Aus dem Institut für Tier-und Umwelthygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Management measures to reduce the prevalence of broiler chickens with ESBL-/pAmpC- producing enterobacteria

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der Freien Universität Berlin

vorgelegt von
Caroline Robé
Tierärztin
aus Potsdam

Berlin 2022

Journal-Nr.: 4335

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List of Abbreviations

AIEC	adherent-invasive *Escherichia coli*
AmpC	AmpC beta–lactamase
AMR	antimicrobial resistance
APEC	avian pathogenic *Escherichia coli*
bla	gene enconding a beta-lactamase
CE	Competitive Exclusion
cfu	colony forming units
CMY	cephamycinase beta-lactamase
CTX-M	cefotaximase-Munich beta-lactamase
DAEC	diffusely adherent *Escherichia coli*
EAEC	enteroaggregative *Escherichia coli*
EARS-Net.	European Antimicrobial Resistance Surveillance Network
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
e.g.	for example
EHEC	enterohemorrhagic *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ESBL	extended-spectrum beta-lactamase
ETEC	enterotoxigenic *Escherichia coli*
ExPEC	extra-intestinal pathogenic *Escherichia coli*
FEC	Fujisawa *Escherichia coli*
HR	hazard ratio
InPEC	intestinal pathogenic *Escherichia coli*
kg/m^2	kilograms per square meter
LB	Luria Bertani broth
MRGN	multiresistant Gram-negative
NMEC	neonatal meningitis-causing *Escherichia coli*
OXA	oxacillinase beta-lactamase
pAmpC	plasmid-mediated AmpC beta–lactamase
p.i.	*post inoculation*

PBP	penicillin binding protein
pKa	acid dissociation constant
RKI	Robert Koch Institute
SePEC	sepsis-associated *Escherichia coli*
SHV	sulfhydryl variable beta-lactamase
ST	sequence type
TEM	Temoneira beta-lactamase
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection
VAG	virulence-associated gene
WGS	whole genome sequencing

1 Introduction

The effective treatment of infectious diseases is a valuable achievement and threatened by the emergence of antimicrobial resistances [1,2]. One main driver of antimicrobial resistances is the dissemination of extended-spectrum beta-lactamase- (ESBL) and plasmid-mediated AmpC beta-lactamase- (pAmpC) producing Enterobacterales. ESBL- and pAmpC- producing Enterobacterales are frequently multi-drug resistant, including resistances against clinically relevant antimicrobials other than cephalosporins [3]. Medical treatments with last resort antibiotics, namely carbapenems, became the “gold standard” for infections with these resistant bacteria in humans [4,5]. As antimicrobial resistance rises, ESBL- and pAmpC-producing Enterobacteriaceae were listed as one of the three most critical “priority pathogens” for developing new and effective antimicrobial agents [6]. Besides the detection in humans [7,8], ESBL- and pAmpC- producing Enterobacterales are detected in animals and the environment [9–11], raising the question of a transfer of resistance genes or resistant bacteria between these compartments in a “One health” approach [12].

For livestock, a high prevalence of ESBL- and pAmpC- producing Enterobacterales is known for broiler production [13,14]. In light of the high prevalence, broiler chickens are considered a reservoir for ESBL- and pAmpC- producing bacteria [15,16]. Investigations showed the presence of these resistant bacteria in the entire broiler production chain from (grand-)parent flocks [17–21], over the hatchery [19,20] and the fattening period [22,23] up to the slaughterhouse level [24] and broiler chicken meat [25,26]. It was demonstrated that day-old broiler chickens are already colonized with ESBL- and pAmpC- producing *Escherichia coli (E. coli)* [27]. As various transmission routes of horizontal and vertical transmission for ESBL- and pAmpC- producing bacteria were revealed [18,20,22,28], the question of the necessary minimal bacterial count for a broiler chickens’ colonization rose. Moreover, there was a growing need for targeted interventions to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing Enterobacterales.

Therefore, this thesis addresses:

i) The determination of the minimal bacterial count for the colonization of day-old broiler chickens with ESBL- and pAmpC- producing *E. coli*.

ii) The investigation of potential intervention measures on the colonization of broiler chickens with ESBL- and pAmpC producing *E. coli* throughout the fattening process.

To achieve the objectives of the study, an ESBL- and pAmpC- colonization model (seeder-bird) for broiler chickens was established (addressed in the first publication: “Low Dose Colonization of Broiler Chickens With ESBL-/ AmpC- Producing *Escherichia coli* in a Seeder-

Bird Model Independent of Antimicrobial Selection Pressure."). The established model was used to investigate distinct intervention measures (regarding the hygiene and the management throughout the fattening process) on their potential to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* (addressed in the second publication: "Impact of different management measures on the colonization of broiler chickens with ESBL- and pAmpC- producing *Escherichia coli* in an experimental seeder-bird model." and in the unpublished data of this thesis).

2 Literature

2.1 Antimicrobial resistance and resistance to beta-lactam antimicrobials

Antimicrobial resistance (AMR) is the ability of microorganisms to counteract a drug treatment and can lead to a failure of medical therapies [29]. At present, we are faced with a rising threat of AMR in both human and veterinary medicine [30]. Within the past century, many advantages in the medical field can be attributed to the discovery of the first antibiotic, Penicillin, by Alexander Fleming in 1928 [31]. With the beginning of the antibiotic era in the 1940s, bacterial infections were successfully treated after Penicillin G became clinically available [32]. New classes of antibiotics got constantly introduced to the market in the “Golden age” of antibiotic development until the 1960s [33]. Together with the coincidental discovery of Fleming, the first resistance mechanism to Penicillin was described already before its use in medical practice [34] and gave rise to the question of antimicrobial resistance development. Now many resistance mechanisms can already be traced back thousands of years and demonstrate that antibiotic resistance is ancient [35,36].

To date, global antibiotic consumption is increasing [37,38]. In human medicine, beta-lactam antibiotics are the most frequently used antimicrobial agents [39], and even after 90 years of discovery, penicillins are of continuing significance worldwide [40]. In veterinary medicine, penicillins are the most frequently consumed antibiotic class after tetracyclines [41,42]. As every treatment with antimicrobials selects for resistant bacteria [43–45], the excessive application of beta-lactam antimicrobials led to an increasing number of beta-lactam resistant bacteria. Between 2015 and 2019, the European Antimicrobial Resistance Surveillance Network (EARS-Net.) recognized a rising number of resistances to beta-lactam antimicrobials in *E. coli*. The EARS-Net. reports that half of the *E. coli* isolates and one-third of the *Klebsiella pneumoniae* isolates are resistant to at least one antimicrobial class and resistance to multiple antimicrobial classes as common [46].

Bacterial resistances are attributed to chromosomal mutations, or they are conveyed by mobile elements such as plasmids [47,48] and can protect bacteria against an antimicrobial treatment. Pathogenic bacteria acquiring resistance genes (e.g. through horizontal gene transfer) can cause severe infections of the host which can, in turn, be difficult to treat. Infections with beta-lactam-resistant Enterobacterales are associated with increased mortality rates, increased time for effective therapy, a prolonged stay in the hospital, and thus higher healthcare costs [40,44,49]. With regard to the high antibiotic consumption, a review on antimicrobial resistance forecasts about ten million deaths caused by AMR each year [50]. The review by O’Neill demands global efforts in a “One Health” context to reduce the unnecessary use of

antimicrobials, improve the surveillance of antimicrobial resistances in humans and animals, and promote new diagnostics and treatment options for diseases.

2.1.1 Beta-lactam antimicrobials

The class of beta-lactam antimicrobials includes penicillins, cephalosporins, carbapenems, and monobactams [51] (Figure 1). The common structure of beta-lactam antimicrobials is a four-membered cyclic amide. Beta-lactam antimicrobials disrupt the cell wall synthesis by blocking the final step of the peptidoglycan cross-linking of both Gram-positive and Gram-negative bacteria [51]. For cross-linking, a transpeptidase (the so-called Penicillin Binding Protein (PBP)) recognizes the terminal D-Ala-D-Ala-fragment of the peptidoglycan pentapeptide as its first substrate. Beta-lactam antimicrobials mimic the terminal peptide structure and form a covalent bond to the PBP. The peptidoglycan synthesis is inhibited and, consequently, ends in cell lysis as the cell wall becomes unstable and the osmotic pressure of the cell can not be maintained.

Based on their effect spectrum in Gram-positive and Gram-negative bacteria, beta-lactam antimicrobials are characterized as narrow-, broad- or extended-spectrum antimicrobials. Cephalosporins belonging to the latter can be divided into five generations [52]. Several bacterial resistance mechanisms against beta-lactam antimicrobials are described, including a reduced permeability of the outer membrane by non-expression of porin genes, efflux pumps that additionally use beta-lactams as substrates, possession of altered or acquired PBPs with low affinity to beta-lactams, and inactivation by beta-lactamases [39,53]. To counter the latter, beta-lactamase inhibitors, such as clavulanic acid and avibactam, were introduced starting from the 1980s [54]. Still, the inhibition of Ambler class C and class B beta-lactamases (see below) by beta-lactamase inhibitors is very limited to nonexistent.

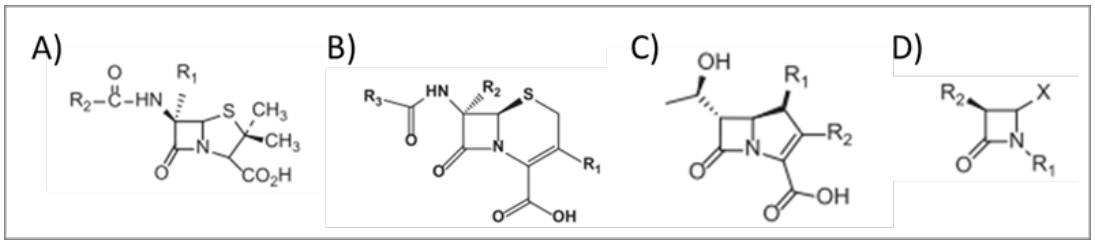

Figure 1. Core structure of beta-lactam antimicrobials adapted from Bush et al. [32].
A) penicillins, B) cephalosporins, C) carbapenems, and D) monobactams.

2.1.2 Beta-lactamases

Beta-lactamases are the primary resistance mechanism against beta-lactam antimicrobials in Gram-negative bacteria [36]. These enzymes can hydrolyze the basic structure of respective antimicrobials, the beta-lactam ring, by dissociating their amide bond (–CO–NH structure). The first chromosomal encoded beta-lactamase-producing *E. coli* was already described in 1940 [34]. The worldwide emergence of beta-lactamases in Gram-negative bacteria can be attributed to the mobilization of the genes to mobile genetic elements like plasmids which encode many different beta-lactamases in combination with only little species barriers during transmission of those elements [36]. In 1965, the first plasmid-encoded beta-lactamase was a TEM-1 (Temoneira) producing *E. coli* isolated from a Greek patient [55], which rapidly disseminated worldwide. SHV (sulfhydryl variable) represents another class of beta-lactamases with its first description of SHV-1 in 1972 [56]. Until the emerge of ESBL-producers, TEM was the most frequently detected beta-lactamase type followed by SHV and OXA (oxacillinase) enzymes [36], able to hydrolyze penicillins and first- and second-generation cephalosporins [32].

Beta-lactamases are classified based on their molecular structure by Ambler [57] or their functional classification by Bush and Jacoby [58,59] (Figure 2). The Ambler classification divides beta-lactamases into four classes. Class A, C, and D include enzymes which possess serine in their active site for beta-lactam hydrolysis, whereas class B enzymes require metal ions for the substrate hydrolysis. With respect to the clinical relevance and effective treatment, Bush and Jacoby consider the substrate and inhibitor profiles for their classification. The major groups of Bush and Jacoby correlate with the molecular classification of Ambler. Group 1 (class C) includes cephalosporinases which are predominantly located on the chromosomes of Enterobacterales and are not inhibited by beta-lactam inhibitors (e.g. clavulanic acid and avibactam). Group 2 (classes A and D) represents the biggest group of beta-lactamases as they include the extended-spectrum beta-lactamases in subgroup 2be, which are susceptible to beta-lactam inhibitors. Group 2 also describes broad-spectrum and inhibitor-resistant beta-lactamases (including some ESBLs) as well as serine carbapenemases. Lastly, group 3 (class B) includes the metallo-beta-lactamases. This group differs structurally and functionally from the other groups by utilizing metal ions (usually a divalent zinc ion) at its active side. Beta-lactamases of group 3 are not inhibited by beta-lactam inhibitors but by metal ion chelators (e.g. EDTA).

