

# 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 extended spectrum beta-lactamase and AmpC beta-lactamase (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 *Escherichia 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 5, broilers were challenged with 0.5 mL with 10<sup>1</sup> or 10<sup>2</sup> cfu/mL *E. coli* encoding the beta-lactamase gene *bla*<sub>CTX-M-1</sub> on an IncI plasmid (CTX-M-1-*E. coli*). Presence and concentration of CTX-M-1-*E. coli*

were determined using cloacal swabs (days 0–14, 16, 19, and 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<sub>10</sub> cfu/g). When challenged on the 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 (hazard ratio between 3.71 × 10<sup>-3</sup> and 3.11), excretion (up to -1.60 log<sub>10</sub> cfu/g), and cecal content (up to -2.80 log<sub>10</sub> cfu/g), 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 1 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 the prevalence of ESBL/pAmpC-producing bacteria in the broiler production chain.

**Key words:** ESBL, pAmpC, antimicrobial resistance, intervention, competitive exclusion

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## INTRODUCTION

Plasmid-mediated extended spectrum beta-lactamase and AmpC beta-lactamase (ESBL/pAmpC)-producing bacteria are resistant to extended spectrum

cephalosporins. ESBL/pAmpC-producing *Escherichia coli* are present in the environment, humans, and animals (Blaak et al., 2015). Although the prevalence has decreased in recent years in different animal divisions (Dorado-Garcia et al., 2016; Hesp et al., 2019; MARAN, 2019), 23.0% of the broilers at slaughter were positive for ESBL/pAmpC-producing *E. coli* in 2018 in the Netherlands (MARAN, 2019). High prevalence of ESBL/pAmpC-producing bacteria in poultry flocks (from 0.3 up to 100%) and poultry products (from 3.3 up to 94.5%) is also reported from several other European countries (Saliu et al., 2017), indicating broilers to be an important source of ESBL/pAmpC-

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producing bacteria. Although the contribution from poultry (meat) to human carriage of ESBL-producing *E. coli* seems less important than initially perceived (Mughini-Gras et al., 2019), all attempts to contribute to reducing emergence and spread of antibiotic resistance in humans and animals are important from a One Health perspective (World Health Organization, 2018). Moreover, direct contact with poultry (e.g., people working or living on a poultry farm) could be a transmission route of ESBL/pAmpC-producing bacteria (Dierikx et al., 2013b; Huijbers et al., 2014, 2015).

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 cross-breeding 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., 2019). Consequently, the introduction of ESBL/pAmpC-producing *E. coli* in a broiler flock can occur at different moments, for example 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., 2019), shows that interventions should be implemented as soon as possible after hatching. Delayed colonization observed in conventional broilers which carried initial *E. coli*, compared to specific pathogen-free (SPF) broilers not carrying *E. coli* upon hatching (Dame-Korevaar et al., 2019), 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 (CE).

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 CE 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 SPF chickens (e.g., Aviguard). Also, some products contain a selection of pre- and probiotics (SYN), the 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 the absence of antibiotics, a single oral supply of a CE product led to reduced cecal content (cfu/g) (Nuotio et al., 2013; Methner et al., 2019), excretion, and transmission (Ceccarelli et al., 2017) upon challenge with a high dose ( $10^5$ – $10^8$  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., 2019), 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 products might be more effective by supplying more of the competitive bacteria.

In this study, we investigated the effect of a 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 3 transmission experiments with contact birds and orally inoculated seeder birds, the effect of 2 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* on the day of hatch (experiment 1) and during the first week of life (day 5, experiments 2 and 3).

## MATERIALS AND METHODS

### *Ethics of Experimentation*

Broilers were observed daily and clinical signs, abnormal behavior, 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 performed in full compliance with all legislations.

## Experimental Design

Three consecutive experiments were conducted (Table 1). In experiment 1 ( $n = 70$  broilers), broilers were challenged on the day of hatch (day 0) with 0.5 mL with  $10^1$  or  $10^2$  cfu/mL CTX-M-1-*E. coli* and the intervention groups received a CE product in drinking water (day 0–14), derived from unselected fermented intestinal bacteria from SPF birds (CEP). In experiments 2 ( $n = 70$  broilers) and 3 ( $n = 80$  broilers), broilers were challenged at day 5 with 0.5 mL with  $10^1$  or  $10^2$  cfu/mL CTX-M-1-*E. coli* and the intervention groups received either CEP or a CE product based on synbiotics containing a selection of SYN.

