.44 (-0.14 – 1.01)
		Day 9	0.15 (-1.73 – 2.03)	0.57 (-0.01 – 1.14)
		Day 10	1.07 (-0.82 – 2.95)	0.83 (0.25 – 1.40)
		Day 11	1.78 (-0.10 – 3.67)	0.72 (0.15 – 1.29)
		Day 12	1.33 (-0.55 – 3.22)	0.66 (0.08 – 1.23)
		Day 13	1.49 (-0.39 – 3.37)	0.40 (-0.17 – 0.97)
		Day 14	1.59 (-0.29 – 3.47)	0.76 (0.19 – 1.34)
		Day 16	1.14 (0.74 – 3.03)	0.33 (-0.24 – 0.90)
		Day 19	1.19 (-0.70 – 3.07)	0.75 (0.18 – 1.32)
		Day 21	2.14 (0.26 – 4.03)	1.10 (0.52 – 1.67)
	Time * Intervention	Day 1 * CEP		1.29 (0.68 – 1.89)
		Day 3 * CEP		-0.77 (-1.38 – -0.17)
		Day 5 * CEP		-1.20 (-1.81 – -0.60)
		Day 6 * CEP		-0.64 (-1.25 – -0.03)
		Day 7 * CEP		-0.60 (-1.20 – 0.01)
		Day 8 * CEP		-0.37 (-0.98 – 0.24)
		Day 9 * CEP		-0.84 (-1.44 – -0.23)

Table S2 continued.

Experiment	Variable		CTX-M-1-*E. coli* (95% CI)	Total *E. coli* (95% CI)
		Day 10 * CEP		-0.87 (-1.48 – -0.27)
		Day 11 * CEP		-0.83 (-1.44 – -0.23)
		Day 12 * CEP		-1.16 (-1.76 – -0.55)
		Day 13 * CEP		-0.59 (-1.20 – 0.02)
		Day 14 * CEP		-1.19 (-1.80 – -0.59)
		Day 16 * CEP		-0.15 (-0.76 – 0.46)
		Day 19 * CEP		-0.79 (-1.41 – -0.18)
		Day 21 * CEP		-1.43 (-2.03 – -0.82)
		Day 1 * SYN		1.02 (0.50 – 1.55)
		Day 3 * SYN		0.27 (-0.25 – 0.80)
		Day 5 * SYN		-0.12 (-0.65 – 0.41)
		Day 6 * SYN	-1.18 (-3.28 – 0.93)	0.02 (-0.50 – 0.55)
		Day 7 * SYN	0.09 (-0.89 – 1.07)	0.43 (-0.10 – 0.95)
		Day 8 * SYN	-0.22 (-0.97 – 0.52)	0.44 (-0.09 – 0.97)
		Day 9 * SYN	-0.21 (-0.96 – 0.53)	-0.05 (-0.58 – 0.48)
		Day 10 * SYN	-1.05 (-1.80 – -0.30)	-0.11 (-0.64 – 0.41)
		Day 11 * SYN	-1.60 (-2.34 – -0.85)	-0.08 (-0.61 – 0.45)
		Day 12 * SYN	-1.35 (-2.08 – -0.62)	-0.02 (-0.55 – 0.50)
		Day 13 * SYN	-1.39 (-2.11 – -0.67)	0.36 (-0.17 – 0.89)
		Day 14 * SYN	-1.46 (-2.18 – -0.74)	-0.17 (-0.69 – 0.36)
		Day 16 * SYN	-1.39 (-2.12 – -0.67)	0.38 (-0.15 – 0.91)
		Day 19 * SYN	-1.06 (-1.79 – -0.35)	-0.05 (-0.58 – 0.48)
		Day 21 * SYN	-1.55 (-2.27 – -0.84)	-0.07 (-0.60 – 0.46)

Table S3 Estimates for cecal content (log_{10} CFU/g cecal content, 95% CI) of CTX-M-1-*E. coli* and total *E. coli* in experiments I (*n*=62, 53), II (*n*=68, 29) and III (*n*=79, 40), using a linear regression model.

Experiment	Variable		Estimate CTX-M-1-*E. coli* (95% CI)	Total *E. coli* (95% CI)
I	Treatment	Control, non-inoculated (intercept total *E. coli*) Control, dose 10^1 (intercept CTX-M-1-*E. coli*)	7.68 (7.35 – 8.01)	8.03 (7.71 – 8.34)
		CEP	-0.71 (-1.06 – -0.37)	-0.36 (-0.63 – -0.08)
	Dose (CFU/mL)	10^1		-0.27 (-0.68 – 0.14)
		10^2	0.46 (0.14 – 0.79)	0.15 (-0.26 – 0.56)
II	Treatment	Control, non-inoculated (intercept total *E. coli*) Control (intercept CTX-M-1-*E. coli*)	7.20 (6.67 – 7.74)	8.00 (7.68 – 8.32)
		CEP	-2.80 (-3.47 – -2.14)	-0.51 (-0.79 – -0.22)
	Dose (CFU/mL)	10^1		0.23 (-0.19 – 0.64)
		10^2		-0.27 (-0.68 – 0.14)
III	Treatment	Control (intercept total *E. coli* and CTX-M-1-*E. coli*)	7.14 (6.44 – 7.84)	7.99 (7.80 – 8.18)
		CEP		-1.50 (-1.76 – -1.24)
		SYN	-1.13 (-1.94 – -0.33)	-0.10 (-0.33 – 0.12)

CHAPTER 6

Competitive exclusion prevents colonization and compartmentalization reduces transmission of ESBL-producing *Escherichia coli* in broilers

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In preparation

Abstract

Extended spectrum β-lactamase (ESBL)-producing bacteria are resistant to extended-spectrum cephalosporins and are common in broilers. Interventions are needed to reduce the prevalence of ESBL-producing bacteria in the broiler production pyramid. This study investigated two different interventions. The effect of a prolonged supply of competitive exclusion (CE) product and compartmentalization on colonization and transmission, after challenge with a low dose of ESBL-producing *E. coli*, in broilers kept under semi-field conditions, were analysed. One-day-old broilers (Ross 308) (*n*=400) were housed in four experimental rooms, subdivided in one seeder (S/C1)-pen and eight contact (C2)-pens. In two rooms, CE product was supplied from day 0 to 7. At day 5, seeder-broilers were inoculated with *E. coli* strain carrying $bla_{CTX\text{-}M\text{-}1}$ on plasmid IncI1 (CTX-M-1-*E. coli*). Presence of CTX-M-1-*E. coli* was determined using cloacal swabs (day 5-21 daily) and cecal samples (day 21). Time until colonization and cecal excretion (log_{10} CFU/gram) were analysed using survival analysis and linear regression. Transmission coefficients within and between pens were estimated using maximum likelihood. The microbiota composition was assessed by 16S ribosomal RNA gene amplicon sequencing in cecal content of broilers on days 5 and 21. None of the CE broilers was CTX-M-1-*E. coli* positive. In contrast, in the untreated rooms 187/200 of the broilers were CTX-M-1-*E. coli* positive at day 21. Broilers in C2-pens were colonized later than seeder-broilers (TR 3.53, 95% CI 3.14 – 3.93). The transmission coefficient between pens was lower than within pens (3.28×10^{-4} day^{-2}, 95% CI 2.41×10^{-4} – 4.32×10^{-4} versus 6.12×10^{-2} day^{-2}, 95% CI 4.78×10^{-2} – 7.64×10^{-2}). The alpha diversity of the cecal microbiota content was higher in CE broilers than in control broilers at days 5 and 21. The supply of a CE product from day 0 to 7 prevented colonization of CTX-M-1-*E. coli* after challenge at day 5, possibly as a result of CE induced effects on the microbiota composition. Furthermore, compartmentalization reduced transmission rate between broilers. Therefore, a combination of compartmentalization and supply of a CE product may be a useful intervention to reduce transmission and prevent colonization of ESBL/pAmpC-producing bacteria in the broiler production pyramid.

Introduction

Extended Spectrum Beta-Lactamase and plasmid AmpC Beta-Lactamase (ESBL/pAmpC)-producing bacteria are resistant to extended-spectrum cephalosporins (ESC). ESBL/pAmpC-producing bacteria are present in humans, animals and the environment (Blaak et al., 2015). Poultry is known as a source of ESBL/pAmpC-producing bacteria and high prevalence in poultry and poultry products have been reported in several European countries, as reviewed by Saliu et al. (2017). ESBL/pAmpC-producing bacteria are present at all levels of the broiler production pyramid (Dierikx et al., 2013a; Agerso et al., 2014; Nilsson et al., 2014; Zurfluh et al., 2014a; Zurfluh et al., 2014b; Projahn et al., 2018). Different routes of transmission within the broiler production pyramid have been described, for example between generations, via the hatcheries, and on and between farms (Dame-Korevaar et al., 2019a). Introduction of ESBL/pAmpC-producing bacteria can occur at several levels of the broiler production pyramid, for example at the farm or at the hatchery. A recent study estimated that, based on the proportional similarity index (PSI), the average transfer of ESBL/pAmpC genes between subsequent generations in the broiler production pyramid is almost 50% (Apostolakos et al., 2019). However, for most of the routes it is unknown to what extent they contribute to the presence of ESBL/pAmpC-producing bacteria in the broiler production pyramid. In the Netherlands, antimicrobial resistance in broilers has decreased significantly since 2010 (Hesp et al., 2019), following the trend of reduced antimicrobial usage. However, additional interventions are needed to further reduce this prevalence in the broiler production pyramid.

Interventions can aim to reduce exposure of broilers to ESBL/pAmpC-producing *Escherichia coli*. This can be done by improving biosecurity. For example hygiene barriers can help reduce exposure to bacteria from the farm environment, or by cleaning and disinfection between production rounds. However, even after cleaning and disinfection, ESBL/pAmpC-producing bacteria might remain in the poultry house and result in colonization of the new flock (Daehre et al., 2018). In addition, housing measures may reduce the prevalence of ESBL/pAmpC-producing *E. coli* in poultry flocks. In turkeys, subdividing the flock was associated with a reduced risk for the presence of resistant *E. coli* in the farm (Jones et al., 2013). Experimental studies showed that spatial separation between infectious and susceptible animals reduced the transmission rate of *Campylobacter* in broilers (van Bunnik et al., 2012) and *Streptococcus suis* in pigs (Dekker et al., 2013). Further, interventions aiming at preventing colonization by ESBL/pAmpC-producing *E. coli* in broilers have been described, such as acid-based feed additives (Roth et al., 2017) or competitive exclusion (CE) products (Nuotio et al., 2013; Ceccarelli et al., 2017; Methner et al., 2019; Chapter 5). CE products are aimed at establishing a natural community of intestinal bacteria to protect broilers from colonization by invaders (Nurmi et al., 1992). In modern broiler production, due to strict hygiene practices in commercial hatcheries, the initial bacterial load to colonize the chicken intestinal tract shortly after hatch is low (Varmuzova et al., 2016; Donaldson

et al., 2017). Eggs are usually disinfected to remove bacterial contamination before placement in the hatcher. Consequently, the chicks are exposed mostly to bacteria from environmental sources rather than parental sources upon hatching. Microbial treatment supplied after hatch has been shown to affect the development of bacterial taxa found in growing chickens (Ballou et al., 2016; Schokker et al., 2017). This suggests that early supply of CE products might influence microbiota composition and act as a possible intervention to prevent colonization by ESBL/pAmpC-producing *E. coli* in young broilers. A single supply of CE product before challenge with a high dose of ESBL-producing *E. coli* has already showed to reduce colonization, cecal and faecal excretion (CFU/gram), as well as transmission of ESBL-producing *E. coli* (Nuotio et al., 2013; Ceccarelli et al., 2017; Methner et al., 2019). Additionally, CE products resulted in a reduced intestinal and cecal excretion (CFU/gram) after challenge with pathogenic *E. coli* (Hofacre et al., 2002). A prolonged supply of CE product via the drinking water to broilers kept in isolators, from day of hatch until day 14 resulted in a delay and even prevention of colonization after challenge with a in the field realistic low dose of ESBL-producing *E. coli* (Chapter 5).

The aim of this study was to determine the effect of interventions on colonization and transmission of ESBL-producing *E. coli* in young broiler chicks kept under semi-field circumstances. Two interventions were included: 1) prolonged supply of CE product from day of hatch until day 7, and 2) compartmentalization of a broiler flock. To investigate the effect of CE product on microbial composition, microbiota in cecal content was assessed before and after challenge by 16S ribosomal RNA (rRNA) gene amplicon sequencing.

Material and Methods

Ethics of experimentation

Broilers were observed daily and the presence of clinical signs, abnormal behaviour and mortality was recorded. The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands) under registration number AVD108002015314; all procedures were done in full compliance with all legislations.

Birds, housing and management

Conventional broiler chicks (Ross 308, *n*=416), from a parent stock flock of 37 weeks of age, were transported directly after hatch to the animal facilities (Utrecht University, Utrecht, the Netherlands). Upon arrival, the broilers were individually tagged, weighed, and randomly divided over four experimental rooms (*n*=104 broilers per room). Each room was subdivided into nine pens, with one seeder (S/C1)-pen in the middle (2 m^2, *n*=24 broilers), surrounded by eight contact (C2)-pens (1 m^2, *n*=10 broilers per pen) (Figure 1). The S/C1-pen was separated from the C2-pens

by a mesh panel (30 cm solid panel at the bottom, 40 cm mesh panel, 10 cm solid panel on top). Feed and water systems were also separated, and strict hygiene measures between pens were taken. No direct contact between the broilers was possible, but small particles (e.g. litter, dust) could be transferred between pens potentially. The C2-pens were separated from each other with wooden panels of 80 cm height, assuming no contact and no spread of particles was possible. At day 5, just before challenge with ESBL-producing *E. coli*, the number of broilers in the S/C1-pen was reduced to 20, by removing the surplus broilers. Ten of the remaining 20 broilers in each S/C1-pen were randomly selected and transported to four separate isolators. In these isolators, the broilers (seeder (S) broilers) were inoculated with CTX-M-1-*E. coli* and after one hour moved back to the original S/C1-pens (see paragraph Challenge). Before the start of the experiment the parent flock, hatchery and research facilities were tested for the absence of ESBL/pAmpC-producing bacteria.

Broilers were housed on fine wood shavings. A standard broiler diet without any antibiotics or coccidiostats, radiated with 9 Gy, was available *ad libitum*. The intervention was supplied in the drinking water (see paragraph Intervention); therefore, drinking water was not available *ad libitum* during the first seven days of the experiment in both intervention and control groups. Five broilers died or were euthanized before the end of the experiment due to causes unrelated to the experiment.

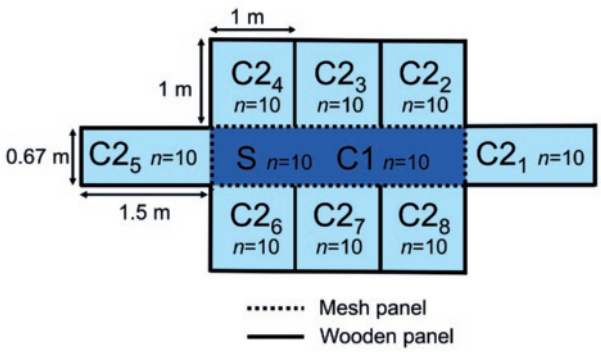

Figure 1 Schematic representation of the experimental set up of one of four broiler rooms (1 – 4). Each room was subdivided in nine pens, with one seeder (S/C1) pen in the middle (2 m^2) (*n*=10 S-broilers, 10 C1-broilers), surrounded by eight contact (C2) pens (1 m^2) (*n*=10 broilers per pen). The S/C1-pen was separated from the C2-pens by 80 cm high mesh panels. The C2-pens were separated from each other by 80 cm high wooden panels.

Intervention

In two of four rooms a competitive exclusion (CE) product was supplied, containing natural, live intestinal microflora derived from specific pathogen free (SPF) chickens and manufactured by fermentation (Aviguard®, MSD Animal Health, the Netherlands). From the moment of arrival in the rooms (day 0, 10:00 a.m.) until day 7, (4:00 p.m.), CE product was supplied in the drinking water, twice per day. Water solutions containing the CE product were prepared in predilution, with a dose level according to recommendations of the manufacturer, i.e. 0.125 gram CE product per 10 broilers. The amount of drinking water was restricted between day 0 and 7, based on the expected water consumption of 10 (C2-pen) and 20 (S/C1-pen) broilers in a pen to ensure that all supplied CE product would be consumed.

***E. coli* challenge**

Broilers were challenged with *E. coli* strain E38.27, which carries the ESBL gene $bla_{\text{CTX-M-1}}$ on an IncI1 plasmid (CTX-M-1-*E. coli*), isolated from conventional healthy broilers at slaughter age and resistant to cefotaxime (Dierikx et al., 2010). Oral inoculation of seeder (S) birds was performed on day 5 at 8:00 a.m. using a 1 mL syringe without a needle with 0.5 mL of 10^2 CFU/mL. The bacterial dilution was measured with the McFarland reader and retrospective colony counting. From one hour after inoculation onwards, 10 contact (C1) birds were exposed to 10 seeder birds, by moving the inoculated seeder birds to the corresponding S/C1-pens containing the contact birds.

Cloacal and cecal samples

Samples were taken using sterile dry cotton swabs (Copan 155C, Copan Diagnostics, USA). Broilers were sampled at day 5 at 4:00 a.m., just before inoculation to confirm absence of ESBL/pAmpC-producing bacteria, and from day 6 until day 21 daily at 8:00 a.m. At day 21, after the last sampling, *post mortem* examination was done within 30 minutes after euthanasia for each broiler. Broilers were weighed and sex was determined, exterior and interior abnormalities were assessed, and ceca were collected and stored on dry ice for further analysis.

Microbiota sample collection and analysis

Cecal content samples were collected from five surplus broilers of the control group and from five surplus broilers of the CE intervention group (n=10) at day 5. At day 21, cecal content of all broilers in the S/C1-pen in all four rooms (n=80) was collected. The closed side of one of the two ceca was cut and cecal content was gently squeezed into a 2 mL sterile cryotube and snap frozen on dry ice and stored at -80 °C for genomic DNA extraction. To determine the microbial composition of the CE product, Aviguard® was suspended in PBS according to the manufacturer's instructions and four aliquots of 2 mL were stored at -80°C for bacterial genomic DNA extraction. The full protocol for DNA extraction and determining microbiota composition was previously described (Kers et al., 2019). Briefly, DNA was extracted from 0.25 gram cecal content or frozen CE product,

using 700 µL of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands). All 94 samples were transferred to a sterile screw-capped 2 mL tube (BIOplastics BV, the Netherlands), used for bead beating. The DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, USA), and the DNA samples were stored at −20°C until further use. Barcoded amplicons covering the variable regions V5-V6 and primers 784F and 1064R were used for 16S rRNA gene-based microbial composition profiling as previously described (Ramiro-Garcia et al., 2016). To ensure high quality sequencing data, synthetic communities of known composition were used as positive controls (Ramiro-Garcia et al., 2016) and nuclease free water as negative controls. Sequencing of resulting libraries was performed on Illumina Hiseq2500 (Eurofins Genomics Germany GmbH). The 16S rRNA data was analysed using NG-tax 2.0 (Ramiro-Garcia et al., 2016). In short, to generate amplicon sequence variants (ASVs), NG-Tax 2.0 employed a fast de novo ASV-picking algorithm. To assign taxonomy the SILVA 128 16S rRNA gene reference database was used (Quast et al., 2013). Raw sequence data were deposited into the Sequence Read Archive (SRA) at the NCBI.