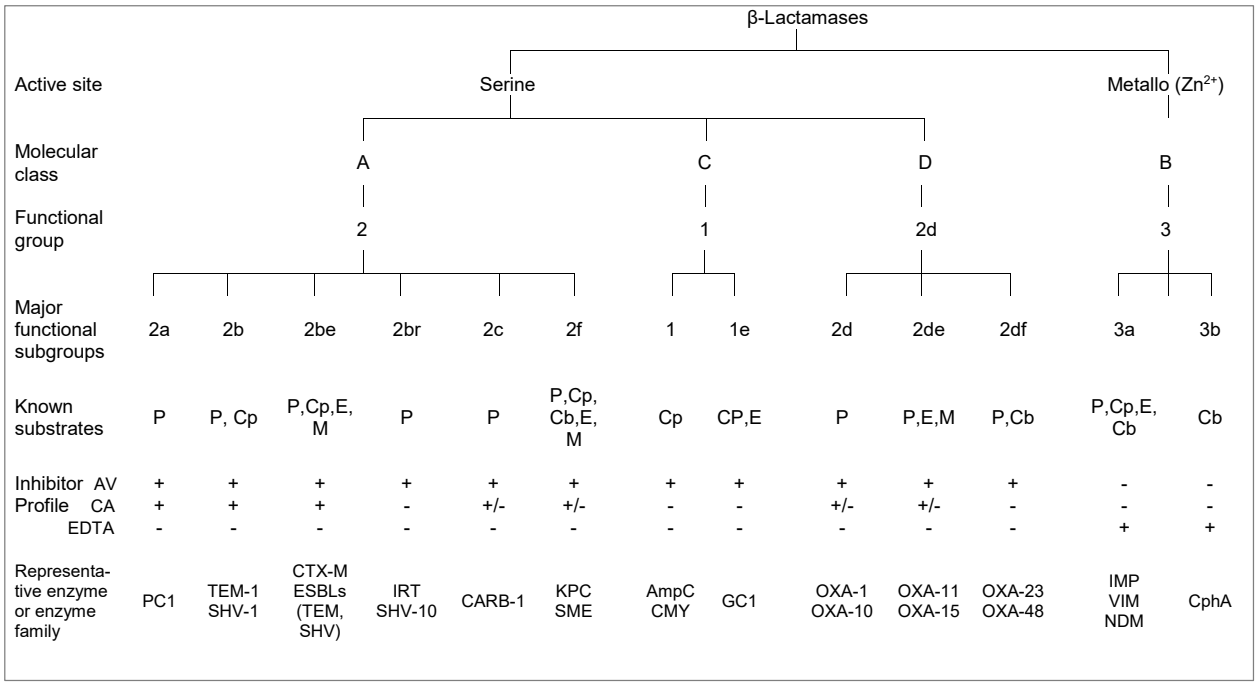

Figure 2. Classification of beta-lactamases based on their molecular structure or their functional classification adapted from Bush [36].

AV = avibactam, CA = clavulanic acid, EDTA = ethylenediaminetetraacetic acid, Cb = carbapenem, Cp = cephalosporin, E = expanded-spectrum cephalosporin, M = monobactam, P = penicillin, PC1 = penicillinase producing *Staphylococcus aureus*, TEM = Temoneira beta-lactamase, SHV = sulfhydryl variable beta-lactamase, CTX-M = cefotaximase-Munich beta-lactamase, ESBL = extended-spectrum beta-lactamase, IRT = inhibitor-resistant TEM enzymes, CARB = carbenicillin-hydrolyzing beta-lactamase, KPC = *Klebsiella pneumoniae* carbapenemase, SME = *Serratia marcescens* enzymes, AmpC = AmpC beta–lactamase, CMY = cephamycinase beta-lactamase, GC1 = beta-lactamase producing *Enterobacter cloacae* strain, OXA = oxacillinase beta-lactamase, IMP = imipenemase beta-lactamase, VIM = Verona integron-encoded metallo-beta-lactamase, NDM = New Delhi metallo-beta-lactamase, CphA = carbapenem-hydrolyzing metallo-beta-lactamase. + = inhibited, - = not inhibited

2.1.2.1 Extended-spectrum beta-lactamases

ESBLs possess an expanded activity against beta-lactam antibiotics and are able to hydrolyze oxyimino-cephalosporins (third- and fourth-generation cephalosporins) as well as monobactams, but not cephamycins or carbapenems [60].

The first ESBLs were variants of TEM and SHV enzymes whose substrate spectrum changed due to amino acid substitutions [61]. The first described ESBL was an SHV-2 producing *Klebsiella ozeanae* in 1983 [62], which differs from SHV-1 in the replacement of glycine by serine at position 238. The first TEM of ESBL type (TEM-3, also known as CTX-M-1) was described soon after [63–65]. Until now, 244 TEM- and 229 SHV variants are described (most of them belonging to ESBLs; http://bldb.eu, assessed on 14.10.2021), each owning only a limited number of amino acid substitutions and causing different phenotypes [66,67].

In the late 1990s, CTX-M (cefotaximase-Munich) beta-lactamases were firstly described and became a dominant contributor in resistances in Gram-negative bacteria from 2000 onwards [68]. In contrast to the ESBLs of TEM and SHV, which evolved from parent enzymes as TEM-1/-2 and SHV-1, the CTX-M family derived from the chromosomes of *Kluyvera* species [69]. Until now, 246 CTX-M beta-lactamases are described (http://bldb.eu, assessed on 14.10.2021) and assigned into five subfamilies: CTX-M-1, -2, -8, -9 and -25 [66,69]. The subfamilies differ in > 10% of their amino acid identity. Within a subfamily, only minor differences of < 5% are present [70]. While the detection of TEM and SHV diminished, the detection of CTX-M beta-lactamases increased with the highest detection rates of CTX-M-14, CTX-M-15, and CTX-M-1 worldwide [45,69,71,72]. The global dissemination of CTX-M is attributed to high-risk clones like *E. coli* sequence type (ST)131, ST648, or ST410 carrying CTX-M-15 beta-lactamases [69,73,74]. High-risk clones are defined as globally distributed pathogens with various AMR determinants. They can cause severe infections in hosts and can be easily transmitted to other hosts, and are often characterized by a combination of antimicrobial resistance and enhanced pathogenicity and fitness [75]. Moreover, in recent years, the chromosomal integration of CTX-M beta-lactamases in certain *E. coli* STs was observed, posing the risk of stable integration of the resistance determinants. However, the reasons why certain STs like ST38 are prone to chromosomal integration of CTX-M remains unclear [76].

Another increasing beta-lactamase family is OXA [60]. Most OXA are assigned to Ambler class D or functional group 2d by Bush and Jakoby and are no ESBLs, as they exhibit activity against oxacillins but not against newer cephalosporins and are only poorly inhibited by clavulanic acid. Some OXA variants (e.g. OXA-2 and -10) show an ESBL-phenotype and are frequently detected in *Pseudomonas aeruginosa* and Enterobacterales [77]. Many OXA exhibit a hydrolytic profile against carbapenems as well, with OXA-48-like carbapenemases as the most important representatives [78].

2.1.2.2 AmpC beta-lactamases

AmpC beta-lactamases (AmpCs) belong to the Ambler class C or functional group 1 by Bush and Jacoby [57–59]. In contrast to ESBLs, AmpCs show an additional efficacy against cephamycins and are not inhibited by older beta-lactamase inhibitors like clavulanic acid. They are inhibited by newer beta-lactamase inhibitors (e.g. avibactam) and show no efficacy against carbapenems. The resistance mechanisms of AmpC beta-lactamases are complex and can be classified into three mechanisms: (I) a chromosomally encoded resistance which can be induced by beta-lactam administration, (II) a non-inducible chromosomal mutation in regulatory AmpC genes leading to a stable derepression, and (III) an acquisition of pAmpC genes [79].

The first detected resistance against beta-lactam antibiotics was a chromosomally encoded AmpC beta-lactamase in 1940 [34]. Forty-nine years after, the first pAmpC, CMY-1 (cephamycinase), was described [80]. At present, the most prominent representative of pAmpC is known to be CMY-2. CMY-2 belongs to the CIT family of pAmpC beta-lactamases, with six families of pAmpC are described: ACC, CIT, DHA, EBC, FOX, and MOX [81,82]. Even though pAmpCs are frequently detected, ESBLs are of greater importance in distributing beta-lactam resistances [82].

2.2 *Escherichia coli*

E. coli is a Gram-negative, facultatively anaerobic, rod-shaped bacterium belonging to the family of Enterobacteriaceae [83] and was discovered by Theodor Escherich in 1885 [84]. It is a constituent of the vertebrates' normal gut flora and one of the early gut colonizers [85]. *E. coli* serves as a reservoir for antimicrobial resistance genes and is characterized by its ubiquitous nature [86]. "It is a truth universally acknowledged that there are only two kinds of bacteria. One is *Escherichia coli*, and the other is not." [87]. This quotation reflects the importance of *E. coli* in different research fields and many AMR surveillance programs (e.g. EFSA [88]) which use *E. coli* as an indicator for antimicrobial resistances caused by the selective pressure of antimicrobial usage [89]. For *E. coli*, resistances to all major antibiotic classes are described, such as resistances to beta-lactam antibiotics, carbapenems, fluoroquinolones, and polymyxins, and multidrug resistance to different classes is frequently detected [90]. The RKI (Robert Koch Institute; Berlin Germany) introduced the term 'MRGN' (multiresistant Gram-negative) in 2012 to categorize clinically relevant and multiresistant Gram-negative bacteria upon their resistance profile to four (classes of) antibiotics: piperacillin, fluoroquinolones, third-generation cephalosporins, and carbapenems [91]. The RKI describes a bacterial resistance of an isolate to more than two of the named antibiotic(s) (classes) as clinically relevant. As a resistance to carbapenems is rare, ESBL-producers are usually categorized as 3-MRGN when additionally resistant to fluoroquinolones. A 4-MRGN strain mostly arises after an acquisition of a carbapenemase by a 3-MRGN strain [92].

As a commensal bacterium, *E. coli* rarely causes diseases. At the same time, pathogenic *E. coli* strains can cause severe infections. Pathogenic *E. coli* strains are traditionally divided into intestinal pathogenic *E. coli* (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC). The classification of these pathotypes is based on various criteria, inter alia, the disease location, the infected host, or the presence of specific genes or virulence factors [93]. InPEC pathotypes are primarily causing diarrhea and are divided into at least six pathotypes: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC),

enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC). A seventh pathotype of adherent-invasive *E. coli* (AIEC) is also described [94,95]. ExPECs are divided into uropathogenic *E. coli* (UPEC), neonatal meningitis-causing *E. coli* (NMEC), sepsis-associated *E. coli* (SePEC), and avian pathogenic *E. coli* (APEC) [94–96]. Since an outbreak in Germany in 2011 caused by a strain harboring characteristics of the pathotypes EAEC and EHEC, the term 'hybrid-pathotype' arose [95,97,98]. Hybrid pathogenic *E. coli* commonly arise from the transfer of virulence-associated genes (VAG) by mobile plasmids or the acquisition of converting phages, including hybrids harboring InPEC- and ExPEC-related genes. [47,95,99]. Due to sequencing technologies, hybrid pathogenic *E. coli* are frequently detected with the most common detection of STEC hybrid pathotypes which may lead to a restructuring of the traditional pathotyping schemes in the future [47,95,99–101]. The course of infection with pathogenic *E. coli* varies widely from a self-limiting infection to a severe disease progression [83]. Of utmost importance are UTIs (urinary tract infections) caused by UPEC strains which account for 90% of community-acquired and 50% of nosocomial UTI in humans and are among the most frequent infectious diseases with 150 million infections annually [102,103]. The most common treatment of UTIs are antibiotics. The excessive antibiotic use led to multiresistant UPEC strains, including 3- and 4-MRGN, which are limiting treatment options [92,104]. In light of the high number of recurrent and severe UTI, prevention strategies and new treatment options are needed [105,106]. As another example, infections with APEC strains causing clinical colibacillosis are leading to massive economic losses in poultry production. It was shown that commensal/"non-outbreak" *E. coli* strains harbor VAGs associated with APEC and can cause clinical signs of colibacillosis [107–109].

2.3 ESBL- and pAmpC- producing *E. coli* in different hosts and the environment

2.3.1 ESBL- and pAmpC producing *E. coli* in the environment and wildlife

As an effect of environmental pollution by humans, ESBL- and pAmpC- producing *E. coli* are frequently detected in environmental samples [110]. A study by Blaak et al. demonstrates the contribution of wastewater to the contamination of surface water in the Netherlands [111]. Similar studies are highlighting the impact of the "human footprint" on the contamination of the environment [112,113], and namely hospitals and livestock farming as two drivers of the dissemination of AMR [12,114–116]. Still, it is noteworthy that areas without human activity can harbor ESBL- producing bacteria, indicating a natural occurrence of antimicrobial-resistant bacteria in the environment [117].