## Birds, Housing, and Management

In all 3 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 1, some of the broilers ( $n = 43$ , randomly selected) were placed temporarily in 2 other isolators; 35 of these broilers were selected for the remainder of the experiment. Five of these 35 birds were moved to isolator 1; thereafter, the remaining birds were inoculated with CTX-M-1-*E. coli* (see the section *E. coli* Challenge below). 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 2 and 3,

upon arrival at day 0 all broilers were randomly distributed over isolators 1 to 7 (experiment 2) or 1 to 8 (experiment 3) (maximum 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 1, 2, and 3, 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 3 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 high efficiency particulate air 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 pm. The intervention was supplied in drinking water (described below). A few broilers died or were euthanized before the end of the experiment due to causes unrelated to the experiment (8 in experiment 1, 2 in experiment 2, and 1 in experiment 3).

## Intervention: CE Product

**Composition** In this study, 2 CE products were used: 1) CE product (CEP) containing unselected, fermented intestinal bacteria, derived from SPF chickens and manufactured by fermentation (Aviguard; MSD Animal Health Nederland, Boxmeer, the Netherlands) (experiments 1, 2, and 3); and 2) a selection of a prebiotic compound and probiotic bacterial strains (SYN): fructo-

**Table 1.** Date, age of parent flock (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 1 ( $n = 70$  broilers), 2 ( $n = 70$  broilers), and 3 ( $n = 80$  broilers).

Experiment	1		2		3	
Date	12 April–3 May 2017		24 May–14 June 2017		23 Oct–13 Nov 2017	
Parent flock <sup>1</sup>	A, 54 wk		A, 60 wk		B, 57 wk	
Day of challenge <sup>2</sup>	Day 0		Day 5		Day 5	
Isolator	<b>Challenge (cfu/mL)<sup>3</sup></b>	<b>Intervention<sup>4</sup></b>	<b>Challenge (cfu/mL)<sup>3</sup></b>	<b>Intervention<sup>4</sup></b>	<b>Challenge (cfu/mL)<sup>3</sup></b>	<b>Intervention<sup>4</sup></b>
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

Abbreviations: CEP, competitive exclusion product; SYN, selection of pre- and probiotics.

<sup>1</sup>In all 3 experiments, the eggs were disinfected with formaldehyde before incubation and in the hatcher before hatching.

<sup>2</sup>Challenge with *Escherichia coli* E38.27 with *bla*<sub>CTX-M-1</sub> on IncI1 plasmid.

<sup>3</sup>Inoculated birds ( $n = 5$  out of 10 in each isolator) received 0.5 mL of the mentioned challenge dose.

<sup>4</sup>Intervention was implemented in all experiments from day 0, 4:00 pm until day 14, 4:00 pm. In experiment 1, supply of the intervention started immediately after challenge.

oligosaccharides and *Enterococcus faecium*, *Bifidobacterium animalis*, *Lactobacillus salivarius* (PoultryStar sol; Biomin Holding GmbH, Getzersdorf, Austria) (experiment 3).

**Supply** The CE products were supplied from the day of hatch (day 0), 4:00 pm, until day 14, 4:00 pm, twice a day, in drinking water. In experiment 1, supply of the intervention started immediately after challenge. Solutions with CEP or SYN in water were prepared in predilution directly before application with a dose according to recommendations of the manufacturer—that is 0.125 g CEP vs. 0.2 g SYN per 10 broilers—and 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 products would be consumed. Control groups received drinking water according to the same schedule, but without any intervention added.

### **E. coli Challenge**

Broilers were challenged with *E. coli* strain E38.27, which carries the ESBL gene *bla*<sub>CTX-M-1</sub> on an IncII 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<sup>1</sup> or 10<sup>2</sup> cfu/mL. Serial dilutions of the *E. coli* strains were prepared on the day of challenge from fresh culture on heart infusion agar 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.

From 1 h after inoculation onward, 5 contact birds were exposed to 5 seeder birds, either by moving the inoculated seeder birds to the isolators containing the contact birds (experiment 1) or by removing the temporal barrier between the inoculated seeder birds and the contact birds within the isolator (experiments 2 and 3). The unchallenged control birds were not inoculated (experiment 1) or received 0.5 mL physiological saline solution (experiments 2 and 3).

### **Cloacal and Cecal Samples**

Samples were taken using sterile dry cotton swabs (MW100, Medical Wire & Equipment, England, during days 0 to 3, and Copan 155C, Copan Diagnostics Inc., Murrieta, CA, from day 4 onward). All birds were sampled just before inoculation to confirm absence of ESBL/pAmpC-producing bacteria (and additionally on days 1 and 3 in experiments 2 and 3), and from the moment of inoculation until day 7 twice a day (8:00 am and 4:00 pm), daily between days 8 and 14, and on days 16, 19, and 21 (8:00 am). On day 21, after the last sampling, post mortem examination was done within at maximum 30 min 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.