ESBL-producing *E. coli* detection

All cloacal samples were enriched in 3 mL Luria Bertani (LB) broth. After overnight incubation at 37°C, 10 µL broth were inoculated on MacConkey plates supplemented with 1 mg/L cefotaxime and incubated overnight at 37°C. *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CTX-M-1-*E. coli*. If visual assessment was not conclusive on the presence of *E. coli*, colonies were selected for further analyses using MALDI-TOF MS (Bruker Daltonik, Germany).

ESBL-producing *E. coli* and total *E. coli* quantification

At day 21, content from one of two ceca of 80 selected broilers from rooms 1 and 2 was collected. For both rooms, selection included all broilers (*n*=20) from the S/C1-pen and additionally 20 broilers from the C2-pens which were excreting CTX-M-1-*E. coli*. Samples were processed as previously described (Dame-Korevaar et al., 2019b). Concentrations of ESBL-producing *E. coli* and total *E. coli* were determined semi-quantitatively. CFU/gram faeces was calculated based on the highest dilution showing growth of typical *E. coli* colonies (Jett et al., 1997) and the weight of the faeces on the swabs or the amount of cecal content collected (Ceccarelli et al., 2017). *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CTX-M-1-*E. coli*. If visual assessment was not conclusive on the presence of *E. coli*, colonies were selected for further analyses using MALDI-TOF MS.

Statistical analysis

Statistical analyses were performed in R, version 3.4.3 (RStudio Team, 2016), using packages “survival”, “phyloseq”, “microbiome” and “vegan”.

Time until colonization

Time until colonization was analysed using parametric survival regression with an accelerated failure time model using a Weibull distribution (Kalbfleisch and Prentice, 2002). The hazard ratio was expected to be non-proportional during the experiment, because of the compartmentalization. This accelerated failure time model models the effect of the variables on the acceleration or deceleration of the time until colonization with CTX-M-1-*E. coli*. Colonization of individual broilers was measured as excretion of CTX-M-1-*E. coli* and time until colonization was defined as the time point of the first cloacal swab of two consecutive cloacal swabs tested positive for CTX-M-1-*E. coli*. If the last swab (day 21) and the ceca tested positive, broilers were assumed to be colonized at day 21. If only the ceca tested positive, broilers were not included as colonized birds within the time span of the experiment.

Microbiota composition

Alpha and beta diversity metrics were calculated and univariate and multivariate statistical analyses were applied to determine differences in the cecal microbiota. Alpha diversity (within sample richness) was determined using Faiths phylogenetic diversity, taking into account the phylogenetic relatedness (Faith, 2007). Differences in alpha diversity were tested using a non-parametric Kruskal-Wallis test. Beta diversity (between sample differences) was determined using weighted and unweighted UniFrac metrics (Lozupone et al., 2007). Principal coordinates analysis (PCoA) was used to visualize the data. To test differences within multivariate community data, non-parametric permutational analysis of variance (PERMANOVA) were used (Anderson, 2001).

Transmission coefficient

The transmission coefficients for within and between pen transmission (β_{within} and $\beta_{between}$) were estimated based on a stochastic multi-pen SI model (Klinkenberg et al., 2002; Velthuis et al., 2007) in which the number of new cases is determined by transmission from excreting (I) birds to susceptible (S) birds for a total population of (N) birds, using maximum likelihood estimation.

The probability (p_K) for a susceptible animal in pen κ to become colonized during time interval Δt is calculated based on the force of infection (*foi*) within the pen and between pens (S/C1-pen to C2-pen):

$$p_K = 1 - e^{-(foi_{within} + foi_{between})\Delta t} \quad \text{Eq. 1}$$

Two models were used in which the *foi* was assumed to be based on direct transmission or on indirect transmission with a build-up of infectivity in the environment. In model 1 *foi* was determined by the proportion of excreting birds in the same pen ($\frac{I_K}{N_K}$) and the proportions of excreting birds in the adjacent pen connected through a mesh panel ($\frac{I_{adj}}{N_{adj}}$) during a time interval Δt:

$$p_K = 1 - e^{-(\beta within \frac{I_K}{N_K} + \beta between \frac{I_{adj}}{N_{adj}})\Delta t} \quad \text{Eq. 2}$$

The unit of β_{within} and $\beta_{between}$ in model 1 is 1/day, and is interpreted as the number of new colonized broilers per day, due to one positive broiler.

In model 2 the $f\,oi$ in pen k was assumed to be a build-up in the environment. The cumulative sum of hours that all excreting birds were excreting in a pen ($cumexcrhours_K$) and in the adjacent pen connected with a mesh panel ($cumexcrhours_{adj}$) up to the beginning of the interval was used as a measure for environmental accumulation:

$$p_K = 1 - e^{-(\beta_{within} cumexcrhours_K + \beta_{between} cumexcrhours_{adj})\Delta t} \quad \text{Eq. 3}$$

The unit of β_{within} and $\beta_{between}$ in model 2 is 1/day^2 and is interpreted as the number of new colonized broilers per day, caused by each day that one positive broiler has been excreting CTX-M-1-*E. coli* (Dekker et al., 2013; Chapter 5).

Cecal excretion levels

The differences in cecal content of total *E. coli* and CTX-M-1-*E. coli* (CFU/gram) were tested using a linear regression model including the variables room, pen, sex, weight at day of hatch, weight at day 21, type of bird (S, C1, C2) and time until colonization. The best fitting model was obtained by backward selection based on AIC value. The correlation between cecal content of CTX-M-1-*E. coli* and time until colonization was tested using Pearson's correlation coefficient.

Results

Time until colonization

Broilers in the intervention rooms (room 3 and 4) were not colonized with CTX-M-1-*E. coli*. In the control groups all broilers in room 1 (n=100), and 87/100 broilers in room 2 were colonized at the end of the experiment (Figure 2). Time until colonization was delayed for broilers in room 2 compared to broilers in room 1 (Time Ratio (TR) 3.00, 95% CI 1.82 – 4.95), and for C2 broilers compared to seeder broilers (TR 3.53, 95% CI 3.14 – 3.93). No difference in time until colonization was observed between seeder and C1 broilers (TR 1.14, 95% CI 1.00 – 1.30). Weight at day of hatch, weight at day 21 and sex did not influence time until colonization (Table 1).

Microbiota composition in cecal content

The alpha diversity (phylogenetic diversity) was higher in cecal content samples of the broilers supplied with CE product (CE broilers) compared to the control broilers on day 5 and day 21 (Figure 3). On day 21 no differences in alpha diversity between the two intervention rooms were observed (X^2 = 1.90, p=0.17), but the control broilers in room 1 had a lower alpha diversity than control broilers from room 2 (X^2 = 4.92, p=0.03). Within rooms, no differences between seeder

and contact (C1) broilers were found. Weight at day of hatch, weight at day 21 and sex did not influence the alpha diversity (data not shown).

In weighted and unweighted UniFrac (wuf and uf) distance based analysis, the supply of the CE product explained 60% (wuf) and 69% (uf) of the variation between the cecal content samples on day 5 (Figure 4, principal coordinate analysis (PCoA), PERMANOVA, wuf: $R^2 = 0.598$, p=0.009, uf: $R^2 = 0.688$, p=0.008). On day 21, application of the CE product explained 46% (wuf) and 51% (uf) of the variation between the cecal content samples (Figure 4, PERMANOVA, uf:$R^2 = 0.461$, $p=1.0\times10^{-4}$, wuf: $R^2 = 0.510$, $p=1.0\times10^{-4}$). Within rooms, being a seeder or contact broiler did not explain any of the variation between the cecal content samples. The variation between the two control groups was larger than between the two intervention rooms (wuf: $R^2 = 0.351$ versus $R^2 = 0.210$).

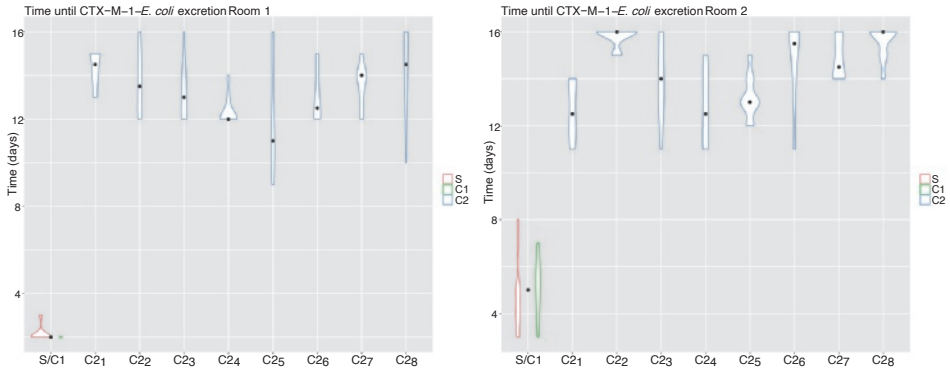

Figure 2 Time until colonization (days) of CTX-M-1-*E. coli* per pen (S/C1, $C2_1$, $C2_2$, $C2_3$, $C2_4$, $C2_5$, $C2_6$, $C2_7$, $C2_8$) and type of bird [seeder (S), contact 1 (C1), contact 2 (C2)] for room 1 (left) and room 2 (right). The violin plot indicates the total range of observations; the black dot indicates the median.

Table 1 Regression coefficients of time until colonization (95% CI) of CTX-M-1-*E. coli* for an accelerated failure time model.

Variable		Accelerated failure time (days, 95% CI)
Baseline survival (Room 1, Seeder, Male)		3.00 (1.82 – 4.95)
Room 2		1.24 (1.15 – 1.33)
Animal type	Contact 1	1.14 (1.00 – 1.30)
	Contact 2	3.53 (3.14 – 3.93)
Sex	Female	0.97 (0.91 – 1.03)
Bodyweight at day 0		1.00 (0.99 – 1.02)
Bodyweight at day 21		1.00 (1.00 – 1.00)

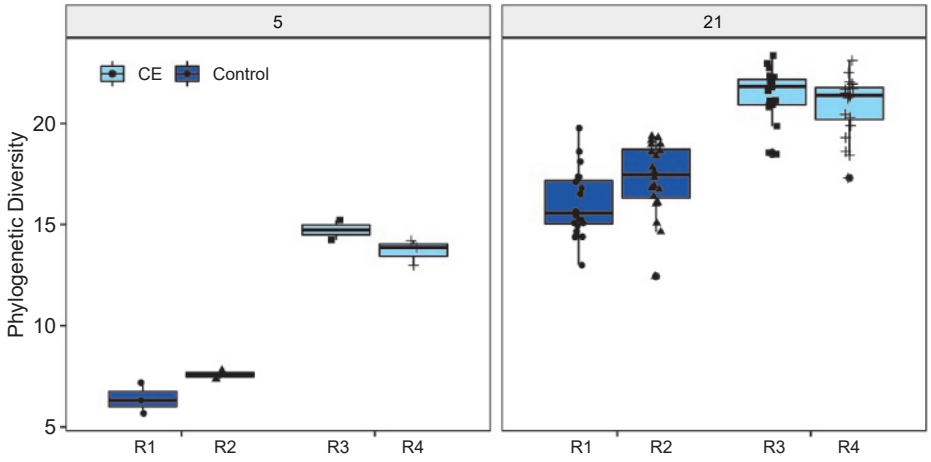

Figure 3 Alpha (phylogenetic) diversity of cecal microbiota at day 5 (*n*=5 broilers per intervention) and day 21 (*n*=40 broilers per intervention), for the control (rooms (R) 1, 2) and intervention groups (rooms (R) 3, 4).

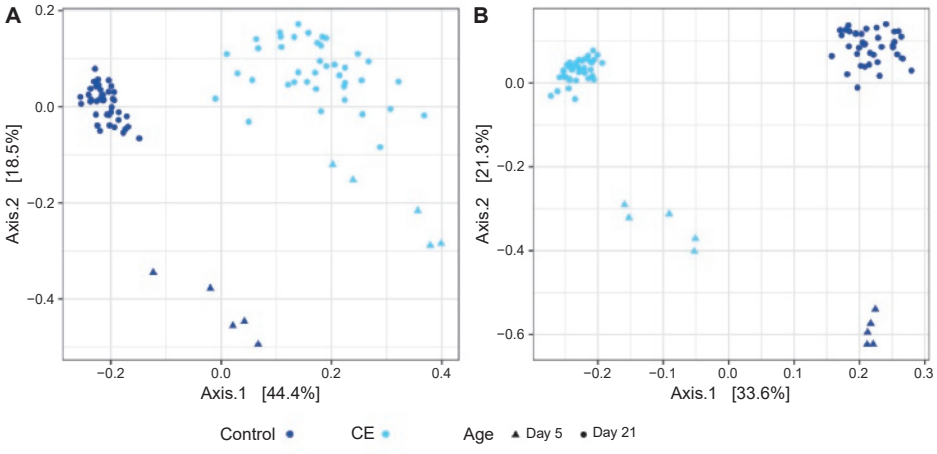

Figure 4 Principal coordinate analysis (PCoA) of microbiota composition based on weighted UniFrac (A) and unweighted UniFrac (B) distances between control (dark blue) and CE (light blue) groups. Different symbols indicate different sampling days: triangles are samples of day 5, and circles are samples of day 21.

The heatmap (Figure 5) shows all genera that significantly differed in relative abundance between CE broilers and control broilers at day 5 and 21. Selection of the first four clusters reveal two clusters with control broilers: one for the broilers of 5 days of age, and one for the broilers of 21 days of age. The other two clusters consist of CE broilers, one cluster contains broilers of both 5

and 21 days of age, while the second cluster contains only CE broilers of 21 days of age.

In the CE product, 22 different genera were identified (Table 2). Of these genera, five were more abundant in CE broilers than in control broilers at day 5: *Collinsella, Eubacterium, Flavonifractor, Lachnoclostridium* and *Lactobacillus*. At day 21, genera *Eubacterium coprostanoligenes, Bacteroides, Collinsella, Enterococcus, Eubacterium, Megamonas, Megasphaera, Slackia* and *Sutterella* were more abundant in CE than in control broilers (Table 2).

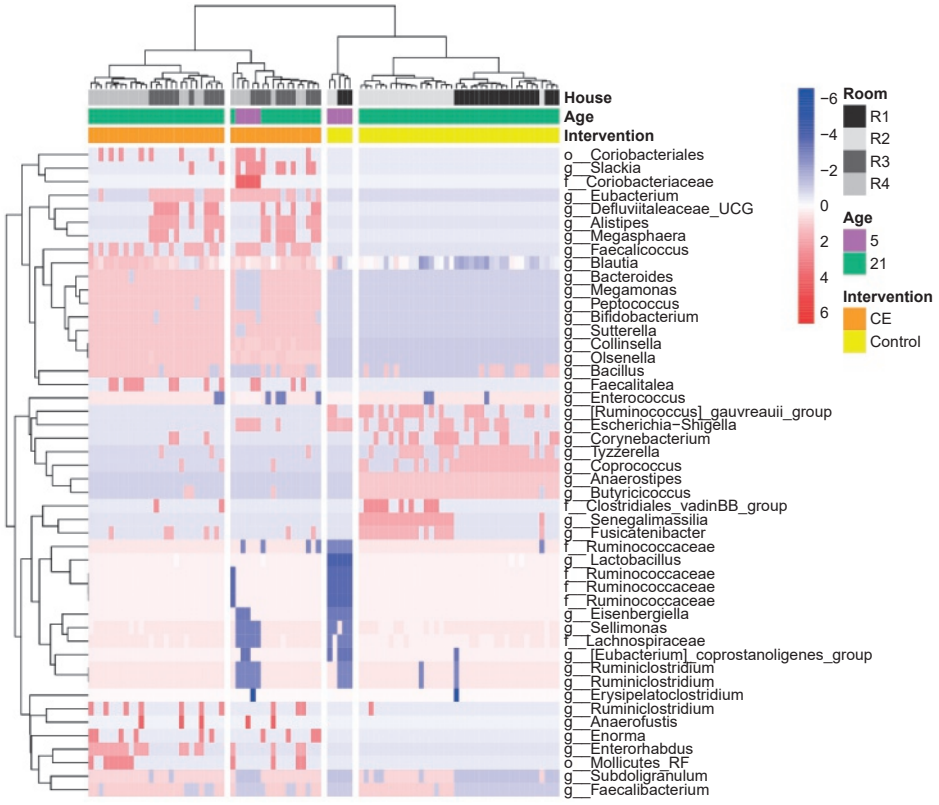

Figure 5 Heatmap representing the abundance of amplicon sequence variants (ASVs) in all individual broiler chickens analysed (*n*=90). Only ASVs that are significantly different at day 5 and day 21 between CE and control are shown (Wilcoxon rank-sum test, adjusted *p*-values are corrected *p*-values for multiple testing, Benjamini-Hochberg, $p<0.05$). Each red, white, or blue rectangle represents the relative abundance of a genus in an individual broiler. Clustering of broilers is based on Ward's minimum variance method and based on weighted UniFrac distances matrix.

Table 2 Relative abundance and standard deviation (SD) of genera that were present in the CE product, and the significantly different relative abundance in cecal content of CE broilers versus control broilers at day 5 (n=10) and 21 (n=80). Results are based on differences of relative abundance tested with Wilcoxon rank-sum test. Adjusted p-values (<0.05) are corrected for multiple testing with Benjamini-Hochberg (BH). - = not detected.