The pollution of the water, sewage systems, landfills, or farm facilities can also serve as a source for disseminating AMR into the wildlife [118]. Wild animals are usually not medicated with antimicrobial agents and can, therefore, act as an indicator of environmental pollution by

antibiotic-resistant bacteria [119]. But it has to be examined whether anthropogenic causes are the only drivers of AMR in wildlife [120]. Conversely, wildlife is considered a reservoir and vector for disseminating and introducing AMR into the livestock and the community [110,121]. A frequent example are wild birds. Wild birds, especially migratory birds, are presumed to pass on acquired resistances over a considerable distance from populated areas even to remote areas and vice versa [122–124]. Wild animals could contaminate animal feed, pasture, urban environments, or reservoirs for drinking water [118,125]. Other animals frequently described as carriers of ESBL- producing bacteria are rodents and small mammals [126], as well as wild boars and foxes [127,128]. The prevalence of ESBL- producing bacteria in wild animals is supposed to be lower than in domesticated animals, but extensive population studies are still limited [129].

2.3.2 ESBL- and pAmpC- producing *E. coli* in companion animals and livestock (with focus on broiler chickens)

In contrast to wild animals, farm animals and companion animals are frequently medicated with antimicrobial agents to treat infectious diseases [42]. Major economic losses are attributed to infections in livestock and led to excessive use of antimicrobial agents. In addition, the common practice of herd treatment in livestock selects for resistant bacteria in both diseased and healthy animals [130]. In companion animals, antimicrobial agents are frequently used without a reliable diagnosis or in the course of a wrong medical indication [131]. Additionally, antimicrobial agents restricted to humans can be used off-label in animals as a last resort during the application of the cascade [131]. As a consequence, antimicrobial-resistant bacteria are commonly detected in farm and companion animals [9,132–134]. Following the first description of an oxyimono-cephalosporinase producing *E.coli* (FEC-1) isolated from the fecal flora of a laboratory dog in 1988 to which various cephem antibiotics were administered before [135], the first ESBL-description in a companion animal (SHV-12 producing *E. coli*) was from a dog suffering from recurrent urinary tract infections in the year 2000 [136].

In livestock, ESBL- and pAmpC- producing *E. coli* are detected in all relevant animal production systems, namely swine, cattle, and poultry [9,47,71]. Poultry, especially broiler chickens, is considered a reservoir for ESBL- and pAmpC- producing bacteria [15,24]. Since the early 2000s, cephalosporin-resistant isolates have been detected in poultry production [137,138] even after the ban on cephalosporins from the use in poultry in the European Union in 2012 [13,130]. Numerous studies provide evidence of ESBL- and pAmpC- producing *E. coli* in the entire broiler production pyramid [13,18,139]. Comprehensive data was generated for (grand-) parent flocks [17,18,140], the hatchery [19,20], the fattening period [22,23] and the slaughterhouse level [15,24] up to the sellable products [14,141]. It was shown that day-old

broiler chickens are already carrying ESBL- and pAmpC- producing *E. coli* [27]. To tackle the colonization of broiler chickens with ESBL- and pAmpC- producing bacteria, their transmission routes were thoroughly investigated [139]. The diversity of vertical and horizontal transfers of ESBL- and pAmpC- producing bacteria in the broiler production was categorized by Dame Korevaar et al. [139] as i) a vertical transmission between generations, ii) a transmission at hatcheries, iii) a horizontal transmission on the farm, iv) a horizontal transmission between farms, and via the environment of farms. The relative contribution of the listed transmission routes can not be named as quantitative data are missing [139].

The detection of ESBL- and pAmpC- producing *E. coli* in environmental samples from broiler chicken stables or adjacent areas is crucial. ESBL- and pAmpC- positive environmental samples include inanimate objects like barn equipment, dust, air, rinsed water, surface water, and soil, as well as animate beings like companion animals, livestock, insects, and birds [22,27,142–146]. The detection of ESBL- and pAmpC- producing *E. coli* in environmental samples points out the dynamic of possible introduction- or dissemination routes of resistant bacteria from or into the broiler stables environment.

2.3.3 ESBL- and pAmpC- producing bacteria in a One Health perspective

One health is an “approach, recognizing that human, animal, and environmental health are linked [and] is essential for developing comprehensive and integrative measures to address antimicrobial resistance” [147].

Many pathogens are zoonotic agents and can infect more than one species [148]. From a global perspective, these agents are not only transmitted between local systems like farms, clinics, wastewater treatment plants, and the nearby environment. The transmission is indeed affected by our interconnected world, including the international trade of goods and animals and international travels, as well as animal migrations [12].

After the first detection of an ESBL- producing bacteria in humans in 1983 [62] and the first outbreak in an intensive care unit in 1986 [63], ESBL- and pAmpC- producing bacteria disseminated in clinical settings and since the 2000s in non-clinical settings worldwide with varying prevalence [77,149,150]. The description of ESBL- and pAmpC- producing bacteria in animals followed soon after [135–138] with frequent detections in livestock, wildlife, and the environment [9,111,117,126–128,132–134,145,146,151]. The most frequently detected beta-lactamase types in livestock and companion animals are CTX-M-1, CTX-M-14, CTX-M-15, SHV-12, TEM-52, and CMY-2. The distribution varies depending on the host and the location. In humans, the most prevalent types are CTX-M-14 and CTX-M-15 without a difference in the spatial distribution [45,71,72,152].

ESBL- and pAmpC- producing *E. coli* are zoonotic agents suspected to be directly transmitted between animals and humans [14,129,153,154]. The transmission seems to be likely, due to the close contact of farmers to livestock in the farm environment or pet owners to the pets in private households [71,129,153,155]. Another possible transmission route is the consumption of contaminated meat [14,141,156,157]. While the broiler meat consumption is increasing [130], ESBL- and pAmpC- producing bacteria are most frequently detected in broiler meat [156,158]. But interestingly, vegetarians are not at a lower risk for ESBL- or pAmpC- carriage than meat-eaters [159].

High-resolution methods as whole genome sequencing (WGS) are needed to investigate suspected isolates' phylogenetic and clonal relationship [160–162]. The low discriminatory power of classical typing methods can lead to wrong interpretations of potential transmission processes between humans and animals [160]. Still, detecting the same ESBL-/ pAmpC-producing enterobacterial clone is not proof of a direct transfer of the resistant bacteria from animals to humans or vice versa as these bacteria are omnipresent in the environment [129]. The evidence for direct transfers is rare [11,160,161], and the extent of transmission between humans and animals is a continuing debate and needs further investigations [14,129].

2.4 Experimental colonization of broiler chickens with *E. coli*

As an enterobacterium, *E. coli* needs to enter the gastrointestinal tract and pass the stomach's acid environment for a following growth and colonization in the moderate acidic environments of the small and large intestine [163–166]. One exception is APEC strains, which are able to colonize via oral and respiratory routes [167]. A spray application or an intratracheal infection is more often described for the experimental colonization with APEC strains via the respiratory route [108,168,169]. Another approach to colonize broiler chickens with APEC or commensal *E. coli* is *in ovo* models [170,171]. As this study deals with the intestinal colonization of broiler chickens with *E. coli*, the focus is on the fecal-oral colonization route. For the oral colonization of broiler chickens, the application into the beak (per os) or instillation into the crop is described for *E. coli* [172–178]. The conducted studies vary in i) the day of inoculation of the broiler chickens (day-old to several week old broiler chickens), ii) the bacterial counts used for the oral *E. coli* administration (10^4 colony forming units (cfu) to 10^{10} cfu), and iii) the number of inoculated broiler chickens per trial (all broilers to various inoculation ratios). The inoculation of only a part of the broiler chickens (seeder-birds) in a trial and placing them together with non-inoculated and susceptible broiler chickens (sentinel-bird) is called ‘seeder-bird’ method. The seeder-bird method is applied for different enteral bacteria, such as *E. coli*, *Salmonella enterica,* and *Campylobacter* species [172,179,180]. This method is used to mirror the route of fecal-oral colonization in experimental settings. The bacterium is shed via fecal droppings

of colonized broiler chickens and taken up by all broiler chickens due to their pecking and feeding behavior, leading to a (re-) colonization with the bacterium. For ESBL- and pAmpC-producing *E. coli*, an inoculation at day one to day five with 10^4 – 10^6 cfu of the bacterial strain is most frequently described using an inoculation of all broiler chickens in a trial or inoculation ratios of 1:2 to 1:6 (seeder:sentinel) [172,176,178,181,182].

2.5 Intervention measures against the colonization of broiler chickens with ESBL- and pAmpC- producing bacteria on farm level

The high complexity of the broiler chicken production system causes a need for interventions at every level of the production chain to reduce the colonization of broiler chickens and to reduce the contamination of retail products with ESBL- and pAmpC- producing bacteria [18]. Interventions at the farm level are necessary to reduce the broiler chickens' colonization throughout a fattening process. The colonization status at the farm level is affected by the ESBL- and pAmpC- status of previous production stages (e.g. broiler breeders or hatchery) and can itself influence the ESBL- and pAmpC- status of subsequent production stages (e.g. slaughterhouse) [18,20,24,139]. Another challenge for implementing effective intervention measures is the diversity of vertical and horizontal transfers of ESBL- and pAmpC- producing bacteria in the broiler production as mentioned above.

Biosecurity measures can prevent the introduction of potentially pathogenic microorganisms into a broiler farm (external biosecurity measures) or can avoid the spread of an introduced microorganism within a broiler farm (internal biosecurity measures) [183]. External biosecurity includes the control of (unauthorized) visitors, companion animals, rodents, wild animals and insects, farm equipment, transport vehicles, as well as feed and water as they can introduce ESBL- and pAmpC- producing bacteria into the farm. Internal biosecurity refers to a strict hygiene concept like a proper cleaning and disinfection regime, protective clothing, no shared equipment for different stables, and includes veterinary care [183,184].

Farm management can affect the health and performance of the broiler chickens and, in turn, the farm's profitability [185–187]. Management factors are also discussed to influence the colonization of broiler chickens with ESBL- and pAmpC- producing bacteria as the general housing system (conventional vs. organic), the breed, the stocking density, the litter or antimicrobial treatments [188,189]. In Germany, broilers are most commonly fattened in an intensive fattening system using the breed Ross 308, a stocking density of 39 kg/m^2 without any enrichment, and conventional feed and water *ad libitum*. Various concepts aim to modulate the microbial composition of the gastrointestinal tract as a pivotal point to enhance the broilers' performance and health status. One approach is the early administration of live bacteria to

newly hatched broiler chickens to protect them from colonization with certain other bacteria [172,190]. These cultures can consist of a single defined strain or a complex and undefined culture, whereas only defined cultures are approved in the European Union [191,192]. Another approach is feed additives like essential oils or acidification of the water, feed, or litter throughout the fattening process to reduce the colonization of broiler chickens with pathogenic or antimicrobial-resistant bacteria, including ESBL- and pAmpC- producing bacteria [193–196]. Although scientific evidence on the efficacy of most of the measures is lacking, several measures are already applied and combined in broiler farming.

3 Publications

3.1 Publication I

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.

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Low Dose Colonization of Broiler Chickens With ESBL-/AmpC-Producing *Escherichia coli* in a Seeder-Bird Model Independent of Antimicrobial Selection Pressure

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Extended-spectrum beta-lactamase- (ESBL-) and AmpC beta-lactamase- (AmpC-) producing Enterobacteriaceae pose a risk for both human and animal health. For livestock, highest prevalences have been reported in broiler chickens, which are therefore considered as a reservoir of multidrug-resistant bacteria. The possibility of transfer to humans either by a close contact to colonized broiler flocks or through contaminated retail meat results in the necessity to develop intervention measures for the entire broiler production chain. In this regard, a basic understanding of the colonization process is mandatory including the determination of the minimal bacterial load leading to a persistent colonization of broiler chickens. Therefore, we conducted a bivalent broiler colonization study close to real farming conditions without applying any antimicrobial selection pressure. ESBL- and AmpC- negative broiler chickens (Ross 308) were co- colonized on their third day of life with two strains: one CTX-M-15-producing *Escherichia coli*-ST410 and one CMY-2/*mcr*-1-positive *E. coli*-ST10. Colonization was assessed by cloacal swabs over the period of the trial, starting 24 h post inoculation. During the final necropsy, the contents of crop, jejunum, cecum, and colon were quantified for the occurrence of both bacterial strains. To define the minimal oral colonization dosage 10^4 to 10^1 colony forming units (cfu) were orally inoculated to four separately housed broiler groups (each $n = 19$, all animals inoculated) and a dosage of already 10^1 cfu *E. coli* led to a persistent colonization of all animals of the group after 3 days. To assure stable colonization, however, a dosage of 10^2 cfu *E. coli* was chosen for the subsequent seeder-bird trial. In the seeder-bird trial one fifth of the animals (seeder, $n = 4$) were orally inoculated and kept together with the non-inoculated animals (sentinel, $n = 16$) to mimic the route of natural infection. After 35 days of trial, all animals were colonized with both *E. coli* strains.

Given the low colonization dosage and the low seeder/sentinel ratio, the rapid spread of ESBL- and AmpC- producing Enterobacteriaceae in conventional broiler farms currently seems inevitably resulting in an urgent need for the development of intervention strategies to reduce colonization of broilers during production.