### **ESBL-producing E. coli Detection**

All cloacal samples except the ones used for quantification of ESBL-producing *E. coli* and total *E. coli* (see below) 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 the MacConkey plates supplemented with cefotaxime were referred to as CTX-M-1-*E. coli*. If visual assessment led to inconclusive results for the presence of *E. coli*, colonies were selected for further analysis using matrix-assisted laser desorption or ionization-time of flight mass spectrometry (Bruker Daltonik, Germany).

### **ESBL-producing E. coli and Total E. coli Quantification**

Cloacal swabs obtained at 8:00 am were weighed before and after sampling to determine the amount of feces collected. The weight of the fecal material on the cloacal swab ranged from 0.01 to 0.43 g. At day 21, content from 1 of 2 ceca was collected. Samples were processed as previously described (Dame-Korevaar et al., 2019). Briefly, each cloacal swab was suspended in 3 mL LB broth. For the ceca, content from 1 of the 2 ceca was collected post mortem and 0.1 to 1.0 g was used to make a 10% dilution in PBS. Then, 200 µL of each suspension was used to prepare 10-fold dilution series, which were inoculated on MacConkey plates with and without 1 mg/L cefotaxime and incubated overnight at 37°C. Concentrations of ESBL-producing *E. coli* and total *E. coli* were determined semi-quantitatively (cfu/g feces), based on the highest consecutive dilution showing growth of typical *E. coli* colonies (Jett et al., 1997) and the weight of the feces on the swabs or the amount of cecal content collected, as previously described (Ceccarelli et al., 2017). The LB broth including the swab was also enriched overnight at 37°C. If no growth of *E. coli* colonies was observed in the dilution series, 10 µL of the overnight enrichment broth was 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 the detection level of the dilution series and the concentration designated as such (see the section Statistical Analysis below).

*E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were ESBL-producing *E. coli*, referred to here as CTX-M-1-*E. coli*. If visual assessment was inconclusive for the presence of *E. coli*, colonies were selected for further analysis using matrix-assisted laser desorption or ionization-time of flight mass spectrometry.

## 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 2 consecutive cloacal swabs tested positive for ESBL/pAmpC-producing *E. coli*. Time until colonization, using the first positive cloacal swab, was analyzed 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<sub>10</sub> cfu/mL LB, as the minimum detection level of the semi-quantitative method was 2 log<sub>10</sub> cfu/mL LB. Results based on negative swab weight (or weight = 0 g) 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 the challenge dose and the intervention on the ESBL-producing *E. coli* and total *E. coli* excretion (log<sub>10</sub> cfu/g) was analyzed using a mixed linear regression model including the variables time, intervention, dose, contact or seeder bird, weight at hatch, weight at day 21, and the interaction between time and intervention. The variable sex was only included for experiments 1 and 2, as in experiment 3 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, and the others as categorical variables. The best fitting model was obtained by backward selection, choosing the model with the lowest Akaike Information Criterion (AIC) value. Models with a difference in AIC of 2 or less were considered to be 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<sub>10</sub> cfu/g) between the control and intervention groups were tested using a linear regression model including the variables intervention, dose, contact or 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 to be of equal fit and the most parsimonious model (lowest number of parameters) was chosen.

**Transmission** The transmission coefficient ( $\beta$ ) was estimated using the data of experiments 2 and 3 based on the stochastic susceptible infectious 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  $\Delta t$  is calculated by  $E(C) = S(1 - e^{-foi \times \Delta t})$ . 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  $\Sigma_{excrhours}$  is the cumulative sum of hours wherein all infectious birds were excreting up to the beginning of the interval. In model 3, the cumulative excretion determined the force of infection ( $foi = \beta_{concentration} \times \Sigma_{excrconcentration}$ ), in which  $\Sigma_{excrconcentration}$  is the cumulative sum of excretion (log<sub>10</sub> cfu/g feces) of all infectious birds. For all 3 models, different assumptions regarding the input data of I-birds were compared, by assuming that I-birds start excreting at the moment of the first positive cloaca swab (basic model) or half an interval before the first positive cloaca swab (alternative model).

**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 the variables intervention, dose, contact or 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 to be of equal fit and the most parsimonious model (lowest number of parameters) was chosen.

## RESULTS

### Time Until Colonization

**Experiment 1: CTX-M-1-*E. coli* Challenge with 10<sup>1</sup> or 10<sup>2</sup> cfu/mL on the Day of Hatch** All broilers were colonized with CTX-M-1-*E. coli* within 24 h after inoculation (Supplementary Table 1). There was no difference in the hazard ratio (HR) between control broilers and CEP broilers, nor between broilers challenged with dose 10<sup>1</sup> and 10<sup>2</sup>. However, isolators 2, 6, and 7 had a higher HR than isolators 3, 4, and 5 (Table 2). Other variables, such as seeder or contact bird, weight on the day of hatch, weight at day 21, and sex, did not influence the time until colonization.