Relative abundance CE product			**Change in relative abundance CE vs control broilers**					
			Day 5 Relative abundance (%)			**Day 21** Relative abundance (%)		
Genera	**Relative abundance (%)**	**SD (%)**	*Control*	*CE*	*p-value*	*Control*	*CE*	*p-value*
[Eubacterium] coprostanoligenes group	0.65	0.22				0.70	1.11	8.05×10^{-07}
Bacteroides	0.47	0.06				-	1.12	9.06×10^{-15}
Blautia	0.30	0.09				18.48	6.64	2.75×10^{-12}
Candidatus_Soleaferrea	0.39	0.06						
Clostridium sensu stricto 1	2.77	0.45	14.67	0.72	0.03	0.04	-	0.04
Clostridium sensu stricto 2	0.72	0.12						
Collinsella	0.53	0.07	-	12.98	0.03	-	4.28	3.34×10^{-15}
Enterococcus	10.80	1.07	31.76	13.40	0.03	0.46	0.94	2.67×10^{-03}
Erysipelatoclostridium	2.53	0.09			0.03	1.84	0.99	0.03
Escherichia-Shigella	0.57	0.02	15.09	0.99	0.03	0.09	3.72×10^{-03}	2.04×10^{-04}
Eubacterium	0.66	0.04	-	3.31	0.03	-	0.20	2.22×10^{-07}
Flavonifractor	1.02	0.14	-	0.79	0.03			
Lachnoclostridium	9.78	0.93	-	1.77	0.03			
Lactobacillus	14.96	1.33	-	10.77	0.03			
Megamonas	1.55	0.56				-	10.36	3.34×10^{-15}
Megasphaera	3.30	0.74				-	0.27	3.82×10^{-05}
Negativicoccus	3.62	0.66						
Oscillibacter	1.94	0.18						
Peptostreptococcus	30.97	4.04						
Sellimonas	1.31	0.38				1.14	0.64	2.60×10^{-04}
Slackia	0.34	0.09				-	0.03	7.57×10^{-03}
Sutterella	1.76	0.21				-	0.99	3.34×10^{-15}
uncultured	4.45	3.56						
unknown	0.08	0.09						

Transmission

Broilers in the intervention rooms (room 3 and 4) were not colonized with CTX-M-1-*E. coli*, and transmission was thus not observed. In the control groups, the transmission coefficient between pens ($\beta_{between}$) was lower than the transmission coefficient within pens (β_{within}) for both models. Model 2, with accumulated environmental transmission, is preferred over model 1, assuming direct transmission (AIC 402.1 vs. 438.1, Table 3). For model 2, $\beta_{between}$ was 3.28×10^{-4} day^{-2} (95% CI 2.41×10^{-4} – 4.32×10^{-4}) and β_{within} was 6.12×10^{-2} day^{-2} (95% CI 4.78×10^{-2} – 7.64×10^{-2}) (Table 3).

Cecal excretion levels

Mean CTX-M-1-*E. coli* (log_{10} CFU/gram) was lower in cecal samples from broilers from C2-pens than from the S/C1-pen, except for pen $C2_6$ and $C2_7$ (Table 4). CTX-M-1-*E. coli* (log_{10} CFU/gram) was lower in cecal samples from broilers kept in room 2 than broilers kept in room 1 (estimate -0.52, 95% CI -0.91 – -0.13 log_{10} CFU/gram). Broilers with a higher bodyweight at day of hatch had slightly higher cecal CTX-M-1-*E. coli* levels (estimate 0.08, 95% CI 0.01 – 0.15 log_{10} CFU/gram). Cecal CTX-M-1-*E. coli* levels are correlated with time until colonization, the shorter the time until colonization, the higher the cecal level (r= -0.60, 95% CI -0.73 – -0.43). Mean *E. coli* levels in cecal content did not differ between rooms or pens.

Table 3 Transmission coefficients (β_{within} and $\beta_{between}$, 95% CI) using an SI-model for transmission based on the proportion of excreting birds (model 1) and the accumulative excretion time (model 2).

Transmission coefficient (β, 95% CI)				
	Unit*	**β_{within} (95% CI)**	**$\beta_{between}$ (95% CI)**	**AIC**
Model 1 proportion excreting birds	day^{-1}	1.31 (1.07 – 1.59)	0.03 (0.02 – 0.04)	438.1
Model 2 accumulative excretion time	day^{-2}	6.12×10^{-2} (4.78×10^{-2} – 7.64×10^{-2})	3.28×10^{-4} (2.41×10^{-4} – 4.32×10^{-4})	402.1

* The unit in model 1 is 1/day, and is interpreted as the number of new colonized broilers due to one positive broiler per day. The unit in model 2 is $1/day^2$ and is interpreted as the number of new colonized broilers caused by each day that one positive broiler has been excreting.

Table 4 Parameter estimates for cecal content levels at day 21 (log_{10} CFU/g cecal content, 95% CI) of CTX-M-1-*E. coli* (*n*=75) using a linear regression model.

Variable	**Estimate CTX-M-1-*E. coli* (95% CI)**
Room 1, pen Seeder/C1 (intercept)	3.95 (0.93 – 6.98)
Room 2	-0.52 (-0.91 – -0.13)
Pen $C2_1$	-1.17 (-1.91 – -0.43)
Pen $C2_2$	-2.28 (-3.23 – - 1.33)
Pen $C2_3$	-1.85 (-2.95 – -0.75)
Pen $C2_4$	-2.01 (-2.64 – -1.39)
Pen $C2_5$	-0.86 (-1.40 – -0.33)
Pen $C2_6$	-0.59 (-1.52 – 0.33)
Pen $C2_7$	0.34 (-0.77 – 1.46)
Pen $C2_8$	-2.69 (-3.49 – -1.88)
Bodyweight day of hatch (day 0)	0.08 (0.01 – 0.15)

Discussion

The supply of CE product via drinking water from day of hatch until day 7 prevented colonization of broilers with ESBL-producing *E. coli* after challenge of seeder birds. In the control group, 93.5% of the broilers were colonized at the end of the experiment. These results are in line with earlier experiments within isolators, in which a continuous supply of CE product during the first 14 days was able to prevent colonization (Chapter 5). In the isolators in which at least one bird was colonized with ESBL-producing *E. coli*, application of CE product reduced the rate of colonization, decreased excretion (CFU/gram) and reduced transmission, as previously shown in studies applying a single supply of CE (Hofacre et al., 2002; Nuotio et al., 2013; Ceccarelli et al., 2017; Methner et al., 2019). The enhanced effect of CE product found in this study compared to these earlier studies could have resulted from the prolonged supply, the longer period between start of CE product and exposure to ESBL-producing *E. coli*, or the moment of challenge with ESBL-producing *E. coli* and the low challenge dose used in our study.

The microbiota composition was more diverse in the CE broilers than in the control broilers on day 5 and 21. This supports the hypothesis that microbial diversity plays a role in preventing colonization. Also successful competitive exclusion of ESBL-producing *E. coli* by specific species being present in the CE broilers could have prevented colonization. Intestinal colonization with microbiota of adult donor hens is associated with increased resistance against colonization, e.g. with *Salmonella* (Varmuzova et al., 2016). In a study where newly hatched layer chicks were exposed to an adult hen, transfer of microbiota occurred within 24 hours of contact and a 1-3 days longer contact period resulted in an even more developed chick microbiota (Kubasova et al., 2019). In our study, supplying a CE product derived from intestinal bacteria of adult chickens possibly increased resistance and prevented colonization with ESBL-producing *E. coli.* The higher diversity observed in broilers at day 5 was maintained during the experiment. At day 21, two weeks after the last supply of the CE product, the intestinal microbiota composition was still more diverse in the CE broilers. Next to genera identified in the CE product, also other genera were found to be different between CE and control groups, indicating that the intestinal microbiota of CE broilers was early and persistently different compared to the composition of the microbiota as observed in the control broilers.

Direct competition between specific bacteria and inoculated *E. coli* in CE broilers might have played a role in preventing colonization, including competition for binding sites or limiting nutrients (Nurmi et al., 1992; Callaway et al., 2008). This could be related also with the production of antimicrobial compounds, including volatile fatty acids, inhibiting or eliminating species that compete for the same niche (Callaway et al., 2008). Some genera were present exclusively in the CE product and in CE broilers, but not in control broilers (Table 2). Due to competition, these genera might have prevented colonization. Next to preventing colonization by *E. coli*, CE products have shown to prevent or reduce colonization of different bacteria, e.g. *Salmonella* (Nakamura

et al., 2002; Ferreira et al., 2003; Luoma et al., 2017; Markazi et al., 2018) and *Campylobacter* (Schneitz and Hakkinen, 2016).

In the control groups compartmentalization resulted in a significantly lower transmission between pens than within pens. Transmission between pens shows that environmental transmission can occur and presence of ESBL/pAmpC-producing bacteria in litter, air or dust plays a role in transmission (Friese et al., 2013; Laube et al., 2013; Laube et al., 2014; Blaak et al., 2014; Blaak et al., 2015; Daehre et al., 2018). Delayed transmission as result of compartmentalization has been described for other pathogens (van Bunnik et al., 2012; Dekker et al., 2013). The effect of compartmentalization can be two-fold: the physical barrier prevents direct contact between broilers, and next to that, during the time needed for transmission between pens to occur, the microbiota of the susceptible broilers might develop further, making it more difficult for ESBL-producing *E. coli* to colonize. In chickens, microbiota in the first week of life contains *Enterobacteriaceae* (Videnska et al., 2014; Ballou et al., 2016; Jurburg et al., 2019) suggesting that *E. coli* can easily colonize during the first week. Older birds might get less susceptible for colonization (Chapter 5), however in our study colonization with ESBL-producing *E. coli* still occurred at 21 days of life, maybe as a result of accumulation of excreted ESBL-producing *E. coli* in the environment. Once transmission between pens occurred, transmission within pens followed rapidly. In room 1, within S/C1-pen transmission occurred very fast: all except one bird were positive at the first sampling after challenge. Therefore, this pen could not be included in the estimation of within pen transmission.

Estimation of the transmission coefficients was done using the proportion of excreting birds (model 1) and the accumulative excretion time (model 2). The model including excretion time fitted better to the observed data, indicating that accumulation of ESBL-producing *E. coli* in the environment most likely plays a role in the transmission within a flock. This increased infectivity by accumulation of the bacteria has been modelled also for other pathogens in pigs and chicken (Lurette et al., 2008; Dekker et al., 2013; van Bunnik et al., 2014). In our model, environmental accumulation is assumed to be unlimited, whereas in practice it is likely that there is a certain maximum, as postulated by Van Bunnik and colleagues that assumed the force of infection to be limited by a maximum exposure capacity for recipient animals (van Bunnik et al., 2014). However, in our study models including a maximum exposure capacity rendered a model that did not converge, which might indicate that the maximum exposure capacity was not yet reached at the end of the experiment.

Conclusions

Overall, our study shows that competitive exclusion is a useful intervention tool to prevent colonization of ESBL-producing bacteria after challenge with a low dose in the first week in a

broiler flock. Transmission within a flock could be delayed by compartmentalization, however as soon as ESBL-producing bacteria are excreted and accumulate in the environment spread to other birds seems inevitable. Therefore, compartmentalization of large flocks into smaller groups of birds, which is for instance more common in breeding flocks at higher levels of the broiler production chain, could be combined to enhance the efficacy of other interventions. CE product could be supplied to young chicks after hatching at different levels of the broiler production pyramid to prevent colonization of birds. The insights provided by this study may provide a basis for further developments towards practically applicable measures to further reduce antimicrobial resistance in poultry.

Acknowledgements

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CHAPTER 7

General discussion

Introduction

The aim of this thesis was to get insight into the processes of colonization and transmission of ESBL/pAmpC-producing bacteria in the broiler production pyramid, and to evaluate possible interventions to reduce the spread of ESBL/pAmpC-producing *E. coli*. In this chapter, the current status of ESBL/pAmpC-producing bacteria in the Netherlands is described, followed by the main findings of this thesis. Then, the effect and possible underlying mechanisms of the studied interventions are discussed. Next, the translation of the results from the animal models to the poultry farm, the application of the interventions and the value of using mathematical models to evaluate the effect of interventions are discussed. Finally, suggestions for further control and the main conclusions of this thesis are given.

ESBL/pAmpC-producing bacteria in the Netherlands

In the Netherlands, the prevalence of ESBL/pAmpC-producing bacteria in broilers has significantly decreased (Dorado-Garcia et al., 2016; Hesp et al., 2019), following the trend in reduction of antimicrobial usage (MARAN, 2019). Based on this, and the observed decreasing prevalence of pAmpC-producing *E. coli* in a parent stock flock without the presence of antibiotics (Chapter 2), one might ask why interventions additional to the reduction of antimicrobial use are needed. However, ESBL/pAmpC-producing bacteria can be present even in absence of antibiotic use, and are able to maintain and spread within animal populations (Huijbers et al., 2016; Ceccarelli et al., 2017); Chapter 2, 4 – 6). These ESBL/pAmpC-positive flocks can, via several routes (Chapter 3), play a role in further spread of ESBL/pAmpC-producing bacteria through the broiler production chain. Moreover, monitoring of antimicrobial usage shows that in 2018 still 77% of the total antimicrobials used in broilers were broad spectrum second choice antimicrobials (MARAN, 2019), including antimicrobials which can select for ESBL/pAmpC-producing bacteria. The use of beta-lactam antibiotics might lead to a steep increase in on-farm prevalence of ESBL/pAmpC-producing bacteria (Dierikx et al., 2013a) and also co-selection might play a role in the occurrence of antimicrobial resistance (Dorado-Garcia et al., 2016). Therefore, caution is required and interventions should be taken to control ESBL/pAmpC-producing bacteria in the broiler production pyramid.

Main findings of this thesis

Process of colonization and transmissio*n*

In the existing literature many observational studies on the occurrence of ESBL/pAmpC-producing

bacteria suggest possible transmission routes of ESBL/pAmpC-producing bacteria (Chapter 3). Four main transmission routes of ESBL/pAmpC-producing bacteria in the broiler production pyramid are categorized (Chapter 3): 1) vertical between generations, 2) at hatcheries, 3) horizontal on farm, and 4) horizontal between farms and via the environment of farms. However, due to the lack of quantitative information in the literature it remains unclear to what extent the different routes contribute to the occurrence of ESBL/pAmpC producing bacteria.

Colonization of birds can occur at young age; several studies have observed young birds carrying ESBL/pAmpC-producing *E. coli* shortly after arrival at the farm, followed by a steep increase in prevalence during the first days (Dierikx et al., 2013a; Huijbers et al., 2016). In a broiler parent stock, a high prevalence of pAmpC-producing *E. coli* was observed at day 7 of the rearing period. Surprisingly, pAmpC-producing *E. coli* prevalence in the parent flock decreased from 91% in week 1 to 0% in week 21, and was not detected in the offspring, although it was still present in the environment (Chapter 2). A decrease of ESBL/pAmpC-producing *E. coli* has been described earlier in organic broiler flocks (Huijbers et al., 2015b; Huijbers et al., 2016; van Hoek et al., 2018) and recently also in parent stock (Apostolakos et al., 2019), but at lower prevalence. The different gene-plasmid combinations might have influenced the inability of ESBL/pAmpC-producing *E. coli* to persist.

To get more insight into the process of colonization of young broilers, a dose-response study was conducted. In this study conventional (Ross 308) and specific pathogen free (SPF) broilers were challenged with ESBL/pAmpC-producing *E. coli* (Chapter 4). A higher challenge dose of ESBL/pAmpC-producing *E. coli* resulted in a shorter time until colonization, but even the lowest dose (0.5 mL of 10^1 CFU/mL) resulted in colonization of SPF broilers within 24 hours. A higher challenge dose resulted in a higher probability of colonization as result of initial inoculation. Conventional broilers showed a delay in colonization compared to SPF broilers, which might be caused by competition between initially present bacteria and the inoculated *E. coli*. The results showed that the presence of only a few ESBL/pAmpC-producing bacteria within a poultry house or hatchery can lead to colonization of some of the broilers, and subsequently transmission will lead to a high prevalence of colonized broilers in the flock.

Effect of interventions

Transmission of ESBL/pAmpC-producing *E. coli* can occur via several routes (Chapter 4) and ESBL/pAmpC-producing bacteria can be present in the environment in and around poultry houses (Laube et al., 2013; Zurfluh et al., 2014a; Zurfluh et al., 2014b; Huijbers et al., 2016; Daehre et al., 2018). Therefore, sufficiently reducing the exposure of birds to ESBL/pAmpC-producing bacteria is difficult and interventions aiming to prevent colonization are needed. Earlier studies described reduced colonization, excretion and transmission after supply of a competitive exclusion (CE, containing intestinal bacteria) product. However, a single supply of CE product could not prevent colonization with ESBL/pAmpC-producing *E. coli* (Nuotio et al., 2013; Ceccarelli et al., 2017; Methner et al.,

2019). Therefore, the effect of a prolonged supply of CE product after challenge with a low dose of ESBL-producing *E. coli* was tested, thereby mimicking the initial stages of colonization in the field (low exposure) and optimizing the potential effect of CE products (prolonged supply) (Chapter 5).

A prolonged supply of CE products prevented colonization of broilers after challenge with 10^2 CFU/mL ESBL producing-*E. coli* at day 5, in some of the isolators. However, if at least one of the broilers was colonized, rapid spread to the other broilers present in the same isolator followed (Chapter 5). Challenge with a lower dose level (10^1 CFU/mL) at day 5 did not lead to colonization of broilers, whereas at age day 0 or 1 challenge with dose level 10^1 CFU/mL did lead to colonization (Chapter 4, 5). This suggests that broilers at age day 5 are less susceptible to colonization than younger broilers. Challenge with 10^1 or 10^2 CFU/mL at day of hatch simultaneously with the supply of CE product did not show any differences in colonization between control and CE broilers (Chapter 5), indicating that the CE product needs time to colonize in the gut before having a protective or reducing effect on colonization of ESBL- producing *E. coli*.

After having observed the effect of CE products under controlled circumstances, a transmission study under semi-field circumstances was carried out. Besides the prolonged supply of CE product, a second intervention was included: compartmentalization (subdividing the flock). If ESBL/pAmpC-producing bacteria are present in a poultry flock, compartmentalization might reduce the transmission, as described for other bacteria (van Bunnik et al., 2012; Dekker et al., 2013). A prolonged supply of CE product prevented colonization of ESBL-producing *E. coli*. In the control flocks, compartmentalization reduced the transmission rate of ESBL-producing *E. coli* within the flock (Chapter 6). The more diverse microbiota compositions of CE broilers at day 5 and 21 (Chapter 6) suggest that the CE product supports the development of the microbiota composition, thereby reducing the susceptibility for colonization with ESBL/pAmpC-producing bacteria.

Interventions affecting colonization and transmission of ESBL/pAmpC-producing *E. coli*

Competitive exclusion

Only a few ESBL/pAmpC-producing bacteria are needed to colonize young broilers, and subsequently transmission will lead to a high prevalence within a flock (Dierikx et al., 2013a; Huijbers et al., 2016; Robé et al., 2019); Chapter 2, 4). Therefore, interventions aiming to prevent colonization with ESBL/pAmpC-producing bacteria should be taken as soon as possible after hatching, before exposure with ESBL/pAmpC-producing bacteria occurs.

Already in the 1970s experiments have been performed aiming to protect young broilers from *Salmonella* colonization. This resulted in the concept of competitive exclusion, defined as “the establishment of adult-type resistance to salmonellas in newly hatched chicks by administering adult intestinal microorganisms” (Nurmi et al., 1992). Although the concept was initially

developed to prevent colonization with *Salmonella*, later also protection against *E. coli, Yersbua* and *Campylobacter* spp. was shown (Nurmi et al., 1992). The expected mechanism of the CE products, resulting in preventing colonization with ESBL/pAmpC-producing *E. coli*, is competition. This might include competition for binding sites or limiting nutrients (Nurmi et al., 1992; Callaway et al., 2008).