Keywords: ESBL, AmpC, *Escherichia coli*, broiler chicken, colonization, antimicrobial selection pressure, seeder bird model

INTRODUCTION

Antimicrobial resistance is an increasing problem in veterinary and human medicine and the spread of extended-spectrum beta-lactamase- (ESBL-) and AmpC beta-lactamase-(AmpC-) producing Enterobacteriaceae was demonstrated in wildlife (Guenther et al., 2011; Alcalá et al., 2016), livestock (Laube et al., 2013; Liebana et al., 2013; Hille et al., 2018), and companion animals (Rubin and Pitout, 2014; Schaufler et al., 2015; Kaspar et al., 2019) as well as in retail meat (Cohen Stuart et al., 2012; Kola et al., 2012) and out of human origin (Pitout, 2013; Valenza et al., 2014; Mazzariol et al., 2017).

Livestock animals, including broiler chickens, show high prevalences (EFSA, 2019) and are considered to be a reservoir of ESBL- and AmpC- producing enterobacteria (Costa et al., 2009; Reich et al., 2013). Recently, it was shown by Projahn et al. (2017) and Daehre et al. (2018b) that transmission of resistant bacteria can take place at a very early stage of the broiler production chain: pseudo-vertical from the parent flock to the eggs in the hatchery and horizontal through the contaminated farm environment 24 h after the placing of the broiler chickens. Genes encoding ESBLs and AmpCs are frequently located on mobile genetic elements such as plasmids (Carattoli, 2013). Commonly detected resistance genes include $bla_{\mathrm{CTX-M-1}}$, $bla_{\mathrm{CTX-M-14}}$, $bla_{\mathrm{CTX-M-15}}$, $bla_{\mathrm{SHV-12}}$, and $bla_{\mathrm{CMY-2}}$ in livestock and companion animals (Ewers et al., 2012). The question of a possible transfer of resistance from animals to humans remains controversial and constitutes a major concern of public health (Cortés et al., 2010; Leverstein-van Hall et al., 2011; Ewers et al., 2012; Dorado-García et al., 2018; Pietsch et al., 2018). Close contact of humans to colonized animals (Dierikx C. et al., 2013; Huijbers et al., 2014) and the consumption of contaminated meat (Vincent et al., 2010; Leverstein-van Hall et al., 2011) are considered to be especially important risk factors, and have been thoroughly investigated.

By contrast, to our knowledge, there have not been any studies investigating the minimal bacterial load that is necessary for a stable colonization of broiler chickens (Daehre et al., 2018b). Therefore, the aim of our study was to define the minimal colonization dosage of broiler chickens with ESBL- and AmpC- producing *Escherichia coli (E. coli)* without applying any antimicrobial selection pressure. In the second step, a seeder-bird colonization model that mimics real farming conditions was conducted for the investigation of the spread of ESBL- and AmpC- producing *E. coli* in flocks. These investigations paved the way for the follow-up studies that are currently being conducted at our institute and aim to examine potential intervention strategies regarding hygiene- and management measures to reduce the colonization of broiler chickens with ESBL- and AmpC- producing Enterobacteriaceae.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the National Animal Protection Guidelines. The protocol was approved by the German Animal Ethics Committee for the protection of animals of the Regional Office for Health and Social Affairs Berlin ("Landesamt für Gesundheit und Soziales," LAGeSo, registration number G 0193/16).

Housing Conditions

All trials were conducted at the experimental facilities of the Center for Infection Medicine of the Freie Universität Berlin, department of Veterinary Medicine. The animals were kept in controlled rooms with attached separate lockrooms for changing of clothes and shoes. Before starting the trial, rooms were cleaned and disinfected with hydrogen peroxide fumigation and the complete experimental setup was tested for the absence of ESBL-/AmpC- producing bacteria (see "ESBL-/AmpC- status prior to the trial"). An individual ventilation was achieved by using HEPA filter. The study was structured in two parts: a colonization dosage part followed by a seeder-bird colonization model.

For each trial, eggs of the breed Ross 308, received from a commercial hatchery in Germany, were hatched in-house for 21 days. The first disinfection of the eggs using formaldehyde gas was performed at the hatchery and the second disinfection with WESSOCLEAN® K 50 Gold Line containing 2.37% hydrogen peroxide and 0.015% peracetic acid (Wesso AG, Hersbruck, Germany) following the transportation to the experimental facilities before incubation in a separate hatcher. After hatch, broilers were conventionally housed in with a stocking density of 39 kg/m^2, fresh litter once at the beginning of the trial, no enrichment and conventional feed and water *ad libitum*. The feeding regime included a starter feed and a grower feed with coccidiostats (decoquinate and narasin/nicarbazin). The finisher feed did not include any coccidiostats and was fed 5 days prior to necropsies. The feed did not contain any antimicrobial agents.

First, four colonization dosage trials were performed. Each of the four groups consisted of 19 chickens. Every group was kept separately in an experimental room for a period of 2 weeks in aviaries to define the minimal oral colonization dosage. Following these colonization dosage experiments, 20 chickens were kept in floor keeping for the seeder-bird model trial. For the seeder-bird trial, four chickens were orally inoculated (seeder birds) with

the minimal oral colonization dosage determined previously and kept together with 16 non-inoculated animals (sentinel birds). During the seeder-bird trial, the chickens were kept up to a weight of two kilograms, corresponding to the duration of 5 weeks.

In the later course of the colonization dosage trial using 10^2 cfu/*E. coli* (day 11) and the seeder-bird trial (day 16) each time one animal died without any sign of infection or illness.

Bacterial Colonization Strains

Two avian *E. coli* strains were used for co-colonization of the birds. Both strains were originally isolated from healthy chickens during a previous research project on ESBL- producing *E. coli* in chickens in 2011 (Falgenhauer et al., 2016; Hering et al., 2016). Both strains were investigated using whole-genome analysis as described in Falgenhauer et al. (2016). One strain is an ESBL- producing *E. coli* {CTX-M-15 [chromosomally encoded (Arredondo-Alonso et al., 2018)]; multilocus sequence type ST410; phylogenetic group B1; internal number 10716; published as R56 in Falgenhauer et al. (2016)} with resistances to cephalosporins and enrofloxacin, and the other strain is an AmpC- producing *E. coli* {CMY-2 [plasmid encoded (Arredondo-Alonso et al., 2018)]; ST10; phylogenetic group A; internal number 10717}, resistant to cephalosporins and colistin, as shown in **Table 1**. In addition, phenotypic antimicrobial resistance analysis was performed by using VITEK 2 system (bioMérieux, Marcy-l'Étoile, France). These avian commensal *E. coli* strains were selected because of their resistance profile, representative for the situation in German chicken production and their ability to colonize broiler chickens digestive tract.

Microorganisms were stored at −80°C in Luria Bertani broth (LB; Carl Roth, Karlsruhe, Germany) containing 20% glycerol (Carl Roth, Karlsruhe, Germany). For the preparation of the bacterial suspension both bacterial strains were streaked out on columbia agar containing 5% sheep blood (Oxoid, Wesel, Germany) and were incubated for 24 h at 37°C. Overnight cultures originating from a single colony from each strain were grown in 5 ml LB broth. On the following colonization day, 40–60 µl of these bacterial suspensions were seeded in 5 ml of fresh LB broth each until the desired optical density of 0.04 (OD_{600}) was obtained. The bacterial cultures were grown at 37°C and 200 rounds per minute (rpm) to the optical density of 1.0 (OD_{600}), corresponding to 1×10^8 colony forming units (cfu) per ml. One ml per culture was centrifuged at 7,000 rpm for 10 min at 4°C, the supernatant was removed and the cells were resuspended in 1 ml phosphate buffered saline (PBS; Phosphate Buffered Saline tablets, Oxoid, Wesel, Germany). The desired colonization dose was adjusted via a 10-fold dilution series in PBS. For co-colonization, both dilutions were mixed, placed on ice, and used for inoculation of broilers within 30 minutes. The concentration of the colonization dose was verified by direct plating of appropriate dilutions of the inoculum.

Oral Colonization of Broilers

On the third day of life, broilers were orally inoculated into the crop with 200 µl of bacterial suspension in PBS containing a mixture of both bacterial strains in equal parts. For the colonization dosage trials, all animals of the group were orally inoculated, starting with a colonization dosage of 10^4 cfu *E. coli*/200 µl followed by 10^3, 10^2, and 10^1 cfu *E. coli*/200 µl in the following experiments to verify the cfu that is necessary to colonize all animals within 24 h *post inoculation* (*p.i.*).

In contrast to the colonization dosage trials, not all animals of the seeder-bird model trial were orally inoculated. The inoculation was performed at the ratio of 1:5 (seeder: sentinel), resulting in four seeders, which are expected to colonize the 16 non-inoculated, susceptible broilers (sentinels) over the period of the trial. From the beginning of the trial, the seeder- and sentinel birds were housed in together. Similarly to the dosage trials, all inoculated animals should be colonized within 24 h *p.i.*

Samplings and Analyses

Samples were initially processed on chromogenic agar (CHROMagar Orientation, Mast Diagnostica, Reinfeld, Germany) for a rapid identification of *E. coli* colonies. Confirmation of single bacterial *E. coli* colonies with a typical morphology and of all untypical colony morphologies was performed using MALDI- TOF (MALDI Microflex LT® and Biotyper database®; Bruker Daltonics, Bremen, Germany). Phenotypic antimicrobial resistance analysis of randomly picked colonies of the cecum samples obtained in necropsy was performed by using VITEK 2 system to confirm their identity as the inoculated ESBL- and AmpC- strains.

ESBL-/AmpC- Status Prior to the Trial

Before starting a trial, the complete experimental setup was tested for the absence of ESBL-/AmpC- producing bacteria after the introduction of the litter, feed, and water into the experimental room. Walls/doors, floor, table, heating lamps, feeding-, and

TABLE 1 | Characteristics of *E. coli* strains used for colonization dosage trials ($10^1 - 10^4$ cfu *E. coli*) and seeder-bird colonization model.

Strain	Origin	MLST	Phylogenetic group	ESBL-/AmpC type	None ESBL-/AmpC resistance genes	Phenotypic resistances
10716	Chicken	ST410	B1	$bla^{*}_{CTX-M-15}$	*aadA1**, *aac*(3)-*IIa**, $aadA5^{+}$, $aadB^{+}$, *mph*(A)$^{+}$, *catA1**, $floR^{+}$, $sul1^{+}$, *tet*(A)$^{+}$, $dfrA17^{+}$	ATM, CAZ, CIP, CTX, GM, PIP, TZP, SXT, TM
10717	Chicken	ST10	A	bla^{+}_{CMY-2}	bla^{+}_{TEM-1}, $aadA1^{+}$, $aadA2^{+}$, $mcr\text{-}1^{+}$, $cmlA1^{+}$, $sul3^{+}$	ATM, CAZ, CTX, CST, PIP, TZP

*10716 = ESBL- E. coli R56, 10717 = AmpC- E. coli G148-1 (Falgenhauer et al., 2016; Hering et al., 2016); *chromosomally encoded; +plasmid encoded [determination using mlplasmids (Arredondo-Alonso et al., 2018)]; ATM, aztreonam; CAZ, ceftazidim; CIP, ciprofloxacin; CTX, cefotaxime; CST, colistin; GM, gentamicin; PIP, piperacillin; TZP, piperacillin/tazobactam; TM, tobramycin; SXT, trimethoprim/sulfamethoxazole.*

drinking troughs were sampled with sterile and moistened gauze swabs. For moistening, 5 ml PBS were used. In addition, 5 g of litter, and feed were collected. Following the sampling, the gauze swabs, litter, and feed were transferred into 50 ml LB broth in each case and incubated for 24 h at 37°C before 10 µl were streaked out on chromogenic agar containing 2 µg/ml cefotaxime (AppliChem, Darmstadt, Germany) and incubated for 24 h at 37°C.

Directly after hatching, the absence of ESBL and AmpC-producing bacteria in the egg shells was confirmed. Five egg shells were crushed, transferred into 50 ml LB broth and incubated for 24 h at 37°C before 10 µl were streaked out on chromogenic agar containing 2 µg/ml cefotaxime and incubated for 24 h at 37°C.

The absence of ESBL and AmpC in the 1 day-old broiler chickens was confirmed by cloacal swabs of all individually tagged animals. Swabs were transferred into reaction tubes containing 500 µl PBS and were thoroughly vortexed. Fifty microliter were streaked out onto chromogenic agar containing 2 µg/ml cefotaxime and incubated for 24 h at 37°C.