**Experiment 2: CTX-M-1-*E. coli* Challenge with 10<sup>1</sup> or 10<sup>2</sup> cfu/mL at Day 5** Broilers challenged with 10<sup>1</sup> 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<sup>2</sup> cfu/mL CTX-M-1-*E. coli* were colonized within 48 (control) or 144 h (CEP) after inoculation (Figure 1 and Table 3). CEP broilers had a lower HR (HR isolator 6: 0.08, 95% CI 0.02–0.42 and isolator 7: 3.71 × 10<sup>-3</sup>, 95% CI 2.71 × 10<sup>-4</sup> to 0.05) than the control isolator (Table 2). Factors seeder or contact bird, weight on the day of hatch, weight at day 21, sex, and the total *E. coli* excretion (log<sub>10</sub> cfu/g feces) just before inoculation (day 5) did not influence the time until colonization.

**Table 2.** Hazard ratio (95% CI) of time until colonization for experiments 1 (n = 53), 2: dose 10<sup>2</sup> (n = 29), and 3 (n = 40); broilers were challenged with CTX-M-1-*Escherichia coli* at day 0 in experiment 1, and at day 5 in experiments 2 and 3.

Experiment	Variable		HR <sup>2</sup> (95% CI)	
1	Isolator <sup>1</sup>	2 (10 <sup>1</sup> – none, reference)	1	
		3 (10 <sup>1</sup> – CEP)	0.25 (0.09–0.72)	
		4 (10 <sup>1</sup> – CEP)	0.24 (0.08–0.68)	
		5 (10 <sup>2</sup> – none)	0.27 (0.09–0.76)	
		6 (10 <sup>2</sup> – CEP)	0.74 (0.25–2.20)	
		7 (10 <sup>2</sup> – CEP)	0.89 (0.29–2.71)	
		Seeder or contact bird	Seeder (reference)	1
		Contact	0.67 (0.34–1.30)	
		Body weight at day 0 (hatch)	0.99 (0.91–1.09)	
		Body weight at day 21	1.00 (1.00–1.01)	
		Sex	Male (reference)	1
			Female	0.91 (0.43–1.91)
2	Isolator <sup>1</sup>	5 (10 <sup>2</sup> – none, reference)	1	
		6 (10 <sup>2</sup> – CEP)	0.08 (0.02–0.42)	
		7 (10 <sup>2</sup> – CEP)	3.71 × 10 <sup>-3</sup> (2.71 × 10 <sup>-4</sup> –0.05)	
	Seeder or contact bird	Seeder (reference)	1	
		Contact	1.09 (0.40–2.98)	
		Body weight at day 0 (hatch)	0.93 (0.800–1.09)	
		Body weight at day 21	1.00 (0.99–1.00)	
		Sex	Male (reference)	1
			Female	0.64 (0.25–1.60)
		Total <i>E. coli</i> (cfu/g feces) day 5	1.06 (0.64–1.74)	
	3	Isolator <sup>1</sup>	2 (10 <sup>2</sup> – none, reference)	1
			6 (10 <sup>2</sup> – SYN)	0.07 (0.01–0.39)
7 (10 <sup>2</sup> – SYN)			1.29 (0.39–4.28)	
8 (10 <sup>2</sup> – SYN)			3.11 (0.97–10.05)	
Seeder or contact bird		Seeder (reference)	1	
		Contact	0.43 (0.20–0.94)	
		Body weight at day 0 (hatch)	1.03 (0.94–1.13)	
		Body weight at day 21	1.00 (0.99–1.00)	
	Total <i>E. coli</i> (cfu/g feces) day 5	1.18 (0.80–1.74)		

Abbreviations: CEP, competitive exclusion product; HR, hazard ratio; SYN, selection of pre- and probiotics.

<sup>1</sup>Isolator number, dose level of challenge (10<sup>1</sup> or 10<sup>2</sup>), and intervention (none, CEP, or SYN).

<sup>2</sup>HR, indicating the ratio between the hazard of colonization with CTX-M-1-*E. coli* in the mentioned group and the reference group. A ratio of <1 indicates a smaller hazard, and a ratio of >1 indicates a higher hazard.