In mammals, the ability of intestinal microbiota inhibiting colonization by invading microbes is described as "colonization resistance" (van der Waaij et al., 1971). Colonization resistance consists of direct microbe-microbe interactions, and the more complex indirect host-microbe interactions, for example by immune responses (Lawley and Walker, 2013). Colonization resistance is associated with a stable and diverse microbiota composition (Lawley and Walker, 2013; Browne et al., 2017). Earlier it was described that the diversity of the microbiota composition increases with age (Lu et al., 2003; Richards et al., 2019) and that the prevalence of ESC-resistant *E. coli* decreases with age (Chauvin et al., 2013; Huijbers et al., 2015b; Braykov et al., 2016; Huijbers et al., 2016; van Hoek et al., 2018); Chapter 2). A higher phylogenetic diversity was observed in CE broilers, which could have contributed to preventing colonization of ESBL/pAmpC-producing *E. coli* in the CE broilers (Chapter 6). Moreover, higher dose levels were needed to colonize older birds than younger ones (day 5 versus day 0 or 1) (Chapter 5). These results indicate that CE products stimulate the natural development of the microbiota composition, and thus the colonization resistance, making young birds less susceptible to colonization with ESBL/pAmpC-producing *E. coli*.

Specific microbe-microbe interactions could have played a role in the observed prevention of colonization, for example by competing for specific binding sites or nutrients (Nurmi et al., 1992; Callaway et al., 2008). This could also be related with the production of antimicrobial compounds, including volatile fatty acids, inhibiting or eliminating species that compete for the same niche (Callaway et al., 2008). The results of the dose response study suggest that *E. coli* present in conventional broilers delays colonization of the inoculated *E. coli* strain (Chapter 4). Likely, also other bacteria species were initially present in the conventional broilers, but their detection was not included in the culturing methods. Fitness costs of the plasmid-carrying *E. coli* strain might play a role in competition (Andersson and Hughes, 2010). However, the theory that in absence of selective pressure the plasmid-carrying strain will be outcompeted by the plasmid-free strain, does not always hold (Lopatkin et al., 2017; Fischer et al., 2019). Possibly plasmid transfer occurred (Chapter 4) and this might have contributed to the rapid transmission of ESBL/pAmpC-producing *E. coli* between broilers (Chapter 4 - 6). The presence of specific genera in the CE broilers which were absent in control broilers shows that the CE product does impact the composition of the microbiota (Chapter 6). These genera might have competed with the inoculated *E. coli* for the same niche or nutrients. Earlier studies have shown a reducing or preventing effect of CE products on colonization of bacteria such as *Salmonella* (Nakamura et al., 2002; Ferreira et al., 2003; Luoma et al., 2017; Markazi et al., 2018) and *Campylobacter* (Schneitz and Hakkinen, 2016), showing that the effect of the CE product includes, but is not limited to, competition with *E. coli*.

The use of prebiotics might provide a competitive advantage to bacteria acting as competitive excluders (Callaway et al., 2008). However, the synbiotic product (including pre- and a selection of probiotics) did not provide better results (i.e. prevention of colonization) than the competitive exclusion product derived from faeces of adult hens (Chapter 5).

Competitive exclusion products as artificial hen

The development of the microbiota composition of young birds as described in recent studies (Jurburg et al., 2019; Richards et al., 2019) is related to the modern broiler production. Young birds are hatched in absence of the hen, and due to strict hygiene practices in commercial hatcheries, only a limited number of natural intestinal bacteria are present in the environment to colonize the bird intestinal tract (Varmuzova et al., 2016; Donaldson et al., 2017). Experimental studies including just hatched chickens in absence and presence of a hen showed that the microbiota of an adult hen was transferred within 24 hours. Within a week young chickens had a composition similar to adult birds (Kubasova et al., 2019). However, care should be taken when inoculating young birds with cecal content from adult donor hens, as pathogens might be present or overgrowth of certain genera, resulting in a shift of microbial populations, can occur (Polansky et al., 2015). Defined cultures such as synbiotics or cultures originating from faeces from SPF birds (Chapter 5, 6) are aimed to avoid these negative effects.

Compartmentalization

Competitive exclusion products could prevent colonization of birds (Chapter 5, 6). However, when one of the birds becomes colonized with ESBL/pAmpC-producing *E. coli*, this might result in spread within the flock (Chapter 4, 5). Housing measures such as compartmentalization can decrease transmission within a flock (Chapter 6). The compartmentalization described in Chapter 6 consisted of a 30 cm solid panel and 40 cm mesh panel on top. The effect of compartmentalization might be two-fold: first, preventing direct contact between excreting and susceptible broilers, and secondly, during the time needed to transmit ESBL-producing *E. coli* between compartments, the microbiota of the susceptible broilers might develop further making them less susceptible (see previous paragraph).

Transmission between the pens did occur, demonstrating the important role of the environment in transmission of ESBL/pAmpC-producing *E. coli*, as indicated by earlier studies observing ESBL/pAmpC-producing *E. coli* in dust and litter in poultry houses (Laube et al., 2013; Friese et al., 2013; Laube et al., 2014; Blaak et al., 2014; Blaak et al., 2015; Daehre et al., 2018). Actually, transmission of ESBL/pAmpC-producing *E. coli* occurs via the faecal-oral route, and thus via the environment, but preventing direct contact with faeces of excreting birds reduced the transmission rate (Chapter 6). However, transmission between pens could not be prevented, indicating that once ESBL/pAmpC-producing *E. coli* is present in a poultry house, additional interventions are needed to successfully limit the spread of ESBL/pAmpC-producing bacteria.

From animal models to the farm

In this thesis the processes of colonization and transmission were studied in intensively cleaned and disinfected isolators (Chapter 4), to ensure an ESBL/pAmpC-producing *E. coli* free environment. The delayed colonization in conventional broilers compared to SPF broilers showed that conventional broilers should be used when testing interventions intended for implementation in practice. *E. coli* was used as reference bacteria, since this is considered as a useful indicator bacteria for monitoring antimicrobial resistance (Hesp et al., 2019).

The consistent results of the dose-response study led to an animal model used to test interventions (Chapter 5) and could also be applied under semi-field circumstances (Chapter 6). Moreover, the probabilistic model (Chapter 4) gave insight into the process of colonization; inoculation did not always lead to colonization, showing the contribution of transmission in these type of experiments performed in small groups of broilers (Line et al., 2008).

Although the experiments were conducted in isolators (Chapter 4, 5), the conventional broilers were hatched in a hatchery and transported to the research facilities, to mimic hatching, collection, transport and arrival at the farm of just hatched birds in actual broiler production. Housing conditions can affect the microbiota composition (Kers et al., 2019), and thus the effect of the tested interventions. However, the promising effect of the interventions in broilers kept in isolators were confirmed under circumstances closer to the field situation (Chapter 6).

The (seeder-)broilers were exposed to ESBL/pAmpC-producing *E. coli* by individual oral inoculation. In practice however, the first bird needs to actively pick up the ESBL/pAmpC-producing bacteria from the environment to get colonized. This includes an additional step in the process of colonization, compared to the process studied in Chapter 4. On the other hand, in practice there might be long term exposure (e.g. in the poultry house), and not only once, via inoculation. The birds in the animal model were rapidly colonized, as was observed in birds at a farm (Dierikx et al., 2013a; Huijbers et al., 2016), indicating that the animal model reflects the actual colonization of young birds when using strains originating from broilers (Dierikx et al., 2010). The observed dynamics of ESBL/pAmpC-producing *E. coli* likely included clonal and plasmid transfer (Chapter 4), similar to the naturally occurring transmission process of ESBL/pAmpC-producing *E. coli* in field situations (Huijbers et al., 2016; van Hoek et al., 2018).

The supply of CE product occurred via the drinking water. In the animal experiments, with small groups and frequent observations, all birds did drink the water. Given good farm management, for most birds after arrival at the farm there will be no, or only a short delay until initial feed and water intake. However, a small number of birds will not start eating and drinking at all. If these birds are exposed to ESBL/pAmpC-producing *E. coli*, this might lead to colonization. However, these birds are likely to perish within a few days, making it unlikely that they will have a substantial contribution to excretion and environmental accumulation of ESBL/pAmpC-producing *E. coli*.

The supply of CE product during the first week prevented colonization after challenge at day

5. Day 5 was chosen to simulate introduction of ESBL-producing *E. coli* during the first week at the poultry farm (e.g. horizontal transmission from parallel flocks, transmission from the environment). In practice, exposure to ESBL-producing *E. coli* at the hatchery or poultry farm might occur before day 5 (e.g. horizontal transmission from previous flocks). The time needed for the CE product to colonize and effectively prevent colonization of the inoculated ESBL-producing *E. coli* is unknown. Earlier research showed reduced colonization when challenging broilers with ESBL-producing *E. coli* one day after providing CE product (Nuotio et al., 2013; Ceccarelli et al., 2017), and microbiota transfer from adult hens to chickens is observed within 24 hours (Kubasova et al., 2019). This suggests that the CE product, given the supply started directly after hatch, is effective when exposure to ESBL-producing *E. coli* occurs at day 1 or later.

Compartmentalization included not only spatial separation of birds, but also separation of feed and water systems, and strict hygiene measures between pens (Chapter 6). Entering compartments without e.g. changing boots might facilitate transmission of ESBL/pAmpC-producing *E. coli* (Chapter 3), thus in practice, with less strict hygiene measures, the effect of compartmentalization might be smaller.

Also other variables present in poultry farms might influence colonization and transmission of ESBL/pAmpC-producing bacteria. First of all, in our experiments no antibiotics were used. The use of antibiotics selecting for resistant bacteria might overrule the effect of CE products (Chantziaras et al., 2018). Other factors possibly affecting the presence of resistance bacteria but not included in our experiments are, as described in Chapter 3, type of farming, region, season and farm-related factors as hygiene, acidification of drinking water, feed changes, breed, litter material. Some of these factors might be related with the susceptibility of broilers for colonization, possibly by affecting the microbiota composition (e.g. hygiene, litter material), others might be related with a source of ESBL/pAmpC-producing bacteria (e.g. hatcheries) (Persoons et al., 2011).

Application of interventions

The effect of a prolonged supply of CE products depends on the moment of exposure of ESBL-producing *E. coli* (Chapter 5), indicating that the CE products need time to establish in the gut before protecting broilers from colonization. Therefore, CE products can be useful to prevent colonization of broilers with ESBL/pAmpC-producing at the farm, but it seems difficult to protect broilers from colonization when exposure occurs at the hatchery.

Compartmentalization delayed transmission between pens (Chapter 6), but could not prevent it. Additional interventions are needed to control the spread of ESBL/pAmpC-producing *E. coli*, for example a combination of compartmentalization and competitive exclusion. Once a compartment tested ESBL/pAmpC-producing bacteria positive, CE products could be supplied to all birds within the flock to protect susceptible birds in the adjacent pens from colonization. However,

the results indicate that the effect of CE products lies in supporting the microbiota development, making birds less susceptible for colonization (see "competitive exclusion"). Therefore, the effect of supplying CE products to older birds, with a more established microbiota, might be limited. On the other hand, during periods of stress or disease, the microbiota composition might be affected, becoming more susceptible for colonization of pathogens (Burkholder et al., 2008; Stanley et al., 2012), but possibly also increasing the potential for colonization with bacteria in the competitive exclusion product. In addition, the ability of CE products to prevent colonization might depend on the environmental accumulation of ESBL/pAmpC-producing *E. coli*. The results of the experiments in the isolators (Chapter 5) indicate that as soon as one (or more) birds start to excrete, transmission to other birds is inevitable, despite the prolonged supply of CE products.

Ideally, interventions should be applicable at different levels of the broiler production pyramid. The experiments described in this thesis included broiler chickens. Although microbiota composition may vary between different type of chickens (Kers et al., 2018), also young (grand) parent chickens were observed ESBL/pAmpC-positive just after hatching (Dierikx et al., 2013a; Apostolakos et al., 2019), indicating similar colonization processes.

The supply of CE product via the drinking water can be implemented easily, independent of the type of farm. Compartmentalization of large flocks into smaller groups of birds, will require major adjustments in the design of poultry houses, but is more common in breeding flocks at farms in the top of the broiler production chain. Compartmentalization in combination with other interventions, such as competitive exclusion, will help to reduce (apparent) vertical transmission. In broiler farms implementation of compartmentalization will be more complicated.

The success of implementation of interventions depends also on the willingness of individual farmers. It lies outside the scope of this thesis to discuss the socio-economic aspects of farmers' decisions, but investments will be needed to implement the interventions. However, for individual farmers currently there is no direct economic impact (positive or negative) related with the ESBL/pAmpC-status of delivered animals or animal products. On the other hand, both the supply of CE product and compartmentalization might not exclusively prevent or reduce the spread of ESBL/pAmpC-producing bacteria but also be effective against other invaders (Nakamura et al., 2002; Ferreira et al., 2003; van Bunnik et al., 2012; Dekker et al., 2013; Schneitz and Hakkinen, 2016; Luoma et al., 2017; Markazi et al., 2018).

Use of models to evaluate interventions

Mathematical models are useful tools to get insight into the population dynamics of, in our study, ESBL/pAmpC-producing *E. coli*, and to interpret the data (de Jong, 1995). In addition to only reporting observed colonization data (Robé et al., 2019), the models used in this thesis allow to estimate probabilities of colonization as result of inoculation or transmission (Chapter 4) and

transmission coefficients (Chapter 5, 6). Transmission coefficients in itself give valuable information on time scale of transmission, but can also be used to predict the effect of interventions in larger populations. Simulation models can be used to extrapolate findings to larger populations such as broiler flocks, or even in the broiler production pyramid, taking into account the differences between animal experiments and poultry farms as discussed in the previous paragraph.

In this thesis, two different models estimating transmission coefficients were used. The force of infection (*foi*) was determined by either 1) the proportion of excreting birds or 2) by the cumulative excretion time (or level) (Chapter 5, 6). The first method is often used in transmission studies, and seems useful when observing transmission within groups, e.g. in isolators. However, this model has the disadvantage that the *foi* is maximized when all broilers are excreting. Based on the mechanism of *E. coli* transmission, via the faecal-oral route, and the survival of *E. coli* in the environment (Merchant et al., 2012; Friese et al., 2013), it seems biologically more relevant to take into account accumulation of the excreted ESBL-producing *E. coli* in the environment to estimate transmission rates. Therefore, in the second method the sum of the excretion time is included as a measure for the accumulation of the excreted ESBL-producing *E. coli* in the environment. This leads to an increasing *foi* during the experiment. In the experiment including panels separating pens, the second model fitted the data best, indicating that accumulation of ESBL-producing *E. coli* in the environment is relevant in between-pen transmission (Chapter 6).

Suggestions for further control of ESBL/pAmpC-producing bacteria in the poultry production pyramid

The different transmission routes, the presence of ESBL/pAmpC-producing bacteria throughout the broiler production chain, and the finding that young birds are susceptible for colonization (Chapter 4) exemplify the need to intervene in the spread of ESBL/pAmpC-producing bacteria as soon as possible after hatching. CE products supplied from the moment of arrival at the farm can be effective when exposure to ESBL/pAmpC-producing *E. coli* occurs during the first week. Intervene at an even earlier stage, at the hatchery, might be helpful to reduce prevalence of colonized flocks (Plaza Rodriguez et al., 2018). As the bacteria in the CE product need time to establish in the gut before providing a protective effect (Varmuzova et al., 2016; Ceccarelli et al., 2017), it seems difficult to protect birds from colonization when exposure occurs at the hatchery. An earlier study aiming to determine the effect of CE prior to hatching, was inconclusive with respect to the protective effect of dipping eggs in CE product, since ESBL/pAmpC-producing *E. coli* was not persistently present on the eggs surfaces collected from poultry farms (Oikarainen et al., 2019). Supply of CE products as soon as possible upon hatching could also be done in alternative hatching systems such as on farm-hatching.

In addition to the interventions studied in this thesis, other interventions might help to reduce

the prevalence of ESBL/pAmpC-producing bacteria in the broiler production chain. Housing measures can influence the risk on resistant *E. coli*, e.g. the type of litter (Persoons et al., 2011; Boulianne et al., 2016). In general, biosecurity measures can play an important role in reducing horizontal between flock transmission, as described for *Campylobacter* (Katsma et al., 2007; Newell et al., 2011). Examples of increased biosecurity include disinfecting the floor between production rounds (Mo et al., 2016) and wearing gloves by farm personnel (Jones et al., 2013), which were effective in reducing the risk on the occurrence of ESC resistant *E. coli*. The results showing that the CE product prevents colonization, provided that it had time to colonize in the gut before exposure occurs, indicate the need for a strict biosecurity. This should ensure that broilers are not exposed to ESBL/pAmpC producing bacteria directly upon arrival at the farm. In practice however, even after cleaning and disinfection, a few ESBL/pAmpC-producing bacteria might remain in the poultry house and result in colonization of the new flock (Daehre et al., 2018).

The studied interventions could be applied not only in broilers, but also in higher levels of the broiler production pyramid. Preventing young (grand)parents birds from colonization with ESBL/pAmpC-producing bacteria, will reduce (apparent) vertical transmission between generations, and the spread throughout the production pyramid.

Main conclusions of this thesis

Transmission of ESBL/pAmpC-producing bacteria in the broiler production chain occurs via several routes, and young birds are rapidly colonized, even with a low dose of ESBL/pAmpC-producing *E. coli*. Therefore, interventions aiming to prevent colonization should be taken as soon as possible after hatch. A prolonged supply of CE product can protect young birds from colonization, if supplied before exposure to ESBL/pAmpC-producing *E. coli*. Transmission of ESBL/pAmpC-producing *E. coli* due to environmental accumulation within a flock, can be reduced by compartmentalization, but compartmentalization will not prevent spread of ESBL/pAmpC-producing *E. coli* completely.

Recommendations

Since ESBL/pAmpC-producing *E. coli* is present throughout the broiler production pyramid, e.g. at the hatchery, at farms and the environment, and transmission occurs via several routes, it is recommended to take interventions at different levels of the pyramid, targeting different transmission routes. This will include aiming to reduce (apparent) vertical transmission of ESBL/pAmpC-producing *E. coli* between generations and prevent colonization of birds at farms. The interventions studied in this thesis can form a basis for further developments towards practically applicable measures to further reduce antimicrobial resistance. Knowledge on the attribution of the different transmission routes is needed to further design the most effective intervention strategies to reduce the prevalence of ESBL/pAmpC-producing bacteria in the broiler production pyramid.

Bibliography

Abraham, E.P., Chain, E., 1940. An enzyme from bacteria able to destroy penicillin. Nature 146, 837.