Sampling During the Trial

Colonization of broilers was analyzed via cloacal swabs during the trial starting 24 h and 72 h *p.i.* and followed by samplings of each individual three times in the second week of a trial, followed by two samplings a week up to the end of a trial. Swabs were transferred into reaction tubes containing 500 µl PBS and were thoroughly vortexed. Fifty microliter were streaked out onto agar and incubated for 24 h at 37°C for semiquantitative analysis. A subjective measurement using eight categories from zero ("no growth") to seven ("overgrown") was applied for the evaluation of the colonization (**Figure S1**) and a set of four chromogenic agar plates was used. One plate without selective media for the total count of *E. coli* colonies (positive control). One plate containing 2 µg/ml cefotaxime and 4 µg/ml enrofloxacin (Sigma-Aldrich, Steinheim, Germany) for the growth of the ESBL- *E. coli* 10716 and another plate containing 2 µg/ml cefotaxime and 7 µg/ml colistin (Carl Roth, Karlsruhe, Germany) for the growth of the AmpC- *E. coli* 10717. A fourth plate contained all three antibiotics in the given concentrations (negative control).

Finalization of the Trial

Following each trial, a necropsy of all broilers was performed on day 14 in the colonization dosage trials and on day 35 in the seeder-bird model trial. Contents of crop, jejunum, cecum, colon as well as organ samples of liver and spleen were analyzed using the plate set described above.

The samples of crop, jejunum, cecum, and colon were quantified. First, up to one gram of the content was weighed out in a reaction tube and PBS was added at the ratio of 1:2, followed by a dilution series in PBS, depending on the expected bacterial growth. Finally, 100 µl each of the suspension were plated on the plate set, as described above. For quantification, a minimum of two different dilution levels were counted.

The organ samples of liver and spleen were qualitatively analyzed via direct processing. For this, the organs were cut in half with sterile scissors, pressed on the agar and spread out with an inoculation loop (Selbitz et al., 2015). All samples were incubated for 24 h at 37°C.

Statistical Analysis

Sample size calculation was based on the hypothesis that the logarithmic mean values in the colonized ceca were equal in each of the different colonization dosage groups (NCSS PASS 14.0). Ceca were selected because ceca are known to be the reservoir for ESBL- *E. coli*. Equivalence was defined as follows: the 95% confidence interval (CI) of the investigated group is within one log unit cfu/g cecal content compared to the mean value of the minimal colonization dosage group. Standard deviation of log 0.9 cfu/g cecal content was assumed. To ensure alpha error of 0.05 and power of 0.90, 19 animals per group were required. Twenty animals were used for the seeder-bird colonization model to have a comparable group size for the defined inoculation ratio of 1:5 (4 seeder birds: 16 sentinel birds).

Statistical analysis was performed by using Excel 2013 (Microsoft Corporation, Washington, USA) and SPSS Statistics 25 (IBM, New York, USA). To obtain normally distributed data, all bacteriological results were log10 transformed. CI of proportions were calculated using Clopper-Pearson method.

To compare the level of colonization in between the four colonization dosage groups, equivalence testing (Thrusfield, 2007) was performed. For this test, a margin of one log unit difference between the groups was defined acceptable to be equal. It was tested whether the margins of the 95% CI are within the range of one log unit.

Differences between different groups regarding the logarithmic bacteriological mean values of the samples were investigated using *t*-test for independent samples: first, the colonization levels between the colonization dosage group 10^2 cfu/*E. coli* and the seeder-bird colonization model were investigated separately for the locations crop, jejunum, cecum, and colon. Second, the results of the seeder- and sentinel birds of the seeder-bird model were compared. The probability level of 0.05 was used to denote significance.

RESULTS

Colonization Dosage

By applying a series of colonization dosages ranging from 10^4, 10^3, and 10^2 to 10^1 cfu *E. coli*, we were able to demonstrate that a dosage of 10^2 cfu *E. coli* per animal led to an almost complete colonization of a broiler group within 24 h *p.i* (**Figure S1**). Even a dosage of 10^1 cfu *E. coli* was sufficient to colonize the majority of the animals within 24 h *p.i.* For the colonization dosage trials, all animals in a group were orally inoculated with both bacterial strains on their third day of life and the successful colonization was initially assessed via cloacal swabs 24 h *p.i.* For colonization dosages 10^4 and 10^3 cfu *E. coli*, all animals were colonized within 24 h *p.i.* up to the end of the trial. Continuing with a dosage of 10^2 cfu *E. coli*, only one animal was tested negative 24 h *p.i.* for one of the strains (AmpC- *E. coli* 10717, 95%CI: 73.97–99.87%). At the second sampling 72 h *p.i.*, however, all animals inoculated with 10^2 cfu *E. coli* were colonized over the entire period of the trial. The dosage trials were completed with 10^1 cfu *E. coli* resulting in

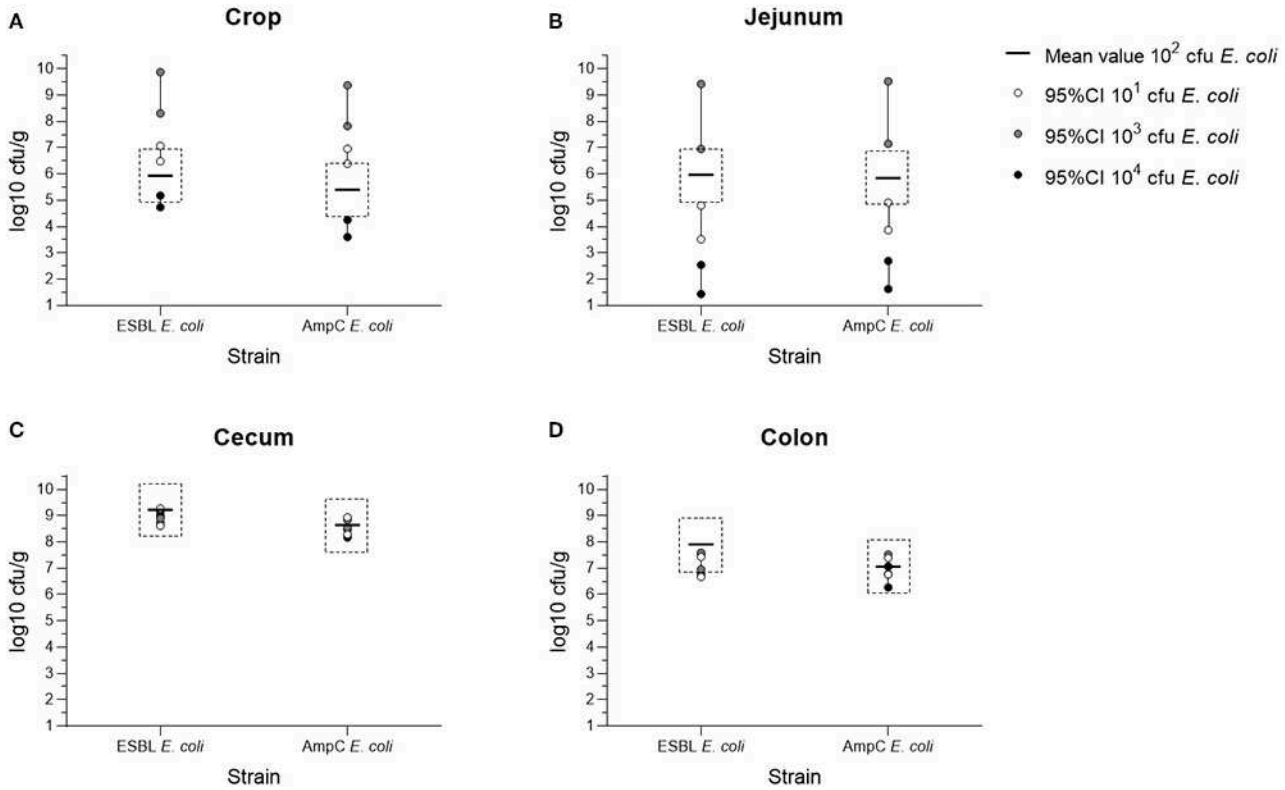

FIGURE 1 | Equivalence testing of ESBL- *E. coli* 10716 and AmpC- *E. coli* 10717 in samples of crop **(A)**, jejunum **(B)**, cecum **(C)**, and colon **(D)** in between the four colonization dosage groups (10^1-10^4 cfu/*E. coli*) at necropsy. 95%CI = 95% confidence interval; all data shown are log10 transformed (log10 cfu/g); margin of < 1 log of 95%CI of dosage groups 10^1, 10^3, and 10^4 cfu/*E. coli* to the mean value of the minimal colonization dosage group (10^2 cfu/*E. coli*) shows equivalence.

a colonization rate of 84% (ESBL- *E. coli* 10716, 95%CI: 60.42–96.62%) and 95% (AmpC- *E. coli* 10717, 95%CI: 73.97–99.87%) of the animals positive for the strains 24 h *p.i.* In this group, complete colonization was detectable within 72 h *p.i.* up to the end of the trial. Based on the results obtained from cloacal swabs during the trial, a minimal dosage of 10^2 cfu *E. coli* was necessary to colonize a broiler group in the given experimental setup within 24 h *p.i.*

The bacterial counts of the cecum samples obtained at the necropsies of all four trials (10^4 to 10^1 cfu *E. coli*) were of equal value in the equivalence test. In contrast to this, samples of crop, jejunum, and colon were non- equivalent (**Figure 1**). The mean colonization values of the digestive tract samples for both bacterial strains are summarized in **Figure 2** and **Table S1**. Overall, maximum values were attained in cecum samples with mean values between 8.84 to 9.22 log10 cfu ESBL- *E. coli*/g cecal content and 8.32 to 8.69 log10 cfu AmpC- *E. coli*/g cecal content, except for the content of crop and jejunum in the dosage trial using 10^3 cfu *E. coli*. Compared to the cecum samples, lower mean values occurred in colon samples and lowest mean values were evident in the content of crop and jejunum (**Table S1**). Independent from the original colonization dosage, the results of cecum samples were equivalent with a deviation less than one log unit to the values of the colonization dosage of 10^2 cfu *E. coli* and every broiler was colonized with both bacterial strains 2 weeks *p.i.* For samples of crop, jejunum and colon, equivalence between dosages was not shown. The values of crop and jejunum samples differed in each trial. Highest values were given in the dosage trial using 10^3 cfu *E. coli*, lowest values occurred while using 10^4 cfu *E. coli*. In contrast to this, less variation was given in colon samples with mean values between 7.05 to 7.91 log10 cfu ESBL- *E. coli*/g colon content and 6.67 to 7.15 log10 cfu AmpC- *E. coli*/g colon content, but without showing equivalence.

In conclusion, the colonization of the cecum with both bacterial strains 2 weeks after inoculation with 10^2 cfu *E. coli* was equal to the colonization after inoculation with 10^4, 10^3, and 10^1 cfu *E. coli*. In contrast to these correlating results of cecum samples obtained at necropsy, we observed lower percentages of colonized animals 24 h after inoculation by using 10^1 cfu *E. coli* compared to the other tested dosages. Based on these results, a dosage of 10^2 cfu *E.coli* was chosen for the following seeder-bird colonization model trial balancing between a low dose, comparable to the situation in the course of natural infection and a colonization of the animals during the trial.

Seeder-Bird Colonization Model

A ratio of 1:5 orally inoculated (seeder) to non- inoculated broilers (sentinel) and a dosage of 10^2 cfu ESBL-/AmpC- *E. coli* were sufficient to colonize an entire broiler group