### Experiment 3: CTX-M-1-*E. coli* Challenge with 10<sup>2</sup> 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 2 control isolators were colonized within 56 h after inoculation. The broilers in one of the SYN isolators (isolator 5) were not colonized, the broilers in the other 3 SYN isolators were all colonized within 336 h after inoculation (Figure 1 and Table 3). Although one of the SYN isolators showed a lower HR 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 on the day of hatch, 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 HR (HR 0.43, 95% CI 0.20–0.94) than seeder birds. The variable sex was not analyzed, as only female broilers were included in experiment 3.

### Excretion

**Experiment 1: 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<sub>10</sub> cfu/g feces) than male birds, and broilers challenged with either 10<sup>1</sup> or 10<sup>2</sup> cfu/mL CTX-M-*E. coli* excreted slightly lower concentrations of total *E. coli* than non-inoculated broilers (–0.81, 95% CI –1.14 to –0.48 vs. –0.85, 95% CI –1.19 to –0.51 log<sub>10</sub> cfu/g feces, Supplementary Table 2). Concentrations of CTX-M-1-*E. coli* in cecal content were lower in CEP broilers than control broilers (–0.71, 95% CI –1.06 to –0.37 log<sub>10</sub> cfu/g cecal content) and higher in broilers receiving dose 10<sup>2</sup> than dose 10<sup>1</sup> (0.46, 95% CI 0.14–0.79 log<sub>10</sub> cfu/g cecal content). Total *E. coli* concentrations in cecal content were slightly lower in CEP broilers than control broilers (–0.36, 95% CI –0.63 to –0.08 log<sub>10</sub> cfu/g cecal content, Supplementary Table 3).

**Experiment 2: Excretion of CTX-M-1-*E. coli* and Total *E. coli*** Broilers challenged with 10<sup>1</sup> cfu/mL CTX-M-1-*E. coli* did not excrete CTX-M-1-*E. coli* during the experiment. CEP broilers challenged with 10<sup>2</sup> cfu/mL excreted lower concentrations of CTX-M-1-*E. coli* (–0.89, 95% CI –1.33 to –0.45 log<sub>10</sub> cfu/g feces) than control broilers. Female birds excreted slightly higher concentrations of CTX-M-1-*E. coli* (0.48, 95% CI 0.04–0.92 log<sub>10</sub> cfu/g feces) than male birds. CEP broilers

**Table 3.** Detection (+/–) and quantification ( $\log_{10}$  cfu/g feces) of CTX-M-1-*Escherichia coli* in broilers in experiments 2 (dose  $10^2$  cfu/mL) and 3, determined at n hours p.i. at days 5 to 7 (8:00 am and 4:00 pm), 8 to 14, 16, 19, and 21 (8:00 am).

Exp	Iso	Dose	Intervention	Bird ID	Seeder or contact	hours p.i.	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
							0	8	24	32	48	56	72	96	120	144	168	192	216	264	336	384
2	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	336	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	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	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	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	5.22
	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	5.12
	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	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	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	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	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	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	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	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	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	3.33
	6	$10^2$	CEP	206	Contact	–	–	–	–	–	–	2.63	3.52	4.57	4.00	5.70	2.52	– <sup>2</sup>	–	–	–	–
	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	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	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	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	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	4.33
	7	$10^2$	CEP	242	Seeder	–	+	–	–	–	–	–	–	4.00	2.78	3.78	4.63	3.63	5.00	4.36	3.44	3.44
	7	$10^2$	CEP	277	Seeder	–	–	–	–	–	–	–	–	–	2.78	2.70	2.57	3.63	3.57	3.48	4.30	4.30
	7	$10^2$	CEP	291	Seeder	–	–	–	–	–	–	–	–	–	2.57	2.78	2.40	2.40	3.63	3.63	4.33	4.33
	7	$10^2$	CEP	298	Seeder	–	–	–	–	–	–	–	–	–	2.63	5.57	4.88	4.63	5.70	3.63	4.44	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	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	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	5.25
	7	$10^2$	CEP	263	Contact	–	–	–	–	–	–	–	2.57	2.52	2.70	4.33	3.30	3.36	3.70	2.44	2.40	2.40
	7	$10^2$	CEP	284	Contact	–	–	–	–	–	–	–	–	–	2.63	3.48	3.57	5.57	2.70	3.78	3.88	3.25
3	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	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	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	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	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	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	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	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	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	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	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	5.48
	6	$10^2$	SYN	329	Seeder	–	–	–	–	–	–	–	–	–	–	–	2.48	2.27	2.57	3.06	5.40	5.40
	6	$10^2$	SYN	337	Seeder	–	–	–	–	–	–	–	–	–	–	–	–	2.48	–	2.33	4.48	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	5.36
	6	$10^2$	SYN	313	Contact	–	–	–	–	–	–	–	–	–	1.85	2.15	2.40	2.15	2.70	2.15	3.36	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	3.48