Agerso, Y., Jensen, J.D., Hasman, H., Pedersen, K., 2014. Spread of extended spectrum cephalosporinase-producing escherichia coli clones and plasmids from parent animals to broilers and to broiler meat in a production without use of cephalosporins. Foodborne Pathog. Dis. 11, 740-746.

Alvarez-Fernandez, E., Dominguez-Rodriguez, J., Capita, R., Alonso-Calleja, C., 2012. Influence of housing systems on microbial load and antimicrobial resistance patterns of escherichia coli isolates from eggs produced for human consumption. J. Food Prot. 75, 847-853.

Amadi, V.A., Watson, N., Onyegbule, O.A., MatthewBelmar, V., Avendano, E., Tiwari, K., Sharma, R., Hariharan, H., 2015. Antimicrobial resistance profiles of escherichia coli recovered from feces of healthy free-range chickens in grenada, west indies. Int. J. Curr. Microbiol. Appl. Sci. 4, 168-175.

Amerah, A.M., Peron, A., Zaefarian, F., Ravindran, V., 2011. Influence of whole wheat inclusion and a blend of essential oils on the performance, nutrient utilisation, digestive tract development and ileal microbiota profile of broiler chickens. Br. Poult. Sci. 52, 124-132.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32-46.

Andersson, D.I., Hughes, D., 2010. Antibiotic resistance and its cost: Is it possible to reverse resistance? Nature Reviews Microbiology 8, 260.

Antilles, N., GarciaMigura, L., Joensen, K.G., Leekitcharoenphon, P., Aarestrup, F.M., CerdaCuellar, M., Hendriksen, R.S., 2015. Audouin's gull, a potential vehicle of an extended spectrum beta -lactamase producing salmonella agona. FEMS Microbiol. Lett. 362, 1-4.

Apostolakos, I., Mughini-Gras, L., Fasolato, L., Piccirillo, A., 2019. Assessing the occurrence and transfer dynamics of ESBL/pAmpC-producing escherichia coli across the broiler production pyramid. PLoS One 14, e0217174.

Aw, Y.K., Loong, M., YueChii, X., LeeKok, M., NgSian, Y., Mae, L., 2015. Antimicrobial resistance of escherichia coli, salmonella and enterococci isolated from surface of conventional broiler eggs, "kampung" chicken eggs and carrying trays from wet markets in selangor, malaysia. Malays. J. Microbiol. 11, 109-115.

Ballou, A.L., Ali, R.A., Mendoza, M.A., Ellis, J.C., Hassan, H.M., Croom, W.J., Koci, M.D., 2016. Development of the chick microbiome: How early exposure influences future microbial diversity. Front. Vet. Sci. 3, 2.

Baron, S., Jouy, E., Larvor, E., Eono, F., Bougeard, S., Kempf, I., 2014. Impact of third-generation-cephalosporin administration in hatcheries on fecal escherichia coli antimicrobial resistance in broilers and layers. Antimicrob. Agents Chemother. 58, 5428-5434.

Baron, S., Jouy, E., Touzain, F., Bougeard, S., Larvor, E., de Boisseson, C., Amelot, M., Keita, A., Kempf, I., 2016. Impact of the administration of a third-generation cephalosporin (3GC) to one-day-old chicks on the persistence of 3GC-resistant escherichia coli in intestinal flora: An in vivo experiment. Vet. Microbiol. 185, 29-33.

Baron, S., Le Devendec, L., Touzain, F., Jouy, E., Lucas, P., de Boisseson, C., Larvor, E., Kempf, I., 2018. Longitudinal study of escherichia coli plasmid resistance to extended-spectrum cephalosporins in free-range broilers. Vet. Microbiol. 216, 20-24.

Blaak, H., Hamidjaja, R.A., van Hoek, A.H., de Heer, L., de, R.H., Schets, F.M., 2014. Detection of extended-spectrum beta-lactamase (ESBL)-producing escherichia coli on flies at poultry farms. Appl. Environ. Microbiol. 80, 239-246.

Blaak, H., van, H.A., Hamidjaja, R., van der, P.R., Kerkhof-de, H.L., de Roda, H.A., Schets, F., 2015. Distribution, numbers, and diversity of ESBL-producing E. coli in the poultry farm environment. PloS one 10, e0135402.

Bonnedahl, J., Stedt, J., Waldenstrom, J., Svensson, L., Drobni, M., Olsen, B., 2015. Comparison of extended-spectrum beta-lactamase (ESBL) CTX-M genotypes in franklin gulls from canada and chile. PLoS One 10, e0141315.

Borges, C.A., Cardozo, M.V., Beraldo, L.G., Oliveira, E.S., Maluta, R.P., Barboza, K.B., Werther, K., Avila, F.A., 2017. Wild birds and urban pigeons as reservoirs for diarrheagenic escherichia coli with zoonotic potential. J. Microbiol. 55, 344-348.

Borjesson, S., Bengtsson, B., Jernberg, C., Englund, S., 2013a. Spread of extended-spectrum beta-lactamase producing escherichia coli isolates in swedish broilers mediated by an incl plasmid carrying bla(CTX-M-1). Acta Vet. Scand. 55, 3-3.

Borjesson, S., Egervarn, M., Lindblad, M., Englund, S., 2013b. Frequent occurrence of extended-spectrum beta-lactamase- and transferable ampc beta-lactamase-producing escherichia coli on domestic chicken meat in sweden. Appl. Environ. Mi-

crobiol. 79, 2463-2466.

Bortolaia, V., Bisgaard, M., Bojesen, A.M., 2010. Distribution and possible transmission of ampicillin- and nalidixic acid-resistant escherichia coli within the broiler industry. Vet. Microbiol. 142, 379-386.

Boulianne, M., Arsenault, J., Daignault, D., Archambault, M., Letellier, A., Dutil, L., 2016. Drug use and antimicrobial resistance among escherichia coli and enterococcus spp. isolates from chicken and turkey flocks slaughtered in quebec, canada. Can. J. Vet. Res. 80, 49-59.

Bradford, P.A., 2001. Extended-spectrum beta-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14, 933-51.

Braykov, N.P., Eisenberg, J.N., Grossman, M., Zhang, L., Vasco, K., Cevallos, W., Munoz, D., Acevedo, A., Moser, K.A., Marrs, C.F., Foxman, B., Trostle, J., Trueba, G., Levy, K., 2016. Antibiotic resistance in animal and environmental samples associated with small-scale poultry farming in northwestern ecuador. mSphere 1, 21.

Brower, C.H., Siddhartha Mandal, Shivdeep Hayer, Mandeep Sran, Asima Zehra, Patel, S.J., Ravneet Kaur, Leena Chatterjee, Savita Mishra, Das, B.R., Parminder Singh, Randhir Singh, Gill, J.P.S., Ramanan Laxminarayan, 2017. The prevalence of extended-spectrum beta-lactamase-producing multidrug-resistant escherichia coli in poultry chickens and variation according to farming practices in punjab, india. Environ. Health Perspect. 125, EHP292.

Browne, H.P., Neville, B.A., Forster, S.C., Lawley, T.D., 2017. Transmission of the gut microbiota: Spreading of health. Nat. Rev. Microbiol. 15, 531-543.

Buck, J.d., Pasmans, F., Immerseel, F.v., Haesebrouck, F., Ducatelle, R., 2004. Tubular glands of the isthmus are the predominant colonization site of salmonella enteritidis in the upper oviduct of laying hens. Poult. Sci. 83, 352-358.

Bui, T.K.N., Bui, T.M.H., Ueda, S., Le, D.T., Yamamoto, Y., Hirai, I., 2017. Potential transmission opportunity of CTX-M-producing escherichia coli on a large-scale chicken farm in vietnam. J. Glob. Antimicrob. Resist 13, 1-6.

Burkholder, K.M., Thompson, K.L., Einstein, M.E., Applegate, T.J., Patterson, J.A., 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to salmonella enteritidis colonization in broilers. Poult. Sci. 87, 1734-1741.

Callaway, T.R., Edrington, T.S., Anderson, R.C., Harvey, R.B., Genovese, K.J., Kennedy, C.N., Venn, D.W., Nisbet, D.J., 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. Anim. Health. Res. Rev. 9, 217-225.

Carattoli, A., 2009. Resistance plasmid families in enterobacteriaceae. Antimicrob. Agents Chemother. 53, 2227-2238.

Carmo, L., Nielsen, L., da Costa, P.M., Alban, L., 2014. Exposure assessment of extended-spectrum beta-lactamases/AmpC beta-lactamases-producing escherichia coli in meat in denmark. Infect. Ecol. Epidemiol. 4, 22924.

Ceccarelli, D., Kant, A., van Essen-Zandbergen, A., Dierikx, C., Hordijk, J., Wit, B., Mevius, D.J., Veldman, K.T., 2019. Diversity of plasmids and genes encoding resistance to extended spectrum cephalosporins in commensal escherichia coli from dutch livestock in 2007-2017. Front. Microbiol. 10, 76.

Ceccarelli, D., van Essen-Zandbergen, A., Smid, B., Veldman, K.T., Boender, G.J., Fischer, E.A.J., Mevius, D.J., van der Goot, J. A., 2017. Competitive exclusion reduces transmission and excretion of extended-spectrum-beta-lactamase-producing escherichia coli in broilers. Appl. Environ. Microbiol. 83, 3439.

Chantziaras, I., Smet, A., Filippitzi, M.E., Damiaans, B., Haesebrouck, F., Boyen, F., Dewulf, J., 2018. The effect of a commercial competitive exclusion product on the selection of enrofloxacin resistance in commensal E. coli in broilers. Avian Pathol. 47, 443-454.

Chauvin, C., Le Devendec, L., Jouy, E., Le Cornec, M., Francart, S., Marois-Crehan, C., Kempf, I., 2013. National prevalence of resistance to third-generation cephalosporins in escherichia coli isolates from layer flocks in france. Antimicrob. Agents Chemother. 57, 6351-6353.

Chen, P., Hung, C., Huang, P., Chen, J., Huang, I., Chen, W., Chiou, Y., Hung, W., Wang, J., Cheng, M., 2016. Characteristics of CTX-M extended-spectrum beta -lactamase-producing escherichia coli strains isolated from multiple rivers in southern taiwan. Appl. Environ. Microbiol. 82, 1889-1897.

Cohen Stuart, J., van, d.M., Voets, G., Scharringa, J., Fluit, A., Hall, M.L., 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int. J. Food Microbiol. 154, 212-214.

Colomer-Lluch, M., Imamovic, L., Jofre, J., Muniesa, M., 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. Antimicrob. Agents Chemother. 55, 4908-4911.

Daehre, K., Projahn, M., Semmler, T., Roesler, U., Friese, A., 2018. Extended-spectrum beta-lactamase-/AmpC beta-lactamase-producing enterobacteriaceae in broiler farms: Transmission dynamics at farm level. Microb. Drug Resist. 24, 511-518.

Dame-Korevaar, A., Fischer, E.A.J., Stegeman, A., Mevius, D., van Essen-Zandbergen, A., Velkers, F., van der Goot, J., 2017. Dynamics of CMY-2 producing E. coli in a broiler parent flock. Vet. Microbiol. 203, 211-214.

Dame-Korevaar, A., Fischer, E.A.J., van der Goot, J., Stegeman, A., Mevius, D., 2019a. Transmission routes of ESBL/pAmpC producing bacteria in the broiler production pyramid, a literature review. Prev. Vet. Med. 162, 136-150.

Dame-Korevaar, A., Fischer, E.A.J., van der Goot, J., Velkers, F., van den Broek, J., Veldman, K., Ceccarelli, D., Mevius, D., Stegeman, A., 2019b. Effect of challenge dose of plasmid-mediated extended-spectrum β-lactamase and AmpC β-lactamase producing escherichia coli on time-until-colonization and level of excretion in young broilers. Veterinary Microbiology 239, 108446.

DANMAP, 2015. DANMAP 2014 - use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in denmark.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiology and molecular biology reviews : MMBR 74, 417-433.

D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D., 2006. Sampling the antibiotic resistome. Science 311, 374.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457.

de Been, M., Lanza, V.F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., Hu, J., Lei, Y., Li, N., Tooming-Klunderud, A., Heederik, D.J., Fluit, A.C., Bonten, M.J., Willems, R.J., de la Cruz, F., van Schaik, W., 2014. Dissemination of cephalosporin resistance genes between escherichia coli strains from farm animals and humans by specific plasmid lineages. PLoS Genet. 10, e1004776.

de Jong, M. C. M., 1995. Mathematical modelling in veterinary epidemiology: Why model building is important. Preventive Veterinary Medicine 25, 183-193.

Dekker, N., Bouma, A., Daemen, I., Klinkenberg, D., van Leengoed, L., Wagenaar, J.A., Stegeman, A., 2013. Effect of spatial separation of pigs on spread of streptococcus suis serotype 9. PLoS One 8, e61339.

Diarra, M.S., Silversides, F.G., Diarrassouba, F., Pritchard, J., Masson, L., Brousseau, R., Bonnet, C., Delaquis, P., Bach, S., Skura, B.J., Topp, E., 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, clostridium perfringens and enterococcus counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in escherichia coli isolates. Appl. Environ. Microbiol. 73, 6566-6576.

Dierikx, C.M., Van der Goot, J. A., Smith, H.E., Kant, A., Mevius, D.J., 2013a. Presence of ESBL/AmpC -producing escherichia coli in the broiler production pyramid: A descriptive study. PLoS ONE 8, e79005.

Dierikx, C.M., van der Goot, J., Fabri, T., van Essen-Zandbergen, A., Smith, H., Mevius, D., 2013b. Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing escherichia coli in dutch broilers and broiler farmers. J. Antimicrob. Chemother. 68, 60-67.

Dierikx, C.M., van der Goot, J., van Essen-Zandbergen, A., Mevius, D.J., 2018. Dynamics of cefotaxime resistant escherichia coli in broilers in the first week of life. Vet. Microbiol. 222, 64-68.

Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H., Mevius, D., 2010. Increased detection of extended spectrum beta-lactamase producing salmonella enterica and escherichia coli isolates from poultry. Vet. Microbiol. 145, 273-278.

Dolejska, M., Matulova, M., Kohoutova, L., Literak, I., Bardon, J., Cizek, A., 2011. Extended-spectrum beta-lactamase-producing escherichia coli in turkey meat production farms in the czech republic: National survey reveals widespread isolates with bla(SHV-12) genes on IncFII plasmids. Lett. Appl. Microbiol. 53, 271-277.

Donaldson, E.E., Stanley, D., Hughes, R.J., Moore, R.J., 2017. The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs. PeerJ

5, e3587.

Dorado-Garcia, A., Mevius, D.J., Jacobs, J.J., Van Geijlswijk, I.M., Mouton, J.W., Wagenaar, J.A., Heederik, D.J., 2016. Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the netherlands. J. Antimicrob. Chemother. 71, 3607-3619.

Dorado-Garcia, A., Smid, J.H., van Pelt, W., Bonten, M.J.M., Fluit, A.C., van den Bunt, G., Wagenaar, J.A., Hordijk, J., Dierikx, C.M., Veldman, K.T., de Koeijer, A., Dohmen, W., Schmitt, H., Liakopoulos, A., Pacholewicz, E., Lam, T. J. G. M., Velthuis, A.G., Heuvelink, A., Gonggrijp, M.A., van Duijkeren, E., van Hoek, A. H. A. M., de Roda Husman, A. M., Blaak, H., Havelaar, A.H., Mevius, D.J., Heederik, D.J.J., 2018. Molecular relatedness of ESBL/AmpC-producing escherichia coli from humans, animals, food and the environment: A pooled analysis. J. Antimicrob. Chemother. 73, 339-347.

Dutil, L., Irwin, R., Finley, R., Ng, L.K., Avery, B., Boerlin, P., Bourgault, A.M., Cole, L., Daignault, D., Desruisseau, A., Demczuk, W., Hoang, L., Horsman, G.B., Ismail, J., Jamieson, F., Maki, A., Pacagnella, A., Pillai, D.R., 2010. Ceftiofur resistance in salmonella enterica serovar heidelberg from chicken meat and humans, canada. Emerg. Infect. Dis. 16, 48-54.

Egervarn, M., Borjesson, S., Byfors, S., Finn, M., Kaipe, C., Englund, S., Lindblad, M., 2014. Escherichia coli with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and salmonella on meat imported into sweden. Int. J. Food Microbiol. 171, 8-14.

Evers, E.G., Pielaat, A., Smid, J.H., van Duijkeren, E., Vennemann, F.B., Wijnands, L.M., Chardon, J.E., 2017. Comparative exposure assessment of ESBL-producing escherichia coli through meat consumption. PLoS One 12, e0169589.

Ewers, C., Bethe, A., Semmler, T., Guenther, S., Wieler, L.H., 2012. Extended-spectrum beta -lactamase-producing and AmpC-producing escherichia coli from livestock and companion animals, and their putative impact on public health: A global perspective. Clinical Microbiology and Infection 18, 646-655.

Faith, D.P., 2007. The role of the phylogenetic diversity measure, PD, in bio-informatics: Getting the definition right. Evol. Bioinform Online 2, 277-283.

Ferreira, A.J., Ferreira, C.S., Knobl, T., Moreno, A.M., Bacarro, M.R., Chen, M., Robach, M., Mead, G.C., 2003. Comparison of three commercial competitive-exclusion products for controlling salmonella colonization of broilers in brazil. J. Food Prot. 66, 490-492.

Fischer, E.A.J., Dierikx, C.M., van Essen-Zandbergen, A., Mevius, D., Stegeman, A., Velkers, F.C., Klinkenberg, D., 2019. Competition between escherichia coli populations with and without plasmids carrying a gene encoding extended-spectrum beta-lactamase in the broiler chicken gut. Appl. Environ. Microbiol. 85, 10.1128/AEM.00892-19.

Fischer, E.A., Dierikx, C.M., van Essen-Zandbergen, A., van Roermund, H.J., Mevius, D.J., Stegeman, A., Klinkenberg, D., 2014. The IncI1 plasmid carrying the blaCTX-M-1 gene persists in in vitro culture of a escherichia coli strain from broilers. BMC Microbiol. 14, 77-77.

Fitch, F.M., CarmoRodrigues, M.S., Oliveira, V.G.S., Gaspari, M.V., Santos, A.d., Freitas, J.B.d., Pignatari, A.C., 2016. Beta -lactam resistance genes: Characterization, epidemiology, and first detection of $bla_{CTX-M-1}$ and $bla_{CTX-M-14}$ in salmonella spp. isolated from poultry in brazil - brazil ministry of agriculture's pathogen reduction program. Microbial Drug Resistance 22, 164-171.

Fleming, A., 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzæ. Br. J. Exp. Pathol. 10, 226-236.

Friese, A., Schulz, J., Laube, H., von Salviati, C., Hartung, J., Roesler, U., 2013. Faecal occurrence and emissions of livestock-associated methicillin-resistant staphylococcus aureus (laMRSA) and ESbl/AmpC-producing E. coli from animal farms in germany. Berl. Munch. Tierarztl. Wochenschr. 126, 175-180.