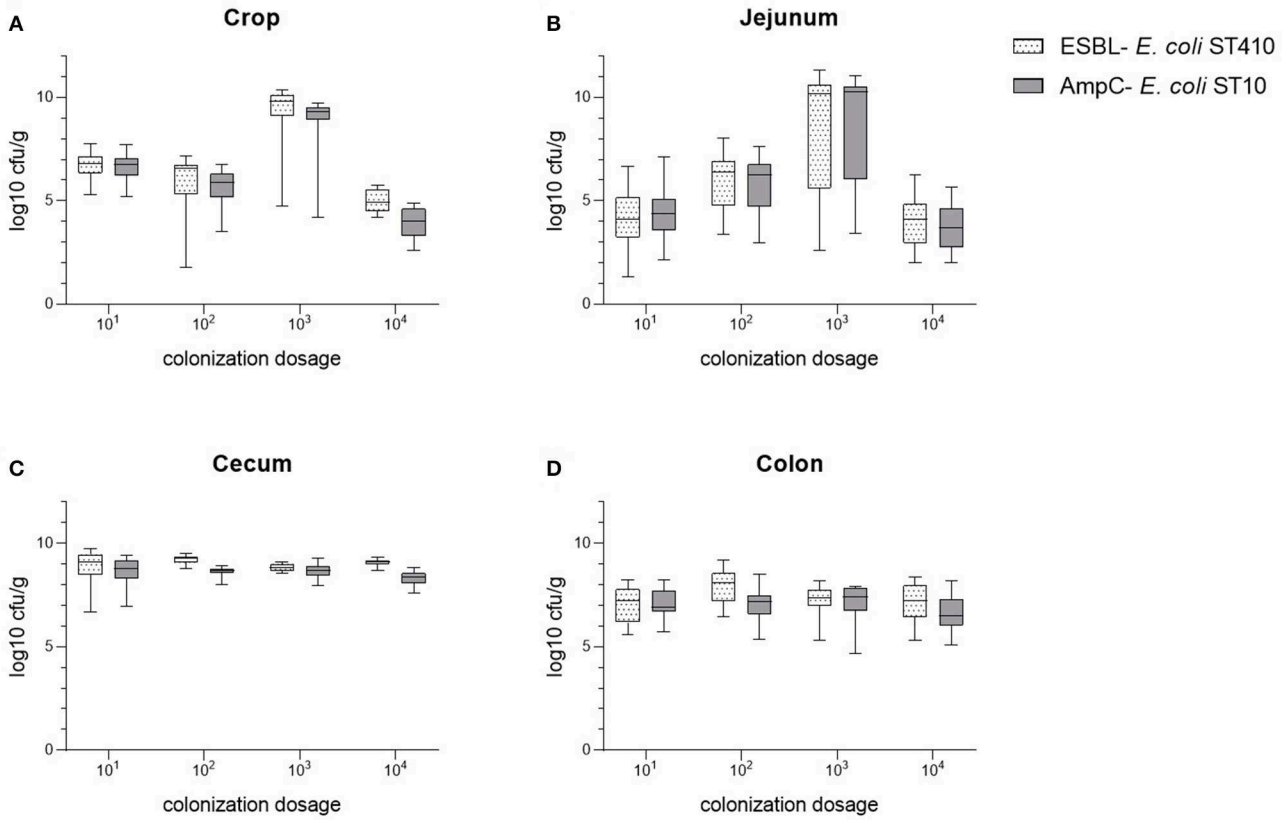

FIGURE 2 | Comparison of the bacterial counts of ESBL- *E. coli* 10716 and AmpC- *E. coli* 10717 in samples of crop **(A)**, jejunum **(B)**, cecum **(C)**, and colon **(D)** in between the four colonization dosage groups (10^1-10^4 cfu/*E. coli*) at necropsy.

within 72 h (**Figure S1**). The seeder birds were completely colonized 24 h *p.i.* with both bacterial strains. At this early time point 69% of the 16 sentinels were tested positive for the ESBL- *E. coli* strain 10716 (95%CI: 41.34–88.98%) and 50% positive for AmpC- *E. coli* 10717 (95%CI: 24.65–75.35%). Three days *p.i.*, at the second sampling, all animals of the broiler group were colonized with both bacterial strains up to the end of the trial. As a result, an oral colonization of one fifth of a broiler group with 10^2 cfu *E. coli* on their third day of life resulted in colonization of the entire broiler group.

At necropsy, cecum colonization of all seeder- and sentinel birds was observed, with mean values of 6.69 log10 cfu ESBL- *E. coli*/g cecal content and 6.57 log10 cfu AmpC- *E. coli*/g cecal content (**Table S1**). Furthermore, no significant difference in bacterial counts between seeder birds and sentinel birds was evident at the end of the trial (*t*-test *p*-values > 0.05). Corresponding to the colonization dosage trials, the maximum values of *E. coli* were found in the cecum irrespective of being a seeder- or sentinel bird. Compared to the cecum samples, reduced values occurred in colon samples and lowest mean values were evident in crop and jejunum samples. Similarly, no association with the origin of the bird as a seeder- or sentinel was observed (t-test *p*-values > 0.05).

However, compared to the dosage trial using 10^2 cfu *E. coli*, the colonization in different parts of the digestive tract of the seeder-bird group was significantly lower (*t*-test *p*-values < 0.001; **Figure 3**). In all necropsy samples taken, we observed lower total numbers of both bacterial strains 5 weeks *p.i.* in the seeder-bird model compared to the initial colonization dosage trial with a duration of 2 weeks. In summary, a comparable colonization of all seeder- and sentinel birds in a group was achieved by an oral colonization dosage of 10^2 cfu *E. coli* in combination with an oral inoculation of one fifth of the broiler chickens on their third day of life.

To conclude, without any antimicrobial selection pressure by antibiotic usage, both commensal ESBL- and AmpC- producing *E. coli* strains colonized all broilers digestive tract independent of being an orally inoculated seeder bird or a susceptible sentinel bird. For all four colonization dosage trials and the seeder-bird trial we did not detect any colonization of liver or spleen with our bacterial strains tested. By analyzing the respective antimicrobial resistance patterns via VITEK 2 of the strains derived from the cecum samples at the necropsies, we did not observe major changes in the resistance profile of the inoculation isolates to the isolates at the end of the trial. The rate of transfer of resistance plasmids to other strains can therefore be considered to be of low importance, as some of our resistance markers were also

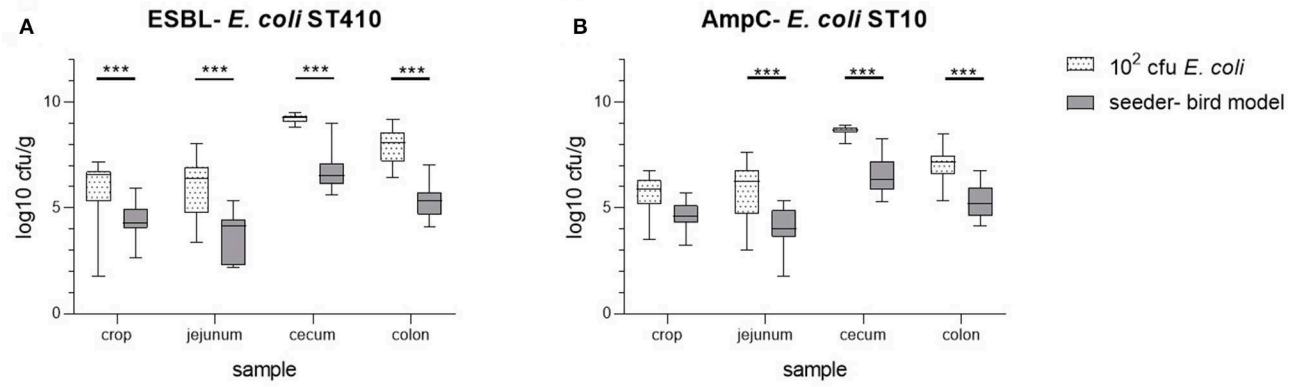

FIGURE 3 | Comparison of the bacterial counts of ESBL- *E. coli* 10716 **(A)** and AmpC- *E. coli* 10717 **(B)** in samples of crop, jejunum, cecum, and colon at necropsy between the minimal colonization dosage group (10^2 cfu/*E. coli*; necropsy on day 14; all animals inoculated on third day of life) and the seeder-bird model (necropsy on day 35; inoculation ratio of 1:5 on third day of life). ***$p < 0.001$ (*t*-test).

chromosomally encoded, such as fluoroquinolone resistance. In addition, we did not observe changes in the morphotypes of the isolates during the trials.

DISCUSSION

Our study points out that (i) a colonization dosage of 10^2 cfu *E. coli* per animal is sufficient for a successful long-time colonization of broiler chickens and (ii) a ratio of 1:5 inoculated seeder birds to non- inoculated sentinel birds is sufficient to colonize a complete broiler group even in the absence of antimicrobial selection pressure. In addition, we also demonstrated that even a very low colonization dosage of 10^1 cfu *E. coli* leads to a colonization of broilers in the given experimental setup using a study design based on parameters of real farming conditions. The animals were treated equally to those in conventional fattening farms. This includes a stocking density of 39 kg/m^2, fresh litter once at the beginning of the trial, no enrichment and conventional feed and water *ad libitum*. Neither the utilized eggs and animals, nor the feed, water or litter were additionally processed for the reduction of germs than usual in the broiler production. In agreement, we received all eggs, litter and feed from a commercial poultry producer in Germany. To ensure comparable conditions in all trials, we used batches from one single producer and all trials were conducted within 6 months. Hence, an interaction between the animals and their surrounding environment including the present bacterial spectrum was possible before inoculation of the broiler chickens with the bacterial strains (Apajalahti et al., 2004; Ballou et al., 2016; Kumar et al., 2018). As the surrounding can be a source of ESBL- and AmpC- acquisition (Dierikx C. M. et al., 2013; Daehre et al., 2018b), we tested the complete experimental setup for the absence of ESBL-/AmpC- producing bacteria prior to the trial to exclude the possibility of a colonization out of the environment.

We inoculated our broiler chickens on the third day of life with two bacterial strains, harboring frequently detected ESBL- and AmpC- resistance profiles in fattening chickens (Ewers et al., 2012; Valentin et al., 2014). This is in accordance with numerous studies assuming a very early colonization of the broilers in the production chain. Projahn et al. (2017) describes a possible transfer of resistant bacteria already in the hatchery and during the transportation of the hatchlings to the fattening farm (Projahn et al., 2018). Furthermore, the fattening stable itself (Daehre et al., 2018b) or the surrounding areas (Laube et al., 2014) are potential sources of the acquisition of resistant bacteria. At the same time, investigations of the ESBL- and AmpC- prevalence of day- old broiler chickens are heterogeneous, varying between 95 and 0% in the analyzed broiler flocks (Laube et al., 2013; Daehre et al., 2018b). Thereby, both studies analyzed seven broiler flocks via cloacal swabs of 20 (Laube et al., 2013) or 40 broiler chickens (Daehre et al., 2018b) and both used a pre-enrichment in LB to determine the ESBL- and AmpC- prevalence. The wide variations may be caused by the selected sampling times. Laube et al. (2013) sampled within the first two days of life. In contrast to this, Daehre et al. (2018b) sampled immediately after placing the broiler chickens into the stable. However, it must be noted, that the overall prevalence of the investigated flocks is higher in the study by Laube et al. (2013). As it is known, compared to other species, the relation between the body length and the length of the gastrointestinal tract of chickens is relatively short with a rapid passage time (Hughes, 2008). To attain a reliable detection of our bacterial strains, we decided for an initial validation of our bacterial strains 24 h after inoculation. At this early time point, more than half of the sentinel birds in the seeder-bird trial had been tested positive for the ESBL- and AmpC- producing strains. This indicates a double passage of the gastrointestinal tract of firstly the seeder birds and following the sentinel birds within 24 h. These results were obtained by direct processing of the cloacal swabs without enrichment. To ensure a strong colonization of the broiler chickens, a direct detection of the bacterial strains is required. At the same time, a swab sampling provides only limited information about the intestinal colonization of the birds. The detection is strongly influenced by the amount of feces on

the swab. Thus, our detection limit is relatively high, depending on the cloacal excretion during the sampling. For this reason, we decided for a minimal colonization dosage of 10^2 cfu *E. coli* even if one broiler chicken was tested negative for one of the bacterial strains 24 h *p.i.*

As this study aims to mimic the route of natural infection with ESBL- and AmpC- producing Enterobacteriaceae, a housing of all broiler chickens immediately from the beginning of the trial in one pen is important. Under real farming conditions, it is not possible to distinguish between colonized and non-colonized birds as well. Consequently, a distinction between colonization with the bacterial strains via inoculation or through the oral uptake out of the contaminated environment was not aimed in our trial. Although we cannot exclude that the colonization via inoculation of the seeder birds might have failed and an oral uptake of droppings from colonized chickens led to a colonization, this does not reduce the importance for the practice. Rather, it reflects the circumstances affecting a colonization under field conditions. Both, the colonization of the sentinel birds as well as the seeder birds is dependent upon the initial colonization of the seeder birds via inoculation. The dependency between seeder birds and sentinel birds and the resulting colonization with ESBL- *E. coli* was described by Ceccarelli et al. (2017) and is neglected in our colonization model. Moreover, besides our housing conditions, the sampling frequency does not allow to draw a conclusion of the transmission rate of ESBL- and AmpC- producing Enterobacteriaceae: our study is focused on the outcome of a practical orientated colonization.

Since the genes encoding for ESBLs and AmpCs are frequently located on plasmids (Carattoli, 2013), the possibility of a horizontal gene transfer (HGT) to other Enterobacteriaceae has to be considered. Even in the absence of antimicrobial selection pressure a transmission of resistance genes is possible (Smet et al., 2011). To ensure a reliable detection of our *E. coli* strains, a chromogenic agar was used and colony morphologies were checked up on their belonging to both strains. Additionally, in all digestive tract samples taken at necropsy, an agar plate without antibiotics was used to quantify the total count of *E. coli* colonies (data not shown). The total count of *E. coli* was congruent with the sum of our quantified ESBL- and AmpC- strains in all trails. Furthermore, out of the cecal content, colonies from the selective agar plates for the growth of the ESBL- and AmpC- *E. coli* strains were randomly selected and checked for their phenotypic antimicrobial resistances using VITEK 2. The profile of the examined colonies corresponded to those of our inoculation strains. The possibility of a HGT to other *E. coli* strains resident in the digestive tract cannot be entirely ruled out, but a colonization based on *in vivo* HGT seems to be unlikely, as in all colonization dosage trials equivalence of the cecum samples was shown. An equal HGT to other *E. coli* strains harboring the same resistance profile in all trials is highly unlikely, taking into consideration the chromosomally encoded resistances of our ESBL- *E. coli* strain.