6	10 <sup>2</sup>	SYN	377	Contact	-	-	-	-	-	-	-	-	-	2.57	2.40	3.18	2.63	2.48	4.30	
6	10 <sup>2</sup>	SYN	385	Contact	-	-	-	-	-	-	-	-	-	2.48	2.33	4.57	4.70	3.33	4.63	
7	10 <sup>2</sup>	SYN	314	Seeder	-	-	2.01	+	2.40	+	4.20	2.36	3.25	4.06	4.30	3.70	4.12	+ <sup>1</sup>	4.25	3.33
7	10 <sup>2</sup>	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 <sup>2</sup>	SYN	354	Seeder	-	-	-	-	-	+	-	3.57	2.30	5.18	4.12	4.36	3.44	3.63	6.27	4.52
7	10 <sup>2</sup>	SYN	370	Seeder	-	-	-	-	-	+	3.27	4.57	3.48	3.27	3.12	3.36	3.40	-	6.44	3.44
7	10 <sup>2</sup>	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 <sup>2</sup>	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 <sup>2</sup>	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
7	10 <sup>2</sup>	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 <sup>2</sup>	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 <sup>2</sup>	SYN	386	Contact	-	-	-	-	-	+	3.27	3.44	+ <sup>1</sup>	5.18	4.05	4.30	4.44	3.70	4.12	5.44
8	10 <sup>2</sup>	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 <sup>2</sup>	SYN	347	Seeder	-	-	2.57	+	2.52	+	3.44	4.25	4.57	3.33	+ <sup>1</sup>	3.52	3.52	2.40	2.44	4.57
8	10 <sup>2</sup>	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 <sup>2</sup>	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 <sup>2</sup>	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 <sup>2</sup>	SYN	307	Contact	-	-	-	-	-	+	4.00	2.15	3.40	6.27	3.33	3.08	3.44	2.70	+ <sup>1</sup>	3.40
8	10 <sup>2</sup>	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 <sup>2</sup>	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 <sup>2</sup>	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 <sup>2</sup>	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

Abbreviations: CEP, competitive exclusion product; Exp, experiment; Iso, isolator; p.i., post inoculation; SYN, selection of pre- and probiotics.

<sup>1</sup>+ 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.

<sup>2</sup>Chick died.



**Table 4.** Transmission coefficients ( $\beta$ , 95% CI) for experiments 2 and 3, using an SI model, for the basic model (assuming I-birds start excreting at the moment of the first positive cloaca swab) and the alternative model (assuming I-birds start excreting half an interval before the first positive cloaca swab).

Transmission coefficient ( $\beta$ , 95% CI)						
	Basic model			Alternative model		
	Model 1 (day <sup>-1</sup> )	Model 2 (day <sup>-2</sup> )	Model 3 (cfu × day) <sup>-1</sup>	Model 1 (day <sup>-1</sup> )	Model 2 (day <sup>-2</sup> )	Model 3 (cfu × day) <sup>-1</sup>
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 ( $C$ ) in model 1:  $E(C) = S \left( 1 - e^{-\beta_{direct} \times \frac{I}{N} \times \Delta t} \right)$ , model 2:  $E(C) = S (1 - e^{-\beta_{time} \times \Sigma_{excretion} \times \Delta t})$ , and model 3:

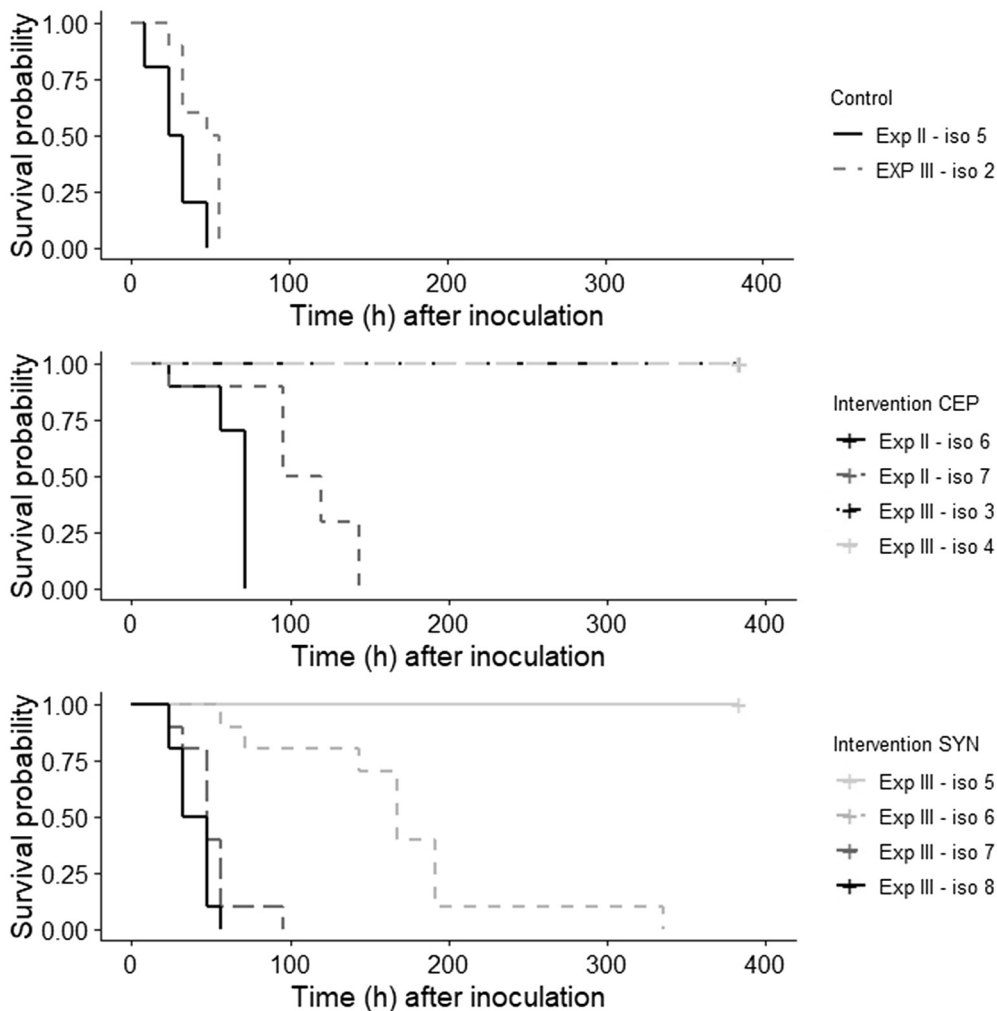
$E(C) = S (1 - e^{-\beta_{concentration} \times \Sigma_{excretion} \times \Delta t})$ . In model 3, cumulative excretion (cfu/g feces) is independent of the number of I-birds and is therefore independent of the assumption regarding the start of excretion.

Statistically different transmission coefficients compared to the control group: \* $P < 0.05$ , \*\* $P < 0.10$ .

Abbreviations: AIC, Akaike Information Criterion; CEP, competitive exclusion product; I-birds, infectious birds; SI model, susceptible infectious model; SYN, selection of pre- and probiotics.

excreted lower or equal concentrations of *E. coli* than control broilers, except at day 1, but without a clear pattern (Supplementary Table 2). 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 to −0.22, vs. −2.80, 95% CI −3.47 to −2.14 log<sub>10</sub> cfu/g cecal content, Supplementary Table 3).



**Figure 1.** Survival curve of time until colonization of CTX-M-1-*Escherichia coli* for experiments 2 and 3, after challenge at day 5 with dose 10<sup>2</sup> cfu/mL. Abbreviations: CEP, competitive exclusion product; Exp, experiment; SYN, selection of pre- and probiotics.

**Experiment 3: 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 onward. 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 (Supplementary Table 2). The concentrations of CTX-M-1-*E. coli* in cecal content of SYN broilers were lower ( $-1.13$ , 95% CI  $-1.94$  to  $-0.33$   $\log_{10}$ /g cecal content) compared to the control broilers. Total *E. coli* concentrations were lower in CEP broilers than in control broilers ( $-1.50$ , 95% CI  $-1.76$  to  $-1.24$   $\log_{10}$ /g cecal content, Supplementary Table 3).

### Transmission

The transmission coefficients ( $\beta_{\text{direct}}$ ,  $\beta_{\text{time}}$ , and  $\beta_{\text{concentration}}$ ) were estimated using the data of experiments 2 and 3. These could not be estimated from experiment 1 because most broilers (seeder and contact) in the control and CEP isolators were colonized already at the first sampling moment (16 h) after inoculation. Also, estimation of the transmission coefficients in the CEP groups in experiment 3 was not possible, because the inoculation did not lead to colonization in the CEP groups.