Gao, L., Hu, J., Zhang, X., Ma, R., Gao, J., Li, S., Zhao, M., Miao, Z., Chai, T., 2014. Dissemination of ESBL-producing escherichia coli of chicken origin to the nearby river water. J. Mol. Microbiol. Biotechnol. 24, 279-285.

Gay, N., Leclaire, A., Laval, M., Miltgen, G., Jego, M., Stephane, R., Jaubert, J., Belmonte, O., Cardinale, E., 2018. Risk factors of extended-spectrum beta-lactamase producing enterobacteriaceae occurrence in farms in reunion, madagascar and mayotte islands, 2016-2017. Vet. Sci. 5, 10.3390/vetsci5010022.

Gould, K., 2016. Antibiotics: From prehistory to the present day. jac 71, 572-575.

Guard-Petter, J., 2001. The chicken, the egg and salmonella enteritidis. Environ. Microbiol. 3, 421-430.

Han, Z., Willer, T., Li, L., Pielsticker, C., Rychlik, I., Velge, P.,

Kaspers, B., Rautenschlein, S., 2017. Influence of the gut microbiota composition on campylobacter jejuni colonization in chickens. Infect. Immun. 85, 10.1128/IAI.00380-17.

Hasan, B., Laurell, K., Rakib, M.M., Ahlstedt, E., Hernandez, J., Caceres, M., Jarhult, J.D., 2016. Fecal carriage of extended-spectrum beta-lactamases in healthy humans, poultry, and wild birds in leon, nicaragua-A shared pool of blaCTX-M genes and possible interspecies clonal spread of extended-spectrum beta-lactamases-producing escherichia coli. Microb. Drug Resist. 22, 682-687.

Hasan, B., Sandegren, L., Melhus, A., Drobni, M., Hernandez, J., Waldenstrom, J., Alam, M., Olsen, B., 2012. Antimicrobial drug-resistant escherichia coli in wild birds and free-range poultry, bangladesh. Emerging infectious diseases 18, 2055-2058.

Hassan, S.A., 2015. Microbiological quality of water offered to livestock assessed by escherichia coli and isolates analysis for potential virulence markers and antibiotic resistance. Int. J. Curr. Microbiol. Appl. Sci. 4, 1037-1048.

Hering, J., Fromke, C., von Munchhausen, C., Hartmann, M., Schneider, B., Friese, A., Rosler, U., Kreienbrock, L., Hille, K., 2016. Cefotaxime-resistant escherichia coli in broiler farms-A cross-sectional investigation in germany. Prev. Vet. Med. 125, 154-157.

Hesp, A., Veldman, K., van der Goot, J., Mevius, D., van Schaik, G., 2019. Monitoring antimicrobial resistance trends in commensal escherichia coli from livestock, the netherlands, 1998 to 2016. Euro Surveill. 24, 10.2807/1560-7917.ES.2019.24.25.1800438.

Hijazi, S.M., Fawzi, M.A., Ali, F.M., Abd El Galil, K. H., 2016. Prevalence and characterization of extended-spectrum beta-lactamases producing enterobacteriaceae in healthy children and associated risk factors. Ann. Clin. Microbiol. Antimicrob. 15, 3.

Hille, K., Fischer, J., Falgenhauer, L., Sharp, H., Brenner, G.M., Kadlec, K., Friese, A., Schwarz, S., Imirzalioglu, C., Kietzmann, M., Von Munchhausen, C., Kreienbrock, L., 2014. On the occurence of extended-spectrum- and AmpC-beta-lactamase-producing escherichia coli in livestock: Results of selected european studies. Berl. Munch. Tierarztl. Wochenschr. 127, 403-411.

Hiroi, M., Matsui, S., Kubo, R., Iida, N., Noda, Y., Kanda, T., Sugiyama, K., Hara-Kudo, Y., Ohashi, N., 2012. Factors for occurrence of extended-spectrum beta-lactamase-producing escherichia coli in broilers. J. Vet. Med. Sci. 74, 1635-1637.

Ho, P., ChunJiao, L., WaiU, L., LingYi, L., ChiKong, L., OiKwan, L., Chow, K., 2015. Prevalence and characterization of hybrid blaCTX-M among escherichia coli isolates from livestock and other animals. Diagn. Microbiol. Infect. Dis. 82, 148-153.

Hofacre, C.L., Johnson, A.C., Kelly, B.J., Froyman, R., 2002. Effect of a commercial competitive exclusion culture on reduction of colonization of an antibiotic-resistant pathogenic escherichia coli in day-old broiler chickens. Avian Dis. 46, 198-202.

Horton, R.A., Randall, L.P., Snary, E.L., Cockrem, H., Lotz, S., Wearing, H., Duncan, D., Rabie, A., McLaren, I., Watson, E., La Ragione, R.M., Coldham, N.G., 2011. Fecal carriage and shedding density of CTX-M extended-spectrum {beta}-lactamase-producing escherichia coli in cattle, chickens, and pigs: Implications for environmental contamination and food production. Appl. Environ. Microbiol. 77, 3715-3719.

Huijbers, P.M., Blaak, H., de Jong, M.C., Graat, E.A., Vandenbroucke-Grauls, C.M., de Roda Husman, A. M., 2015a. Role of the environment in the transmission of antimicrobial resistance to humans: A review. Environ. Sci. Technol. 49, 11993-12004.

Huijbers, P.M., Graat, E.A., Haenen, A.P., van Santen, M.G., van Essen-Zandbergen, A., Mevius, D.J., van Duijkeren, E., van Hoek, A.H., 2014. Extended-spectrum and AmpC beta-lactamase-producing escherichia coli in broilers and people living and/or working on broiler farms: Prevalence, risk factors and molecular characteristics. J. Antimicrob. Chemother. 69, 2669-2675.

Huijbers, P.M., Graat, E.A., van Hoek, A.H., Veenman, C., de Jong, M.C., van Duijkeren, E., 2016. Transmission dynamics of extended-spectrum beta-lactamase and AmpC beta-lactamase-producing escherichia coli in a broiler flock without antibiotic use. Prev. Vet. Med. 131, 12-19.

Huijbers, P.M., van Hoek, A.H., Graat, E.A., Haenen, A.P., Florijn, A., Hengeveld, P.D., van Duijkeren, E., 2015b. Methicillin-resistant staphylococcus aureus and extended-spectrum and AmpC beta-lactamase-producing escherichia coli in broilers and in people living and/or working on organic broiler farms. Vet. Microbiol. 176, 120-125.

Idris, U., Lu, J., Maier, M., Sanchez, S., Hofacre, C.L., Harmon, B.G., Maurer, J.J., Lee, M.D., 2006. Dissemination of fluoroquinolone-resistant campylobacter spp. within an integrated commercial poultry production system. Appl. Environ. Micro-

biol. 72, 3441-3447.

Jacoby, G.A., 2009. AmpC beta-lactamases. Clin. Microbiol. Rev. 22, 161-82.

Jett, B.D., Hatter, K.L., Huycke, M.M., Gilmore, M.S., 1997. Simplified agar plate method for quantifying viable bacteria. BioTechniques 23, 648-650.

Jiang, H.X., Lu, D.H., Chen, Z.L., Wang, X.M., Chen, J.R., Liu, Y.H., Liao, X.P., Liu, J.H., Zeng, Z.L., 2011. High prevalence and widespread distribution of multi-resistant escherichia coli isolates in pigs and poultry in china. Vet. J. 187, 99-103.

Jimenez-Belenguer, A., Domenech, E., Villagra, A., Fenollar, A., Ferrus, M.A., 2016. Antimicrobial resistance of escherichia coli isolated in newly-hatched chickens and effect of amoxicillin treatment during their growth. Avian Pathol. 45, 501-507.

Jones, E.M., Snow, L.C., Carrique-Mas, J.J., Gosling, R.J., Clouting, C., Davies, R.H., 2013. Risk factors for antimicrobial resistance in escherichia coli found in GB turkey flocks. Vet. Rec. 173, 422.

Jurburg, S.D., Brouwer, M.S.M., Ceccarelli, D., van der Goot, J., Jansman, A.J.M., Bossers, A., 2019. Patterns of community assembly in the developing chicken microbiome reveal rapid primary succession. Microbiologyopen e821.

Kalbfleisch, J.D. and Prentice, R.L., 2002. The Statistical Analysis of Failure Time Data. John Wiley and Sons, New York.

Karanika, S., Karantanos, T., Arvanitis, M., Grigoras, C., Mylonakis, E., 2016. Fecal colonization with extended-spectrum beta-lactamase-producing enterobacteriaceae and risk factors among healthy individuals: A systematic review and metaanalysis. Clin. Infect. Dis. 63, 310-318.

Katsma, W.E., De Koeijer, A.A., Jacobs-Reitsma, W.F., Mangen, M.J., Wagenaar, J.A., 2007. Assessing interventions to reduce the risk of campylobacter prevalence in broilers. Risk Anal. 27, 863-876.

Kers, J.G., Velkers, F.C., Fischer, E.A.J., Hermes, G.D.A., Stegeman, J.A., Smidt, H., 2018. Host and environmental factors affecting the intestinal microbiota in chickens. Front. Microbiol. 9, 235.

Kers, J.G., Velkers, F.C., Fischer, E.A.J., Hermes, G.D.A., Lamot, D.M., Stegeman, J.A., Smidt, H., 2019. Take care of the environment: Housing conditions affect the interplay of nutritional interventions and intestinal microbiota in broiler chickens. Animal Microbiome 1, 10.

Kim, S.H., Wei, C.I., Tzou, Y.M., An, H., 2005. Multidrug-resistant klebsiella pneumoniae isolated from farm environments and retail products in oklahoma. J. Food Prot. 68, 2022-2029.

Klinkenberg, D., de Bree, J., Laevens, H., de Jong, M. C. M., 2002. Within- and between-pen transmission of classical swine fever virus: A new method to estimate the basic reproduction ratio from transmission experiments. Epidemiol. Infect. 128, 293-299.

Kluytmans, J.A., Overdevest, I.T., Willemsen, I., Kluytmans-van, d.B., van, d.Z., Heck, M., Rijnsburger, M., Vandenbroucke-Grauls, C.M., Savelkoul, P.H., Johnston, B.D., Gordon, D., Johnson, J.R., 2013. Extended-spectrum beta-lactamase-producing escherichia coli from retail chicken meat and humans: Comparison of strains, plasmids, resistance genes, and virulence factors. Clin. Infect. Dis. 56, 478-487.

Kubasova, T., Kollarcikova, M., Crhanova, M., Karasova, D., Cejkova, D., Sebkova, A., Matiasovicova, J., Faldynova, M., Pokorna, A., Cizek, A., Rychlik, I., 2019. Contact with adult hen affects development of caecal microbiota in newly hatched chicks. PLoS One 14, e0212446.

Laube, H., Friese, A., von Salviati, C., Guerra, B., Kasbohrer, A., Kreienbrock, L., Roesler, U., 2013. Longitudinal monitoring of extended-spectrum-beta-lactamase/AmpC-producing escherichia coli at german broiler chicken fattening farms. Appl. Environ. Microbiol. 79, 4815-4820.

Laube, H., Friese, A., von Salviati, C., Guerra, B., Rosler, U., 2014. Transmission of ESBL/AmpC-producing escherichia coli from broiler chicken farms to surrounding areas. Vet. Microbiol. 172, 519-527.

Laukens, D., Brinkman, B.M., Raes, J., De Vos, M., Vandenabeele, P., 2016. Heterogeneity of the gut microbiome in mice: Guidelines for optimizing experimental design. FEMS Microbiol. Rev. 40, 117-132.

Lawley, T.D., Walker, A.W., 2013. Intestinal colonization resistance. Immunology 138, 1-11.

Le Devendec, L., Bouder, A., Dheilly, A., Hellard, G., Kempf, I., 2011. Persistence and spread of qnr, extended-spectrum beta-lactamase, and ampC resistance genes in the digestive tract of chickens. Microb. Drug Resist. 17, 129-134.

Leistner, R., Meyer, E., Gastmeier, P., Pfeifer, Y., Eller, C., Dem,

P., Schwab, F., 2013. Risk factors associated with the community-acquired colonization of extended-spectrum beta-lactamase (ESBL) positive escherichia coli. an exploratory case-control study. PLoS One 8, e74323.

Leverstein-van Hall, M.A., Dierikx, C.M., Cohen Stuart, J., Voets, G.M., van den Munckhof, M. P., van Essen-Zandbergen, A., Platteel, T., Fluit, A.C., van de Sande-Bruinsma, N., Scharinga, J., Bonten, M.J., Mevius, D.J., National ESBL surveillance group, 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin. Microbiol. Infect. 17, 873-880.

Li, L., Wang, B., Feng, S., Li, J., Wu, C., Wang, Y., Ruan, X., Zeng, M., 2014. Prevalence and characteristics of extended-spectrum beta-lactamase and plasmid-mediated fluoroquinolone resistance genes in escherichia coli isolated from chickens in anhui province, china. PloS one 9, e104356.

Li, X.Z., Mehrotra, M., Ghimire, S., Adewoye, L., 2007. Beta-lactam resistance and beta-lactamases in bacteria of animal origin. Vet. Microbiol. 121, 197-214.

Line, J., Hiett, K., Conlan, A., 2008. Comparison of challenge models for determining the colonization dose of campylobacter jejuni in broiler chicks. Poult. Sci. 87, 1700-1706.

Livermore, D.M., 2008. Defining an extended-spectrum β-lactamase. Clinical Microbiology and Infection 14, 3-10.

Ljubojevic, D., Puvaca, N., Pelic, M., Todorovic, D., Pajic, M., Milanov, D., Velhner, M., 2016. Epidemiological significance of poultry litter for spreading the antibiotic-resistant strains of escherichia coli. Worlds Poult. Sci. J. 72, 485-494.

Lobanovska, M., Pilla, G., 2017. Penicillin's discovery and antibiotic resistance: Lessons for the future? Yale J. Biol. Med. 90, 135-145.

Lopatkin, A.J., Meredith, H.R., Srimani, J.K., Pfeiffer, C., Durrett, R., You, L., 2017. Persistence and reversal of plasmid-mediated antibiotic resistance. Nature Communications 8, 1689.

Lozupone, C.A., Hamady, M., Kelley, S.T., Knight, R., 2007. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl. Environ. Microbiol. 73, 1576-1585.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J., Lee, M.D., 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. 69, 6816-6824.

Lu, L., Dai, L., Wang, Y., Wu, C., Chen, X., Li, L., Qi, Y., Xia, L., Shen, J., 2010. Characterization of antimicrobial resistance and integrons among escherichia coli isolated from animal farms in eastern china. Acta Trop. 113, 20-25.

Luoma, A., Markazi, A., Shanmugasundaram, R., Murugesan, G.R., Mohnl, M., Selvaraj, R., 2017. Effect of synbiotic supplementation on layer production and cecal salmonella load during a salmonella challenge. Poult. Sci. 96, 4208-4216.

Lurette, A., Belloc, C., Touzeau, S., Hoch, T., Ezanno, P., Seegers, H., Fourichon, C., 2008. Modelling salmonella spread within a farrow-to-finish pig herd. Vet. Res. 39, 49.

Ma, J., Liu, J.H., Lv, L., Zong, Z., Sun, Y., Zheng, H., Chen, Z., Zeng, Z.L., 2012. Characterization of extended-spectrum beta-lactamase genes found among escherichia coli isolates from duck and environmental samples obtained on a duck farm. Appl. Environ. Microbiol. 78, 3668-3673.

MARAN, 2016. Monitoring of antimicrobial resistance and antibiotic usage in animals in the netherlands in 2015.

MARAN, 2018. Monitoring of antimicrobial resistance and antibiotic usage in animals in the netherlands in 2017.

MARAN, 2019. Monitoring of antimicrobial resistance and antibiotic usage in animals in the netherlands in 2018.

Markazi, A., Luoma, A., Shanmugasundaram, R., Mohnl, M., Raj Murugesan, G., Selvaraj, R., 2018. Effects of drinking water synbiotic supplementation in laying hens challenged with salmonella. Poult. Sci. 97, 3510-3518.

Mathys, D.A., Mollenkopf, D.F., Nolting, J., Bowman, A.S., Daniels, J.B., Wittum, T.E., 2017. Extended-spectrum cephalosporin-resistant enterobacteriaceae in enteric microflora of wild ducks. J. Wildl. Dis. 53, 690-694.

Mattiello, S.P., Drescher, G., Barth, V.C., Jr., Ferreira, C.A.S., Oliveira, S.D., 2015. Characterization of antimicrobial resistance in salmonella enterica strains isolated from brazilian poultry production. Antonie Van Leeuwenhoek 108, 1227-1238.

Merchant, L.E., Rempel, H., Forge, T., Kannangara, T., Bittman, S., Delaquis, P., Topp, E., Ziebell, K.A., Diarra, M.S., 2012. Characterization of antibiotic-resistant and potentially pathogenic escherichia coli from soil fertilized with litter of broiler chickens fed antimicrobial-supplemented diets. Can. J. Microbiol. 58, 1084-1098.

Mesa, R.J., Blanc, V., Blanch, A.R., Cortes, P., Gonzalez, J.J., Lavilla, S., Miro, E., Muniesa, M., Saco, M., Tortola, M.T., Mirelis, B., Coll, P., Llagostera, M., Prats, G., Navarro, F., 2006. Extended-spectrum beta-lactamase-producing enterobacteriaceae in different environments (humans, food, animal farms and sewage). J. Antimicrob. Chemother. 58, 211-215.

Methner, U., Friese, A., Rosler, U., 2019. Competitive exclusion: A tool to combat extended-spectrum beta-lactamase-producing escherichia coli strains in chickens. Res. Vet. Sci. 123, 124-128.

Mevius, D., Heederik, D., 2014. Reduction of antibiotic use in animals 'let's go dutch". Journal fur Verbraucherschutz und Lebensmittelsicherheit 9, 177-181.

Mezhoud, H., Boyen, F., Touazi, L.H., Garmyn, A., Moula, N., Smet, A., Haesbrouck, F., Martel, A., Iguer-Ouada, M., Touati, A., 2015. Extended spectrum beta-lactamase producing escherichia coli in broiler breeding roosters: Presence in the reproductive tract and effect on sperm motility. Anim. Reprod. Sci. 159, 205-211.

Mezhoud, H., Chantziaras, I., Iguer-Ouada, M., Moula, N., Garmyn, A., Martel, A., Touati, A., Smet, A., Haesebrouck, F., Boyen, F., 2016. Presence of antimicrobial resistance in coliform bacteria from hatching broiler eggs with emphasis on ESBL/AmpC-producing bacteria. Avian Pathol. 45, 493-500.

Miranda, J.M., Guarddon, M., Vazquez, B.I., Fente, C.A., BarrosVelazquez, J., Cepeda, A., Franco, C.M., 2008. Antimicrobial resistance in enterobacteriaceae strains isolated from organic chicken, conventional chicken and conventional turkey meat: A comparative survey. Food Control 19, 412-416.