Our results confirm the hypothesis that the uptake of only a few bacteria is sufficient to colonize broiler chickens with ESBL- and AmpC- producing Enterobacteriaceae. In fact, an inoculation of 10^1 cfu *E. coli* leads to a colonization of broiler chickens. Due to a continuous cleaning and disinfection regime, the bacterial load at every level of the broiler production chain is supposed to be minimized. Nevertheless, there is still remaining bacteria detectable after cleaning and disinfection (Luyckx et al., 2015). Furthermore, ESBL- and AmpC- producing bacteria are found frequently (Dierikx C. M. et al., 2013; Daehre et al., 2018a). Even if there is no detailed information available about the detected quantities of these resistant bacteria after cleaning and disinfection, it is practically impossible to achieve procedures that will result in such a very low bacterial load, that a colonization of broiler chickens from the subsequent flock does not take place. In addition, the process of colonization with resistant bacteria can be greatly strengthened by applying selection pressure through antimicrobial treatment of the birds during the fattening period. The importance of cleaning and disinfection measures in broiler fattening stables is underlined by Schulz et al. (2016). The study by Schulz et al. points out, that antimicrobial resistant *E. coli* is able to survive for decades in dust samples with concentrations up to 10^4 cfu per gram dust.

In our study, an instillation into the crop with 10^1 cfu *E. coli* led to an equivalent colonization of broiler chickens as the higher tested doses. These data were obtained by inoculating every single animal of the group. To obtain data closer to real farming conditions, we conducted a seeder-bird model by inoculating only one fifth of the animals (seeder birds) in a group with our defined minimal colonization dosage of 10^2 cfu *E. coli*. In literature, different methods of administration of *E. coli* to poultry are described. Besides an oral inoculation into the beak (Wang et al., 2017), a spray application over the nose and eyes of the birds and a treatment of the feeders has been implemented (Huff et al., 2011). A safer method to ensure the uptake of the bacterial suspension is an instillation into the crop. Thereby, to mimic the route of natural bacterial infection in animal trials, the seeder-bird model is frequently used and described for different enteral bacteria, such as *E. coli*, *Salmonella enterica* and *Campylobacter* spec. (Ratert et al., 2015; Schneitz and Hakkinen, 2016; Ceccarelli et al., 2017). The non- inoculated, susceptible sentinel birds mirror the animals naturally infected by the oral uptake of resistant bacteria from their surrounding environment after they were shed by the seeder birds. In the course of this, a variety of different ratios is known and a ratio of 1:5 seeder- to sentinel birds is more often described for the inoculation with Enterobacteriaceae (Methner et al., 2011; de Cort et al., 2015; Kilroy et al., 2015). Using this relation led to a complete colonization of all birds within our group. For us, with regard to future studies, a colonization in combination with an approximation to real farming conditions was the most important. As there is no data available about the number of initially colonized broiler chickens with resistant bacteria through contaminated farm environment, other ratios were not tested. Lower ratios of inoculated to non-inoculated broiler chickens might lead to lower prevalences of the flocks but can still constitute a source for contamination in further steps of the broiler production chain. In this connection, an introduction of resistant bacteria and cross- contamination at slaughterhouse level was proven by von Tippelskirch et al. (2018). As another consequence, remaining bacteria in the fattening stable after

cleaning and disinfection is a source for the colonization of the subsequent broiler flock as described above.

After 35 days of the seeder-bird trial, the bacterial count observed in all necropsy samples taken is similar for all broilers, irrespective of being an artificially inoculated seeder bird or naturally colonized sentinel bird. This clearly shows the impact of the oral (re-) colonization with ESBL- and AmpC-producing Enterobacteriaceae during the ongoing trial. Even if the inoculation of only one fifth of the broilers initially resulted in a higher percentage of colonized seeder birds, observed in the cloacal swabs taken, the oral uptake of resistant bacteria from their surrounding environment seems to be of great importance for the colonization. In this way, all sentinel birds achieved a comparable colonization as the seeder birds over a period of 35 days. Interestingly, the bacterial count in all necropsy samples from the seeder-bird group is significantly lower compared to the dosage trial using 10^2 cfu *E. coli*. A first possible explanation is the different number of ESBL- and AmpC- inoculated broilers per trial. The oral colonization of only one fifth of the broilers in the seeder-bird model initially leads to less shedding of resistant bacteria into the animal housing. Thus, the contamination of the environment with the bacterial strains and in consequence, the oral (re-) colonization of the birds in the seeder-bird group is lower compared to the dosage trial using 10^2 cfu *E. coli*. The lower bacterial contamination of the environment might also explain the lower initial prevalence observed in the colonization dosage trials using 10^1 and 10^2 cfu ESBL- and AmpC- *E. coli* compared to 10^3 and 10^4 cfu. However, a reasonable explanation for the different colonization levels in the seeder-bird trial compared to the trial using 10^2 cfu *E. coli* are the durations of the trials. With regard to strict animal welfare norms, a duration of 2 weeks was found to be adequate to demonstrate a colonization of our chickens with the *E. coli* strains in the colonization dosage trials. After defining the minimal colonization dosage of 10^2 cfu *E. coli*, a duration of 5 weeks was chosen for the subsequent seeder-bird trial, to mimic real farming conditions. This discrepancy between the experimental periods leads to a change in microbial flora of the broilers gastrointestinal tract. Up to an age of 49 days significant differences in the composition of the cecal content for Ross-hybrids were shown by Lu et al. (2003). This is in agreement with other studies postulating a change of the predominant bacteria in broilers at older age (Amit-Romach et al., 2004; Crhanova et al., 2011). These changes in the microbial flora might lead to a competition of ESBL- and AmpC-producers with other bacteria, resulting in a decrease of ESBL- and AmpC- colonization.

As the reservoir for ESBL- and AmpC- producing *E. coli*, the cecum has a predominant role to evaluate the colonization of the broilers in the final necropsy. In contrast to the other samples of the colonization dosage trials taken during necropsy, the values gained from the content of the cecum are comparable between the trials. Whereas a fermentation of the bacteria takes places in both ceca, the content of crop and jejunum do simply reflect the recent uptake of the resistant bacteria out of the surrounding. This is strongly influenced by the feeding and drinking behavior of the broilers immediately before the necropsy. Because of the contamination with feces, an increased pecking in the litter leads to a large intake of bacteria compared to a pecking out of the feeding troughs or drinking and provides a reasonable explanation for the different bacterial counts observed in crop and jejunum samples.

Without applying any antimicrobial selection pressure to the birds, a colonization was achieved in our trials using a low dose of ESBL- and AmpC- producing *E. coli*. The usage of antibiotics in animal farming is discussed controversially, with studies indicating an impact of antibiotic treatment on co-selection and occurrence of resistant bacteria (Costa et al., 2009; Persoons et al., 2011; Dierikx C. M. et al., 2013). In recent years, an increasing number of studies are concluding that the occurrence of ESBL- and AmpC- producing bacteria in fattening chickens is not related to antibiotic treatment (Hiroi et al., 2012; Huijbers et al., 2016; Projahn et al., 2018). Our study reinforces these findings, showing a colonization even of the susceptible sentinel birds using a low dose of ESBL- and AmpC- producing *E. coli* without applying any antimicrobial selection pressure. In support of this, numerous studies are reporting a carriage of ESBL- and AmpC-resistant bacteria in wild bird species that have never been faced with any antibiotics before (Guenther et al., 2012; de Cort et al., 2015; Alcalá et al., 2016). Both of our inoculation strains were derived from a previous research project on ESBL- and AmpC-*E. coli* from chicken farming in Germany, thus presenting actual strains occurring in broiler chicken production (Falgenhauer et al., 2016; Hering et al., 2016). Besides that, ST10 and ST410 were chosen due to their ubiquitous nature as ST10 presents an ancient sequence type often present in livestock farming. ST410 was chosen due to its recent emergence as high risk clone in Germany and worldwide (Falgenhauer et al., 2016; Schaufler et al., 2016, 2019).

As a limitation, we investigated only one animal group per colonization dosage and we have no information on the variation between groups with the same colonization dosage. Although the inoculation of four different colonization dosages verified the general method, a repetition of the equal dosage or inoculation ratio of the broiler chickens might result in higher or lower initial colonization rates. Furthermore, an intra-herd correlation between the broiler chickens of one group is present and the results of the birds within one group are dependent upon each other. The effects shown can be group-specific and through a possible underestimation of variance in our analysis the results cannot be generalized completely.

Nonetheless, we showed a colonization of broiler chickens with a low colonization dosage of 10^2 cfu *E. coli* and a small number of orally inoculated broilers in the seeder-bird model. This might be a feasible explanation for the global spread of ESBL- and AmpC- producing enterobacteria in conventional broiler farms. With regard to an assumed transmission of resistant bacteria to humans through close contact to colonized broilers or the consumption of contaminated retail meat, further steps for the reduction of ESBL- and AmpC-producing bacteria have to be considered. Consequently, based on this practical orientated colonization model, different hygiene- and management measures as well as gut microbiota influencing measures are currently being investigated as potential intervention strategies to reduce the colonization of broilers.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

SG, UR, AB, and AF designed the study. CR, SG, and AB performed the samplings and laboratory work. RM and CR performed the statistical analysis. CR evaluated the final data and wrote the manuscript. All authors have read and approved the final draft of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02124/full#supplementary-material

Figure S1 | Colonization of broiler chickens during the four colonization dosage trials 10^1–10^4 cfu/*E. coli* **(A–D)** and the seeder birds and sentinel birds of the seeder-bird model trial **(E,F)** using a semiquantitative measurement with categories from 0 to 7: 0 = no growth; 1 ≤ 10 cfu *E. coli*; 2 = between 1 and 3; 3 ≤ 100 cfu *E. coli*; 4 = between 3 and 5; 5 > 100 cfu *E. coli*; 6 = between 5 and 7; 7 = agar plate overgrown; d p.i. = days post inoculation.

Table S1 | Mean values and confidence intervals of digestive tract samples from colonization dosage groups (10^1–10^4 cfu/*E. coli*) and seeder-bird model attained at necropsy.

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3.2 Publication II

Robé, C.; Daehre, K.; Merle, R.; Friese, A.; Guenther, S.; Roesler, U. (2021):

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RESEARCH ARTICLE

Impact of different management measures on the colonization of broiler chickens with ESBL- and pAmpC- producing *Escherichia coli* in an experimental seeder-bird model

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Abstract

The colonization of broilers with extended-spectrum β-lactamase- (ESBL-) and plasmid-mediated AmpC β-lactamase- (pAmpC-) producing Enterobacteriaceae has been extensively studied. However, only limited data on intervention strategies to reduce the colonization throughout the fattening period are available. To investigate practically relevant management measures for their potential to reduce colonization, a recently published seeder-bird colonization model was used. Groups of 90 broilers (breed Ross 308) were housed in pens under conventional conditions (stocking of 39 kg/m^2, no enrichment, water and feed *ad libitum*). Tested measures were investigated in separate trials and included (I) an increased amount of litter in the pen, (II) the reduction of stocking density to 25 kg/m^2, and (III) the use of an alternative broiler breed (Rowan x Ranger). One-fifth of ESBL- and pAmpC- negative broilers (n = 18) per group were orally co-inoculated with two *E. coli* strains on the third day of the trial (seeder). One CTX-M-15-positive *E. coli* strain (ST410) and one CMY-2 and mcr-1-positive *E. coli* strain (ST10) were simultaneously administered in a dosage of 10^2 cfu. Colonization of all seeders and 28 non-inoculated broilers (sentinel) was assessed via cloacal swabs during the trials and a final necropsy at a target weight of two kilograms (= d 36 (control, I-II), d 47 (III)). None of the applied intervention measures reduced the colonization of the broilers with both the ESBL- and the pAmpC- producing *E. coli* strains. A strain-dependent reduction of colonization for the ESBL- producing *E. coli* strain of ST410 by 2 log units was apparent by the reduction of stocking density to 25 kg/m^2. Consequently, the tested management measures had a negligible effect on the ESBL- and pAmpC- colonization of broilers. Therefore, intervention strategies should focus on the prevention of ESBL- and pAmpC- colonization, rather than an attempt to reduce an already existing colonization.

and analysis, decision to publish, or preparation of the manuscript.

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Introduction

Antibiotic resistance is an increasing threat to global public health and concerns human- and veterinary medicine [1]. Special attention is required for extended-spectrum β-lactamase (ESBL-) and plasmid-mediated AmpC β-lactamase- (pAmpC-) producing Enterobacteriaceae as they can limit therapeutic options with critically important antimicrobials, such as cephalosporins of 3rd, 4th and 5th generation as well as monobactams [2].