Transmission coefficients ( $\beta_{\text{direct}}$ ,  $\beta_{\text{time}}$ , and  $\beta_{\text{concentration}}$ ), estimated using the assumptions in the alternative model (assuming that I-birds start excreting half an interval before 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 ( $\beta_{\text{time}}$ : CEP:  $0.19 \text{ day}^{-2}$ , 95% CI  $0.04$ – $0.87$ , SYN:  $0.09 \text{ day}^{-2}$ , 95% CI  $0.02$ – $0.40$ , control:  $0.27 \text{ day}^{-2}$ , 95% CI  $0.13$ – $0.49$ ) and model 3 ( $\beta_{\text{concentration}}$ : CEP:  $0.12 [\text{cfu} \times \text{day}]^{-1}$ , 95% CI  $0.15$ – $0.56$ , SYN:  $0.14 [\text{cfu} \times \text{day}]^{-1}$ , 95% CI  $0.02$ – $0.63$ , control:  $0.31 [\text{cfu} \times \text{day}]^{-1}$ , 95% CI  $0.10$ – $0.57$ ). The transmission coefficients ( $\beta_{\text{direct}}$ ,  $\text{day}^{-1}$ ) estimated based on model 1 were not different ( $\beta_{\text{direct}}$ : CEP:  $2.57 \text{ day}^{-1}$ , 95% CI  $0.51$ – $11.47$ , SYN:  $1.58 \text{ day}^{-1}$ , 95% CI  $0.35$ – $6.57$ , control:  $2.19 \text{ day}^{-1}$ , 95% CI  $1.09$ – $3.91$ ) (Table 4). The unit of  $\beta$  in model 2 is  $\text{day}^{-2}$  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  $\beta$  in model 3 is  $(\text{cfu} \times \text{day})^{-1}$  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 g of feces. In addition, a second alternative model was tested including the assumption that I-birds that were not colonized at 32 h after inoculation were S-birds. However, this assumption did not improve the fit of the model (data not shown).

### Performance

There was no effect of CEP on growth (experiments 1, 2, and 3). In experiment 3, SYN broilers had higher

growth (from day of hatch until day 21) than control broilers ( $1021.1$ , 95% CI  $914.1$ – $1128.0$  g vs.  $914.8$ , 95% CI  $866.5$ – $963.1$  g). 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  $884.9$ – $1228.1$  g).

## DISCUSSION

The supply of CE products to broilers during the first 2 wk of life resulted in an increased time until colonization and lower excretion of CTX-M-1-*E. coli* and even in the 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 CE products when challenged on the 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 the 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 ESBL-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 2 did not result in colonization of CTX-M-1-*E. coli* in the control and intervention groups, although the results of experiment 1 and earlier studies showed that with this low dose young broilers could colonize (Dame-Korevaar et al., 2019). However, in this earlier study broilers were challenged at day 1, whereas we challenged them 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 analyze microbiota composition in this study, it is likely that the gut microbiome composition might have played a

role, as 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 the 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 the HR 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 CE, but CEP contains natural, live, fermented intestinal microflora from SPF chickens, whereas SYN contains a prebiotic compound (fructo-oligosaccharides) and probiotic bacterial strains (*E. faecium*, *B. animalis*, and *L. salivarius*). In our study the total concentrations of *E. coli* at day 5, just before inoculation, did not influence the time until colonization. Therefore, the protective effects of the CE products might not be competition between the different *E. coli* strains (initially present, inoculated, and in the supplied intervention), but may do so between other (combinations of) supplied bacteria. Moreover, different mechanisms might have played a role: not only direct CE between specific bacteria, including competition for specific niches or nutrients (Callaway et al., 2008), but also more complex indirect host-microbe interactions, for example immune responses (Lawley and Walker, 2013). It is likely that the 2 CE products may 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 1). This is in line with earlier studies (Ceccarelli et al., 2017), showing that the effect of CE 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 doses 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., 2019).

The prevention of colonization (experiments 2 and 3), and the quick colonization of one seeder bird in both isolators in experiment 2 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 fecal-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 the force of infection based on excretion time of infectious broilers. We suggest using 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 the force of infection based on excretion concentrations, did not improve the fit of the model. However, in both models including the environment CE products reduces the transmission coefficients.

The colonization of ESBL-producing *E. coli* in the broilers' intestinal tract as observed in our experiments is likely a result of both vertical and horizontal (via conjugation) transfer of the plasmids present in the inoculum *E. coli* to other *E. coli* strains, as was suggested in earlier experiments (Dame-Korevaar et al., 2019). This reflects 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 even if only 1 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, and therefore should be applied as soon as possible after hatch, before broilers are exposed to ESBL/pAmpC-producing *E. coli*. Further studies are recommended on the mechanisms behind the dynamical processes in the gut responsible for the CE effects, and to determine the best timing and type of bacterial composition manipulations to optimize these intervention strategies for practical use.

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Conflict of Interest Statement: Daniela Ceccarelli is currently employed by the Research Executive Agency. The authors have no conflict of interest to declare.

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <http://doi.org/10.1016/j.psj.2020.04.025>.

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