Mo, S.S., Kristoffersen, A.B., Sunde, M., Nodtvedt, A., Norstrom, M., 2016. Risk factors for occurrence of cephalosporin-resistant escherichia coli in norwegian broiler flocks. Prev. Vet. Med. 130, 112-118.

Mo, S.S., Norstrom, M., Slettemeas, J.S., Lovland, A., Urdahl, A.M., Sunde, M., 2014. Emergence of AmpC-producing escherichia coli in the broiler production chain in a country with a low antimicrobial usage profile. Vet. Microbiol. 171, 315-320.

Mo, S.S., Sunde, M., Ilag, H.K., Langsrud, S., Heir, E., 2017. Transfer potential of plasmids conferring extended-spectrum-cephalosporin resistance in escherichia coli from poultry. Appl. Environ. Microbiol. 83, 654.

Mollenkopf, D., Cenera, J., Bryant, E., King, C., Kashoma, I., Kumar, A., Funk, J., Rajashekara, G., Wittum, T., 2014. Organic or antibiotic-free labeling does not impact the recovery of enteric pathogens and antimicrobial-resistant escherichia coli from fresh retail chicken. Foodborne Pathog. Dis. 11, 920-9.

Mughini-Gras, L., Dorado-Garcia, A., van Duijkeren, E., van den Bunt, G., Dierikx, C.M., Bonten, M.J.M., Bootsma, M.C.J., Schmitt, H., Hald, T., Evers, E.G., de Koeijer, A., van Pelt, W., Franz, E., Mevius, D.J., Heederik, D.J.J., ESBL Attribution Consortium, 2019. Attributable sources of community-acquired carriage of escherichia coli containing beta-lactam antibiotic resistance genes: A population-based modelling study. Lancet Planet. Health. 3, e357-e369.

Myrenas, M., Slettemeas, J.S., Thorsteinsdottir, T.R., Bengtsson, B., Borjesson, S., Nilsson, O., Landen, A., Sunde, M., 2018. Clonal spread of escherichia coli resistant to cephalosporins and quinolones in the nordic broiler production. Vet. Microbiol. 213, 123-128.

Nakamura, A., Ota, Y., Mizukami, A., Ito, T., Ngwai, Y.B., Adachi, Y., 2002. Evaluation of aviguard, a commercial competitive exclusion product for efficacy and after-effect on the antibody response of chicks to salmonella. Poult. Sci. 81, 1653-1660.

Nakphaichit, M., Thanomwongwattana, S., Phraephaisarn, C., Sakamoto, N., Keawsompong, S., Nakayama, J., Nitisinprasert, S., 2011. The effect of including lactobacillus reuteri KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens. Poult. Sci. 90, 2753-2765.

Netherlands Veterinary Medicines Institute (SDa), 2019. Usage of antibiotics in agricultural livestock in 2018.

Newell, D.G., Elvers, K.T., Dopfer, D., Hansson, I., Jones, P., James, S., Gittins, J., Stern, N.J., Davies, R., Connerton, I., Pearson, D., Salvat, G., Allen, V.M., 2011. Biosecurity-based interventions and strategies to reduce campylobacter spp. on poultry farms. Appl. Environ. Microbiol. 77, 8605-8614.

Nguyen, D.T.A., Kanki, M., Phuc Do Nguyen, Hien Thi Le, Phong Thanh Ngo, Doan Nguyen Minh Tran, Ninh Hoang Le, Chinh Van Dang, Kawai, T., Kawahara, R., Yonogi, S., Hirai, Y., Jinnai, M., Yamasaki, S., Kumeda, Y., Yamamoto, Y., 2016. Prevalence, antibiotic resistance, and extended-spectrum and AmpC beta -lactamase productivity of salmonella isolates from raw meat and seafood samples in ho chi minh city, vietnam. Int. J. Food Microbiol. 236, 115-122.

Nguyen, V.T., Carrique-Mas, J.J., Ngo, T.H., Ho, H.M., Ha, T.T., Campbell, J.I., Nguyen, T.N., Hoang, N.N., Pham, V.M., Wage-

naar, J.A., Hardon, A., Thai, Q.H., Schultsz, C., 2015. Prevalence and risk factors for carriage of antimicrobial-resistant escherichia coli on household and small-scale chicken farms in the mekong delta of vietnam. J. Antimicrob. Chemother. 70, 2144-2152.

Nhung, N.T., Cuong, N.V., Campbell, J., Hoa, N.T., Bryant, J.E., Truc, V.N., Kiet, B.T., Jombart, T., Trung, N.V., Hien, V.B., Thwaites, G., Baker, S., Carrique-Mas, J., 2015. High levels of antimicrobial resistance among escherichia coli isolates from livestock farms and synanthropic rats and shrews in the mekong delta of vietnam. Appl. Environ. Microbiol. 81, 812-820.

Nikaido, H., 2009. Multidrug resistance in bacteria. Annu. Rev. Biochem. 78, 119-146.

Nilsson, O., 2012. Vancomycin resistant enterococci in farm animals - occurrence and importance. Infect. Ecol. Epidemiol. 2, 10.3402/iee.v2i0.16959.

Nilsson, O., Borjesson, S., Landen, A., Bengtsson, B., 2014. Vertical transmission of escherichia coli carrying plasmid-mediated AmpC (pAmpC) through the broiler production pyramid. J. Antimicrob. Chemother. 69, 1497-1500.

Nuotio, L., Schneitz, C., Nilsson, O., 2013. Effect of competitive exclusion in reducing the occurrence of escherichia coli producing extended-spectrum beta-lactamases in the ceca of broiler chicks. Poult. Sci. 92, 250-254.

Nurmi, E., Nuotio, L., Schneitz, C., 1992. The competitive exclusion concept: Development and future. Int. J. Food Microbiol. 15, 237-240.

Obeng, A.S., Rickard, H., Ndi, O., Sexton, M., Barton, M., 2014. Prevalence of antimicrobial resistance in enterococci and escherichia coli in meat chicken flocks during a production cycle and egg layer pullets during rearing. Int. J. Poult. Sci. 13, 489-503.

Oguttu, J.W., Veary, C.M., Picard, J.A., 2008. Antimicrobial drug resistance of escherichia coli isolated from poultry abattoir workers at risk and broilers on antimicrobials. J. S. Afr. Vet. Assoc. 79, 161-166.

Oh, J.Y., Kwon, Y.K., Tamang, M.D., Jang, H.K., Jeong, O.M., Lee, H.S., Kang, M.S., 2015. Plasmid-mediated quinolone resistance in escherichia coli isolates from wild birds and chickens in south korea. Microb. Drug Resist. 22, 69-79.

Oikarainen, P.E., Pohjola, L.K., Pietola, E.S., Heikinheimo, A., 2019. Direct vertical transmission of ESBL/pAmpC-producing escherichia coli limited in poultry production pyramid. Veterinary Microbiology 231, 100-106.

Olsen, R.H., Bisgaard, M., Lohren, U., Robineau, B., Christensen, H., 2014. Extended-spectrum beta-lactamase-producing escherichia coli isolated from poultry: A review of current problems, illustrated with some laboratory findings. Avian pathology : journal of the W. V. P. A 43, 199-208.

Oluduro, A.O., 2012. Antibiotic-resistant commensal escherichia coli in faecal droplets from bats and poultry in nigeria. Vet. Ital. 48, 297-308.

Osman, K.M., Kappell, A.D., Elhadidy, M., ElMougy, F., El-Ghany, W.A.A., Orabi, A., Mubarak, A.S., Dawoud, T.M., Hemeg, H.A., Moussa, I.M.I., Hessain, A.M., Yousef, H.M.Y., 2018. Poultry hatcheries as potential reservoirs for antimicrobial-resistant escherichia coli: A risk to public health and food safety. Sci. Rep. 8, 5859-7.

Ozaki, H., Matsuoka, Y., Nakagawa, E., Murase, T., 2017. Characteristics of escherichia coli isolated from broiler chickens with colibacillosis in commercial farms from a common hatchery. Poult. Sci. 1-8.

Paivarinta, M., Pohjola, L., Fredriksson-Ahomaa, M., Heikinheimo, A., 2016. Low occurrence of extended-spectrum beta-lactamase-producing escherichia coli in finnish food-producing animals. Zoonoses Public. Health. 63, 624-631.

Parker, D., Sniatynski, M.K., Mandrusiak, D., Rubin, J.E., 2016. Extended-spectrum beta -lactamase producing escherichia coli isolated from wild birds in saskatoon, canada. Lett. Appl. Microbiol. 63, 11-15.

Persoons, D., Dewulf, J., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Catry, B., Butaye, P., Haesebrouck, F., 2010. Prevalence and persistence of antimicrobial resistance in broiler indicator bacteria. Microbial drug resistance (Larchmont, N.Y.) 16, 67-74.

Persoons, D., Haesebrouck, F., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Catry, B., Berge, A.C., Butaye, P., Dewulf, J., 2011. Risk factors for ceftiofur resistance in escherichia coli from belgian broilers. Epidemiol. Infect. 139, 765-771.

Plaza Rodriguez, C., Correia Carreira, G., Kasbohrer, A., 2018. A probabilistic transmission model for the spread of extended-spectrum-beta-lactamase and AmpC-beta-lactamase-producing escherichia coli in the broiler production chain. Risk

Anal. 38, 2659-2682.

Pohjola, L., Nykasenoja, S., Kivisto, R., Soveri, T., Huovilainen, A., Hanninen, M.L., Fredriksson-Ahomaa, M., 2016. Zoonotic public health hazards in backyard chickens. Zoonoses Public. Health. 63, 420-430.

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F., Rychlik, I., 2015. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. Appl. Environ. Microbiol. 82, 1569-1576.

Poole, T., Crippen, T., 2009. Conjugative plasmid transfer between salmonella enterica newport and escherichia coli within the gastrointestinal tract of the lesser mealworm beetle, alphitobius diaperinus (coleoptera: Tenebrionidae). Poult. Sci. 88, 1553-1558.

Projahn, M., Daehre, K., Roesler, U., Friese, A., 2017. Extended-spectrum-beta-lactamase- and plasmid-encoded cephamycinase-producing enterobacteria in the broiler hatchery as a potential mode of pseudo-vertical transmission. Appl. Environ. Microbiol. 83, 2364.

Projahn, M., Daehre, K., Semmler, T., Guenther, S., Roesler, U., Friese, A., 2018. Environmental adaptation and vertical dissemination of ESBL-/pAmpC-producing escherichia coli in an integrated broiler production chain in the absence of an antibiotic treatment. Microb. Biotechnol. 11, 1017-1026.

Qiao, J., Zhang, Q., Alali, W.Q., Wang, J., Meng, L., Xiao, Y., Yang, H., Chen, S., Cui, S., Yang, B., 2017. Characterization of extended-spectrum beta-lactamases (ESBLs)-producing salmonella in retail raw chicken carcasses. Int. J. Food Microbiol. 248, 72-81.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glockner, F.O., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41, 590.

Ramiro-Garcia, J., Hermes, G.D.A., Giatsis, C., Sipkema, D., Zoetendal, E.G., Schaap, P.J., Smidt, H., 2016. NG-tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. F1000Res 5, 1791.

Randall, L.P., Lodge, M.P., Elviss, N.C., Lemma, F.L., Hopkins, K.L., Teale, C.J., Woodford, N., 2017. Evaluation of meat, fruit and vegetables from retail stores in five united kingdom regions as sources of extended-spectrum beta-lactamase (ESBL)-producing and carbapenem-resistant escherichia coli. Int. J. Food Microbiol. 241, 283-290.

Rasmussen, M.M., Opintan, J.A., Frimodt-Moller, N., Styrishave, B., 2015. Beta-lactamase producing escherichia coli isolates in imported and locally produced chicken meat from ghana. PLoS One 10, e0139706.

Raza, S., Mohsin, M., Madni, W.A., Sarwar, F., Saqib, M., Aslam, B., 2017. First report of $bla_{CTX\text{-}M\text{-}15}$-type ESBL-producing klebsiella pneumoniae in wild migratory birds in pakistan. EcoHealth 14, 182-186.

Reich, F., Atanassova, V., Klein, G., 2013. Extended-spectrum beta-lactamase- and AmpC-producing enterobacteria in healthy broiler chickens, germany. Emerging Infect. Dis. 19, 1253-1259.

Richards, P., Fothergill, J., Bernardeau, M., Wigley, P., 2019. Development of the caecal microbiota in three broiler breeds. Frontiers in Veterinary Science 6, 201.

Robé, C., Blasse, A., Merle, R., Friese, A., Roesler, U., Guenther, S., 2019. Low dose colonization of broiler chickens with ESBL-/AmpC- producing escherichia coli in a seeder-bird model independent of antimicrobial selection pressure. Frontiers in Microbiology 10, 2124.

Roth, N., Mayrhofer, S., Gierus, M., Weingut, C., Schwarz, C., Doupovec, B., Berrios, R., Domig, K.J., 2017. Effect of an organic acids based feed additive and enrofloxacin on the prevalence of antibiotic-resistant E. coli in cecum of broilers. Poult. Sci. 96, 4053-4060.

RStudio Team, 2016. RStudio: Integrated development for R.

Saliu, E.M., Vahjen, W., Zentek, J., 2017. Types and prevalence of extended-spectrum beta-lactamase producing enterobacteriaceae in poultry. Anim. Health. Res. Rev. 1-12.

Samanta, I., Joardar, S.N., Das, P.K., Sar, T.K., 2015. Comparative possession of shiga toxin, intimin, enterohaemolysin and major extended spectrum beta lactamase (ESBL) genes in escherichia coli isolated from backyard and farmed poultry. Iran. J. Vet. Med. 16, 90-93.

Sapkota, A.R., Kinney, E.L., George, A., Hulet, R.M., Cruz-Cano, R., Schwab, K.J., Zhang, G., Joseph, S.W., 2014. Lower prevalence of antibiotic-resistant salmonella on large-scale U.S. conventional poultry farms that transitioned to organic practices. Sci. Total Environ. 476-477, 387-392.

Schaufler, K., Semmler, T., Wieler, L.H., Wohrmann, M., Baddam, R., Ahmed, N., Muller, K., Kola, A., Fruth, A., Ewers, C.,

Guenther, S., 2016. Clonal spread and interspecies transmission of clinically relevant ESBL-producing escherichia coli of ST410--another successful pandemic clone? FEMS Microbiol. Ecol. 92, fiv155.

Schneitz, C., Hakkinen, M., 2016. The efficacy of a commercial competitive exclusion product on campylobacter colonization in broiler chickens in a 5-week pilot-scale study. Poult. Sci. 95, 1125-1128.

Schokker, D., Jansman, A.J., Veninga, G., de Bruin, N., Vastenhouw, S.A., de Bree, F.M., Bossers, A., Rebel, J.M., Smits, M.A., 2017. Perturbation of microbiota in one-day old broiler chickens with antibiotic for 24 hours negatively affects intestinal immune development. BMC Genomics 18, 241-6.

Schwaiger, K., Bauer, J., Holzel, C.S., 2013. Selection and persistence of antimicrobial-resistant escherichia coli including extended-spectrum beta-lactamase producers in different poultry flocks on one chicken farm. Microb. Drug Resist. 19, 498-506.

Shahada, F., Chuma, T., Kosugi, G., Kusumoto, M., Iwata, T., Akiba, M., 2013. Distribution of extended-spectrum cephalosporin resistance determinants in salmonella enterica and escherichia coli isolated from broilers in southern japan. Poult. Sci. 92, 1641-1649.

Siemon, C.E., Bahnson, P.B., Gebreyes, W.A., 2007. Comparative investigation of prevalence and antimicrobial resistance of salmonella between pasture and conventionally reared poultry. Avian Dis. 51, 112-117.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Catry, B., Herman, L., Haesebrouck, F., Butaye, P., 2008. Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal escherichia coli isolates in belgian broiler farms. Antimicrob. Agents Chemother. 52, 1238-1243.

Smith, J.C., Ferreira, A.M., Baak, J.H., Burt, S.A., 2017. Antibiotic resistant bacteria in rodents, birds, and insects. Proceedings of the Ninth International Conference on Urban Pests, 189-192.

Sola-Gines, M., Gonzalez-Lopez, J.J., Cameron-Veas, K., Piedra-Carrasco, N., Cerda-Cuellar, M., Migura-Garcia, L., 2015. Houseflies (musca domestica) as vectors for extended-spectrum beta-lactamase-producing escherichia coli on spanish broiler farms. Appl. Environ. Microbiol. 81, 3604-3611.

Stanley, D., Keyburn, A.L., Denman, S.E., Moore, R.J., 2012. Changes in the caecal microflora of chickens following clostridium perfringens challenge to induce necrotic enteritis. Vet. Microbiol. 159, 155-162.

Stedt, J., Bonnedahl, J., Hernandez, J., Waldenstrom, J., McMahon, B.J., Tolf, C., Olsen, B., Drobni, M., 2015. Carriage of CTX-M type extended spectrum beta-lactamases (ESBLs) in gulls across europe. Acta Vet. Scand. 57, 74-82.

Stokes, M.O., Cottell, J.L., Piddock, L.J., Wu, G., Wootton, M., Mevius, D.J., Randall, L.P., Teale, C.J., Fielder, M.D., Coldham, N.G., 2012. Detection and characterization of pCT-like plasmid vectors for blaCTX-M-14 in escherichia coli isolates from humans, turkeys and cattle in england and wales. J. Antimicrob. Chemother. 67, 1639-1644.

Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M., Macalpine, R., 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88, 2474-2481.

van Bunnik, B.A., Katsma, W.E., Wagenaar, J.A., Jacobs-Reitsma, W.F., de Jong, M.C., 2012. Acidification of drinking water inhibits indirect transmission, but not direct transmission of campylobacter between broilers. Prev. Vet. Med. 105, 315-319.

van Bunnik, B.A., Ssematimba, A., Hagenaars, T.J., Nodelijk, G., Haverkate, M.R., Bonten, M.J., Hayden, M.K., Weinstein, R.A., Bootsma, M.C., De Jong, M.C., 2014. Small distances can keep bacteria at bay for days. Proc. Natl. Acad. Sci. U. S. A. 111, 3556-3560.

van den Broek, J., Heesterbeek, H., 2007. Nonhomogeneous birth and death models for epidemic outbreak data. Biostatistics 8, 453-467.

van der Waaij, D., Berghuis-de Vries, J.M., Lekkerkerk, L., 1971. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J. Hyg. (Lond) 69, 405-411.

van Duijkeren, E., van Overbeek, W.M., Huijbers, P.M., van de Giessen, A. W., van Hoek, A.H., 2015. Inoculation of mice with avian blaCTX-M-1- or blaCMY-2-carrying escherichia coli strains does not lead to long-term colonization. J. Antimicrob. Chemother. 70, 3164-3165.

van Hoek, A. H. A. M., Veenman, C., Florijn, A., Huijbers, P.M.C., Graat, E.A.M., de Greeff, S., Dierikx, C.M., van Duijkeren, E., 2018. Longitudinal study of ESBL escherichia coli carriage on an organic broiler farm. J. Antimicrob. Chemother. 73, 3298-3304.

van Hoek, A.H., Stalenhoef, J.E., van Duijkeren, E., Franz, E., 2016. Comparative virulotyping of extended-spectrum cephalosporin-resistant E. coli isolated from broilers, humans on broiler farms and in the general population and UTI patients. Vet. Microbiol. 194, 55-61.

Van Immerseel, F., Zutter, L.d., Houf, K., Pasmans, F., Haesebrouck, F., Ducatelle, R., 2009. Strategies to control salmonella in the broiler production chain. Worlds Poult. Sci. J. 65, 367-392.

Varmuzova, K., Kubasova, T., Davidova-Gerzova, L., Sisak, F., Havlickova, H., Sebkova, A., Faldynova, M., Rychlik, I., 2016. Composition of gut microbiota influences resistance of newly hatched chickens to salmonella enteritidis infection. Front. Microbiol. 7, 957.

Velkers, F.C., Bouma, A., Stegeman, J.A., de Jong, M.C., 2012. Oocyst output and transmission rates during successive infections with eimeria acervulina in experimental broiler flocks. Vet. Parasitol. 187, 63-71.

Velthuis, A.G., Bouma, A., Katsma, W.E., Nodelijk, G., De Jong, M.C., 2007. Design and analysis of small-scale transmission experiments with animals. Epidemiol. Infect. 135, 202-217.

Vergara, A., Pitart, C., Montalvo, T., Roca, I., Sabate, S., Hurtado, J.C., Planell, R., Marco, F., Ramirez, B., Peracho, V., Simon, M.d., Vila, J., 2017. Prevalence of extended-spectrum- beta -lactamase- and/or carbapenemase-producing escherichia coli isolated from yellow-legged gulls from barcelona, spain. Antimicrob. Agents Chemother. 61, 2071.

Videnska, P., Sedlar, K., Lukac, M., Faldynova, M., Gerzova, L., Cejkova, D., Sisak, F., Rychlik, I., 2014. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. PLoS One 9, e115142.

Wang, J., Ma, Z.B., Zeng, Z.L., Yang, X.W., Huang, Y., Liu, J.H., 2017. The role of wildlife (wild birds) in the global transmission of antimicrobial resistance genes. Zool. Res. 38, 55-80.

Weill, F.X., Lailler, R., Praud, K., Kerouanton, A., Fabre, L., Brisabois, A., Grimont, P.A.D., Cloeckaert, A., 2004. Emergence of extended-spectrum- beta -lactamase (CTX-M-9)-producing multiresistant strains of salmonella enterica serotype virchow in poultry and humans in france. J. Clin. Microbiol. 42, 5767-5773.

Wilkinson, D.M., 1999. Bacterial ecology, antibiotics and selection for virulence. Ecol. Lett. 2, 207-209.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C., Ochman, H., Achtman, M., 2006. Sex and virulence in escherichia coli: An evolutionary perspective. Mol. Microbiol. 60, 1136-1151.

World Health Organization, 2016. Fact sheet antimicrobial resistance number 194.

World Health Organization, 2018. Fact sheet antimicrobial resistance number 194.

Wu, H., Xia, X., Cui, Y., Hu, Y., Xi, M., Wang, X., Shi, X., Wang, D., Meng, J., Yang, B., 2013. Prevalence of extended-spectrum b-lactamase-producing salmonella on retail chicken in six provinces and two national cities in the people's republic of china. J. Food Prot. 76, 2040-2044.

Xu, X., Cui, S., Zhang, F., Luo, Y., Gu, Y., Yang, B., Li, F., Chen, Q., Zhou, G., Wang, Y., Pang, L., Lin, L., 2014. Prevalence and characterization of cefotaxime and ciprofloxacin co-resistant escherichia coli isolates in retail chicken carcasses and ground pork, china. Microbial drug resistance (Larchmont, N.Y.) 20, 73-81.

Yossapol, M., Sugiyama, M., Asai, T., 2017. The occurrence of CTX-M-25-producing enterobacteriaceae in day-old broiler chicks in japan. Journal of Veterinary Medical Science 79, 1644-1647.

Zurek, L., Ghosh, A., 2014. Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. Appl. Environ. Microbiol. 80, 3562-3567.

Zurfluh, K., Jakobi, G., Stephan, R., Hachler, H., Nuesch-Inderbinen, M., 2014a. Replicon typing of plasmids carrying bla CTX-M-1 in enterobacteriaceae of animal, environmental and human origin. Front. Microbiol. 5, 555.

Zurfluh, K., Wang, J., Klumpp, J., Nuesch-Inderbinen, M., Fanning, S., Stephan, R., 2014b. Vertical transmission of highly similar bla CTX-M-1-harboring IncI1 plasmids in escherichia coli with different MLST types in the poultry production pyramid. Front. Microbiol. 5, 519.

Summary

Antimicrobial resistance is considered by the World Health Organization as a serious threat to global public health. ESBL/pAmpC-producing bacteria, being resistant against extended-spectrum cephalosporins, are present in humans, animals, food and the environment. For several countries, a high prevalence of these bacteria has been reported in broilers. Even though in the Netherlands the prevalence of ESBL/pAmpC-producing bacteria in broilers has decreased during the last decade, following the trend of reduction in antimicrobial usage, these bacteria are still frequently found in broilers. Although the attribution of ESBL/pAmpC-producing *E. coli* in broilers to carriage in the human population is estimated to be relatively low, direct contact between animals and humans could be a transmission route. Considering the complex links between different reservoirs, further emergence and spread of antibiotic resistance in humans and animals should be avoided. Therefore, food production chains need to be taken into account to further reduce the prevalence of ESBL/pAmpC-producing bacteria.

The aim of the research described in this thesis is to get insight into the processes of colonization and transmission of ESBL/pAmpC-producing bacteria in broilers, and to evaluate interventions aiming to prevent or reduce colonization and transmission of these bacteria in the broiler production pyramid.

Chapter 2 gives the results of a field study that followed the dynamics of pAmpC-producing *E. coli* in a broiler parent stock flock during the rearing and laying phase. A sharp decrease of pAmpC-producing *E. coli* during the rearing phase was observed. Moreover, no pAmpC-producing *E. coli* was detected in the offspring of the parent flock, although pAmpC-producing *E. coli* was still present in the environment during the laying phase. These observations suggest that, in the absence of antibiotics, this specific gene-plasmid combination was unable to persist on animal level.

Chapter 3 is a literature study on transmission routes of ESBL/pAmpC-producing bacteria in the broiler production pyramid. Transmission can occur throughout the broiler production pyramid via four main transmission routes: 1) vertical between generations, 2) at hatcheries, 3) horizontal on farm, and 4) horizontal between farms and via the environment of farms. Most of the evidence was based on observational studies and quantitative data was limited. These identified gaps in knowledge should be studied further to determine to what extend the different routes contribute to the occurrence of ESBL/pAmpC-producing bacteria in the broiler production pyramid.

To test the effect of possible interventions, information is needed on the process of colonization. This information should be used to develop a reproducible animal model to test interventions.

In **Chapter 4**, a dose-response study was conducted to study the colonization process of ESBL/pAmpC-producing *E. coli*. Specific pathogen free (SPF) and conventional just hatched broilers were challenged with different dose levels of two different *E. coli* strains and gene-plasmid

combinations. The results show shorter time until colonization with higher dose levels. However, even with the lowest dose level (10^1 CFU/mL) young broilers were colonized within 72 hours after inoculation. A probabilistic model estimating the probability of colonization by initial inoculation or transmission showed that higher dose levels have a higher probability of colonization by initial inoculation. Conventional broilers were colonized later than SPF broilers, possibly because of initial non-resistant *E. coli* present before inoculation.

The finding that young broilers are rapidly colonized with low dose levels indicates the need for interventions as soon as possible after hatching. Two interventions were studied in this thesis: 1) competitive exclusion, the supply of intestinal bacteria, and 2) compartmentalization, subdividing the broiler flock.

In **Chapter 5**, a transmission experiment was carried out to test the effect of the intervention competitive exclusion. One-day old conventional broilers were housed in isolators and supplied with a competitive exclusion or synbiotic product from day 0-14. In each isolator, half of the broilers were challenged with ESBL-producing *E. coli* at day 0 or day 5. The results showed that both products supplied before challenge with ESBL-producing *E. coli* reduced colonization, transmission and excretion. Moreover, in some isolators colonization was completely prevented. When challenging at day 5, the lowest dose (10^1 CFU/mL) did not result in colonization of broilers, indicating that older broilers are less susceptible for colonization.
After having observed the promising effects of competitive exclusion in broilers kept under controlled circumstances in isolators, a transmission experiment was carried out under circumstances closer to the field situation.

In **Chapter 6**, two interventions were included: competitive exclusion and compartmentalization of the broiler flock. Conventional broilers were housed under semi-field conditions in compartmentalized rooms. The groups supplied with competitive exclusion (day 0-7) and challenged with ESBL-producing *E. coli* at day 5 were not colonized. In the control groups, not supplied with competitive exclusion, compartmentalization reduced transmission of ESBL-producing *E. coli*, but did not prevent it, indicating the importance of transmission of ESBL-producing *E. coli* via the environment. The microbiome composition was more diverse in the competitive exclusion broilers than in control broilers.

The main conclusions of this thesis are presented in **Chapter 7**. The presence of ESBL/pAmpC-producing *E. coli* at different levels of the broiler producing chain, the different transmission routes, and the rapid colonization of young broilers after challenge with only low dose levels exemplify the need for interventions as soon as possible after hatch. Competitive exclusion products could be useful to protect broilers from colonization at the farm, if supplied before exposure to ESBL/pAmpC-producing bacteria. Compartmentalization reduced environmental transmission, but did

not prevent it. Ideally, combinations of interventions should be applied at different levels of the broiler production pyramid targeting different transmission routes. This should include aiming to prevent (apparent) vertical transmission between generations and prevent colonization of birds at farms. Knowledge on the attribution of the different transmission routes will help to further design the most effective intervention strategies to reduce the prevalence of ESBL/pAmpC-producing bacteria in the broiler production pyramid.

Samenvatting

Antimicrobiële resistentie wordt door de Wereldgezondheidsorganisatie (WHO) beschouwd als een belangrijke bedreiging van de volksgezondheid. ESBL/pAmpC-producerende bacteriën zijn resistent tegen breedspectrum cefalosporinen, en zijn aanwezig in mensen, dieren, voedsel en de omgeving. In diverse landen is een hoge prevalentie van deze bacteriën gevonden in vleeskuikens. Hoewel in Nederland, met de afname van het antibioticagebruik, de prevalentie van ESBL/pAmpC-producerende bacteriën in vleeskuikens in de afgelopen tien jaar is afgenomen, worden deze bacteriën nog vaak aangetroffen in vleeskuikens. Hoewel de bijdrage van ESBL/pAmpC-producerende *E. coli* in vleeskuikens aan dragerschap in mensen relatief laag lijkt te zijn, kan direct contact tussen dieren en mensen een transmissieroute van deze bacteriën zijn. Gezien de complexe verbanden tussen verschillende reservoirs moet verdere verspreiding van antibioticaresistentie in mensen en dieren voorkomen worden. Daarom moeten voedselproductieketens deel uit maken van de aanpak om de prevalentie van ESBL/pAmpC-producerende bacteriën verder te verlagen.

Het doel van het onderzoek beschreven in dit proefschrift is om inzicht te krijgen in de processen van kolonisatie en transmissie van ESBL/pAmpC-producerende bacteriën en om het effect van mogelijke interventies op het voorkomen of verminderen van kolonisatie en transmissie van deze bacteriën in de vleeskuikenketen te bepalen.

Hoofdstuk 2 bevat de resultaten van een veldstudie waarin de dynamiek van pAmpC-producerende *E. coli* in een ouderdierkoppel tijdens de opfok- en legperiode gevolgd is. De prevalentie van pAmpC-producerende *E. coli* nam sterk af tijdens de opfokperiode. Bovendien is er geen pAmpC-producerende *E. coli* gevonden in de nakomelingen van dit ouderdierkoppel, hoewel de bacteriën nog wel aanwezig waren in de omgeving tijdens de legperiode. De resultaten suggereren dat in afwezigheid van antibiotica, deze specifieke gen-plasmide combinatie niet in staat was om de dieren blijvend te koloniseren.

Hoofdstuk 3 is een literatuurstudie naar de transmissieroutes van ESBL/pAmpC-producerende bacteriën in de vleeskuikenketen. Transmissie kan plaatsvinden in de vleeskuikenketen via vier belangrijke routes: 1) verticale transmissie tussen generaties, 2) op de broederijen, 3) horizontaal op het bedrijf, en 4) horizontaal tussen bedrijven en hun omgeving. Het meeste bewijs is gebaseerd op observationele studies en kwantitatieve data was beperkt aanwezig. Deze hiaten in kennis moeten verder bestudeerd worden om de relatieve bijdrage van de verschillende routes aan het voorkomen van ESBL/pAmpC-producerende bacteriën in de vleeskuikenketen te kunnen bepalen.

Om het effect van mogelijke interventies te kunnen testen, is informatie over kolonisatieproces nodig. Deze informatie moet gebruikt worden om een reproduceerbaar diermodel te ontwikkelen om interventies te testen.

In **hoofdstuk 4** is een dosis-respons studie beschreven om het kolonisatieproces van ESBL/pAmpC-producerende *E. coli* te kunnen bestuderen. Specifieke pathogeenvrije (SPF) en reguliere

eendagskuikens zijn geïnoculeerd met verschillende doseringen van twee verschillende *E. coli* stammen met verschillende gen-plasmide combinaties. Hogere doseringen leidden tot een kortere tijd tot kolonisatie, maar zelfs met de laagste dosering (10^1 CFU/mL) werden de jonge vleeskuikens binnen 72 uur na inoculatie gekoloniseerd. Een model waarmee de kans op kolonisatie door inoculatie en door transmissie geschat werd liet zien dat hoe hoger de dosering, des te groter de kans op kolonisatie door initiële inoculatie is. Reguliere vleeskuikens werden later gekoloniseerd dan SPF kuikens, mogelijk omdat de SPF kuikens al gekoloniseerd waren met niet-resistente *E. coli* voorafgaand aan de inoculatie.

De snelle kolonisatie van jonge vleeskuikens na inoculatie met een lage dosering laat zien dat interventies zo snel mogelijk na het uitkomen toegepast moeten worden. Twee interventies zijn bestudeerd in dit proefschrift: 1) *competitive exclusion*, het verstrekken van darmbacteriën, en 2) compartimentalisatie, het onderverdelen van de vleeskuikenkoppel.

In **hoofdstuk 5** is een transmissie experiment uitgevoerd om het effect van de eerste interventie te testen: *competitive exclusion*. Reguliere eendagskuikens werden gehuisvest in isolatoren en kregen een *competitive exclusion* of een synbiotisch product van dag 0-14. Op dag 0 of dag 5 werd in elke isolator de helft van de kuikens geïnoculeerd met ESBL-producerende *E. coli*. Kolonisatie, transmissie en excretie van ESBL-producerende *E. coli* namen af, mits de producten voorafgaand aan de blootstelling met ESBL-producerende *E. coli* werden gegeven. In sommige isolatoren werd kolonisatie voorkomen. De laagste dosering (10^1 CFU/mL) leidde bij inoculatie op dag 5 niet tot kolonisatie, wat suggereert dat oudere vleeskuikens minder gevoelig zijn voor kolonisatie dan jongere kuikens.

Op basis van de veelbelovende resultaten van *competitive exclusion* in vleeskuikens onder gecontroleerde omstandigheden in de isolatoren, is een transmissie experiment uitgevoerd onder omstandigheden die meer op de praktijksituatie lijken.

In **hoofdstuk 6** zijn twee interventies beschreven: *competitive exclusion* en compartimentalisatie van de vleeskuikenkoppel. Reguliere vleeskuikens werden gehuisvest onder semi-praktijkomstandigheden in gecompartimentaliseerde ruimten. De kuikens die *competitive exclusion* van dag 0-7 kregen en op dag 5 werden blootgesteld aan ESBL-producerende *E. coli* werden niet gekoloniseerd. In de vleeskuikens in de controlegroepen leidde compartimentalisatie tot een vertraging van de transmissie van ESBL-producerende *E. coli*, maar werd het niet voorkomen, wat het belang van transmissie van ESBL-producerende *E. coli* via de omgeving laat zien. De microbioom compositie van de *competitive exclusion* kuikens was meer divers dan van de controle kuikens.

De algemene conclusies van dit proefschrift worden gepresenteerd in **hoofdstuk 7**. De aanwezigheid van ESBL/pAmpC-producerende *E. coli* in verschillende schakels van de vleeskuikenketen, de

verschillende transmissieroutes en de snelle kolonisatie van jonge vleeskuikens na blootstelling aan slechts lage doseringen laten zien dat interventies zo snel mogelijk na uitkomst toegepast moeten worden. *Competitive exclusion* kan bruikbaar zijn om kolonisatie van kuikens op het bedrijf te voorkomen, mits toegediend voordat blootstelling aan ESBL/pAmpC-producerende bacteriën plaatsvindt. Compartimentalisatie vermindert transmissie via de omgeving, maar kan het niet voorkomen. Combinaties van interventies zouden moeten worden toegepast in verschillende schakels van de vleeskuikenketen, gericht op verschillende transmissieroutes. Dit zou zowel het voorkomen van verticale transmissie tussen generaties, als het voorkomen van kolonisatie van kuikens op het bedrijf moeten omvatten. Meer kennis van de bijdrage van de verschillende transmissieroutes is nodig om effectieve interventiestrategieën te ontwikkelen en de prevalentie van ESBL/pAmpC-producerende bacteriën in de vleeskuikenketen te verminderen.

About the author

Anita Dame-Korevaar was born on 11 October 1988 in Graafstroom (Goudriaan, the Netherlands). After finishing secondary school at CSG Oude Hoven, Gorinchem in 2006, she started studying Animal Sciences at Wageningen University. During her study, she specialized in animal health and completed a master thesis in Adaptation Physiology and Quantitative Veterinary Epidemiology. After obtaining her MSc degree in 2011, she worked a few years as a researcher in the feed-additive industry. In 2015, Anita started her PhD project on the transmission and interventions to reduce ESBL-producing bacteria in the broiler production pyramid. The most important results of this research, conducted at the Faculty of Veterinary Medicine, department of Farm Animal Health, at Utrecht University, are described in this thesis. In 2019, she obtained the postgraduate master degree in Epidemiology, with a specialization in Veterinary Epidemiology, at Utrecht University. Currently, Anita is working as a researcher/veterinary epidemiologist at Wageningen Bioveterinary Research, Lelystad.

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