Broiler chickens are considered as a reservoir for ESBL- and pAmpC- producing bacteria, it with frequently confirmed high prevalence [3, 4]. These antibiotic-resistant bacteria are detected at every level of the broiler production chain. Comprehensive data on ESBL- and pAmpC- detection are available for parent- and grandparent stocks [5], the hatchery level [6], the fattening period [7, 8], the slaughterhouse level [9], and the commercial product [10]. Various transmission routes are described for ESBL- and pAmpC- producing bacteria. Dame-Korevaar et al. [11] reviewed transmission routes in the broiler production chain and categorized them as 1) vertical transmission between generations, 2) transmission at hatcheries, 3) horizontal transmission on the farm, 4) horizontal transmission between farms, and via the environment of farms. A transmission can lead to colonization of young broiler chickens through the oral uptake of the resistant bacteria. The spread of ESBL- and pAmpC- producing *Escherichia coli (E. coli)* in the broiler production chain can partly be attributed to the very low colonization dosage. Recent findings have shown that 10^1 to 10^2 colony forming units (cfu) of orally administered ESBL- and pAmpC- producing *E. coli* can already lead to colonization of broiler chickens [12, 13]. Cleaning and disinfection procedures can lower the risk of transmission of cephalosporin-resistant *E. coli* from one flock to the subsequent flock in broiler fattening farms [14]. Nevertheless, a reduction of the microbial load through intense cleaning and disinfection processes cannot eliminate ESBL- and pAmpC- producing bacteria and the recirculation of resistant bacteria from earlier production rounds could play a role in contaminating the consecutive flock [5].

Different measures to influence the microbial composition of the broiler chickens' guts and to affect the colonization of broiler chickens with ESBL- and pAmpC- producing Enterobacteriaceae are discussed in literature. One approach is the direct modulation of the broiler chickens' microbiota to influence the early colonization with ESBL- and pAmpC- producing bacteria. For example, probiotics such as commercial Competitive Exclusion cultures and phytobiotics showed promising results to reduce the ESBL- and pAmpC- colonization of broiler chickens [15–18]. Another approach is to modify the conventional housing conditions during the fattening process. A modification of the conventional conditions by an environmental enrichment is applied frequently in broiler production to increase animal welfare. However, information on the practical application and the economics of the production systems is often lacking certainty [19]. The conventional conditions for broiler production in Germany most commonly include the broiler breed Ross 308, no environmental enrichment during the fattening process, a stocking density of 39 kilograms per square meter (kg/m^2), and water and feed *ad libitum*. Due to the variety of study designs, no clear statement on the impact of practically relevant management measures on the colonization of broiler chickens with ESBL- and pAmpC- producing Enterobacteriaceae is possible. We aimed to investigate different management measures which are taken during the fattening process on their potential to reduce the spread of ESBL- and pAmpC- producing bacteria. We investigated the replacement of the broiler breed Ross 308 with the broiler breed Rowan x Ranger, increase of the common litter quantity of one kg/m^2 to three kg/m^2, and reduction of the common stocking density of 39 kg/m^2 to 25 kg/m^2. In our study, the investigated management measures had a negligible impact on the ESBL- and pAmpC- colonization of the broiler chickens.

Materials and methods

Ethics statement

This study was carried out following the National Animal Protection Guidelines. The protocol was approved by the German Animal Ethics Committee for the protection of animals of the Regional Office for Health and Social Affairs Berlin ("Landesamt für Gesundheit und Soziales", LAGeSo, permission number G 0193/16). All applicable national and institutional guidelines of the Freie Universität Berlin for the care and use of animals were followed. Experimental treatments of animals were classified as to lead to no worse than minor discomfort in the animals due to low pain of very short duration and were approved by LAGeSo.

Housing conditions

All trials were set up successively in the experimental facilities of the Center for Infection Medicine of the Department for Veterinary Medicine, Freie Universität Berlin, using controlled rooms with respective ventilation and HEPA filtration of the exhaust air. Before each trial, related room was disinfected using hydrogen peroxide fumigation, and the absence of ESBL- and pAmpC- producing bacteria was confirmed. Before entering the room, daily clothes and shoes were changed with trial specific clothing in an attached separate anteroom.

In accordance with our recently established seeder-bird colonization model [12], a control group, having the broiler breed Ross 308 (mixed gender), was kept in a pen until the broilers reached a target weight of two kilograms (= 36 days (d) of trial). Ninety broiler chickens were housed under conventional conditions, with one-fifth of them (n = 18) being orally co-inoculated with 10^2 cfu of one ESBL- and one pAmpC- producing *E. coli* on the third day of the trial (Fig 1). Said conventional conditions included a stocking density of 39 kg/m^2 corresponding to 4.6 m^2 for 90 broiler chickens, fresh litter once at the beginning of the trial (one kg/m^2), no environmental enrichment, and conventional feed and water *ad libitum*. The light regime was set to 11 hours of light and 13 hours of dark with a dimming period of 30 minutes. The floor temperature was decreased from 28˚C (d 1–6), over 26˚C (d 7–13) and 24˚C (d 14–20) to 22˚C (d 21 to the end of the trial) with a relative humidity of 55%. The broiler chickens were fed a commercial starter feed (d 1–7), grower feed, and a finisher feed (five days before finalization of each trial) from a commercial broiler producer. Except for the finisher feed, coccidiostats were included in the feed (decoquinate and narasin/nicarbazin). Neither in the control nor the intervention groups antimicrobial agents were administered.

The intervention groups (I-III, Fig 1) were kept under the same conditions as the control group, altering only one management measure in each group. To evaluate their impact on the colonization of the broiler chickens with ESBL- and pAmpC- producing *E. coli*,

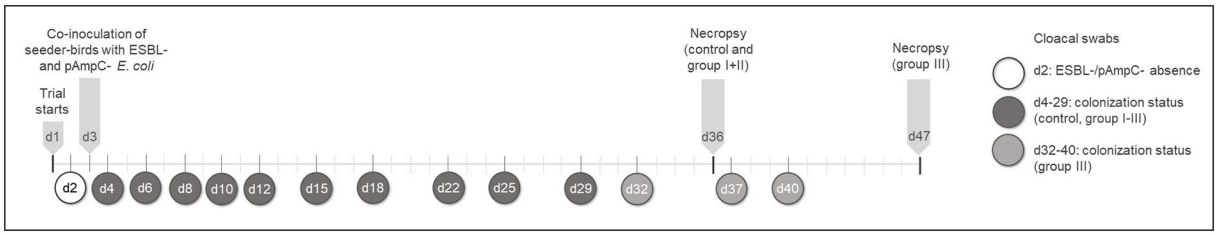

Fig 1. Study design of the experimental groups (control group, increased litter (I), reduced stocking density (II), and alternative breed (III)). d = day of trial.

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1. the litter quantity in kg/m^2 was tripled from one kg/m^2 to three kg/m^2 over the course of the trial. We procured the litter which consisted of pelletized straw granules from a commercial broiler producer. The broiler breed (Ross 308, mixed gender), the number of broiler chickens (n = 90), the stocking density of 39 kg/m^2, and the trial period of 36 days remained unchanged.
2. the common stocking density of 39 kg/m^2 in Germany [20] was reduced to 25 kg/m^2, as described in the Commission Regulation (EC) No. 543/2008 [21]. For this, the effective area of the experimental setup was enlarged from 4.6 m^2 to 7.2 m^2, whilst the broiler breed (Ross 308, mixed gender), the number of broiler chickens (n = 90), the litter quantity of one kg/m^2 and the trial period of 36 days remained unchanged.
3. the broiler breed Rowan x Ranger (mixed gender) was included. In contrast to Ross 308, this breed is included in 'slow-growing' broiler concepts with an extended fattening period. The duration of the trial was set to 47 days so that the broilers reach the target weight of two kg. The number of broiler chickens per trial (n = 90), the stocking density of 39 kg/m^2, and the litter quantity of one kg/m^2 remained unchanged.

For each trial, we procured eggs from a commercial hatchery. The first disinfection using formaldehyde gas was already performed in the hatchery. After the transportation to the experimental facilities, the second liquid disinfection of the eggs was performed using WESSO-CLEAN® K 50 Gold Line containing 2.37% hydrogen peroxide and 0.015% peracetic acid (Wesso AG, Hersbruck, Germany). Following the second disinfection, the eggs for each experimental group were hatched in-house for 21 days. The eggs were incubated from day 1 to day 18 at 37.8˚C and 60% relative humidity in a setter. From day 18 to day 21, the eggs were incubated at 37.2˚C and 80% relative humidity for hatching.

Before the trial, the absence of ESBL- and pAmpC- producing bacteria was confirmed in the eggshells and in the one-day-old broiler chickens as well as in the experimental room and on the equipment as described in our seeder-bird colonization model [12].

ESBL- and pAmpC- colonization strains

We used two avian *E. coli* strains to co-colonize our broiler chickens. One ESBL- producing *E. coli* strain (CTX-M-15; multilocus sequence type (ST) 410; phylogenetic group B1; internal number 10716) published as R56 by Falgenhauer et al. [22] and one pAmpC- producing *E. coli* strain (CMY-2, mcr-1; ST10; phylogenetic group A; internal number 10717). Both strains were isolated in a previous research project [23] and were recently used to establish our seeder-bird colonization model [12]. In this colonization model, both commensal *E. coli* strains colonized the digestive tracts of broilers with high bacterial counts and without causing any clinical signs.

The bacterial suspension for the inoculation of the broiler chickens with the ESBL- and pAmpC- *E. coli* strains was prepared, according to Robé et al. [12].

Oral ESBL- and pAmpC- inoculation of the broilers

On the third day of trial, one-fifth of the broilers (n = 18) were orally co-inoculated with 10^2 cfu *E. coli* of both bacterial strains in equal parts (Fig 1). These 18 seeder-birds were randomly selected prior to the trial and for the inoculation, 200 μl of the bacterial suspension were administered into the crop via a crop needle. After inoculation, the broiler chickens were immediately put back into the pen to the 72 non-inoculated broiler chickens (sentinel-birds). Regardless of the subsequent colonization status of the seeder-birds after inoculation, all broilers were kept together in one experimental setup to mimic real farming conditions.

Samplings and analyses

The sample processing was identical to the one which was described in our seeder-bird colonization model [12]. Chromogenic orientation agar (CHROMagar Orientation, Mast Diagnostica, Reinfeld, Germany) was used for a reliable identification of the *E. coli* colonies. To confirm the ESBL-/ pAmpC- absence in the experimental room and the newly hatched broiler chickens, the agar was supplemented with two μg/ml cefotaxime (AppliChem, Darmstadt, Germany). In order to process all other samples, a set of four chromogenic agar plates which has proven suitable for our study was used. The total count of *E. coli* colonies was determined using an agar plate without selective media (positive control). For the detection of the ESBL- *E. coli* 10716, one plate was supplemented with two μg/ml cefotaxime and four μg/ml enrofloxacin (Sigma- Aldrich, Steinheim, Germany). For the detection of the pAmpC- *E. coli* 10717, one agar plate was supplemented with two μg/ml cefotaxime and seven μg/ml colistin (Carl Roth, Karlsruhe, Germany). The fourth agar plate contained all three antibiotics in the given concentrations (negative control). All samples were incubated for 24 h at 37˚C. Every untypical *E. coli* colony morphology was further analyzed using MALDI- TOF (MALDI Microflex LT® and Biotyper database®; Bruker Daltonics, Bremen, Germany).

Colonization status during the trial. The colonization of all 18 seeder-birds and 28 selected sentinel-birds with both *E. coli* strains was monitored over the entire period of each trial via cloacal swabs. The investigated birds were randomly selected before the trial and repeatedly sampled during the trial, first of which was done 24 h *post-inoculation* (*p.i.*). Groups using the breed Ross 308 (control group, increased amount of litter in the pen (I), and reduced stocking density (II)) were sampled for ten times during the 36 days of trial. The group using the breed Rowan x Ranger (III) was sampled 13 times during the 47 days of trial (sampling 1 = 24 h *p.i.*, 2 = 72 h *p.i.*, 3–5 = second week of trial, 6–7 = third week of trial, 8–9 = fourth week of trial, 10–11 = fifth week of trial, 12–13 = sixth week of trial (Fig 1)). To evaluate the colonization status of the broiler chickens, the swabs were immediately transferred into reaction tubes containing 500 μl PBS, thoroughly vortexed, and 50 μl were streaked out onto the chromogenic agar set as described above.

Colonization status at necropsy. A necropsy was performed at a target weight of two kilograms for all sampled seeder- and sentinel-birds (= d 36 (control, I-II) and d 47 (III); Fig 1). For sedation, ketamine hydrochloride (43 mg/kg body weight, Ketamin 10%, Bremer Pharma GmbH, Warburg, Germany), xylazine hydrochloride (1.75 mg/kg body weight, Xylavet 20mg/ml, cp-pharma, Burgdorf, Germany), and midazolam hydrochloride (0.85 mg/kg body weight, Midazolam 5mg/ml, Braun, Melsungen, Germany) were administered into the pectoral muscle. Animals were sacrificed by an intracardiac injection of tetracaine hydrochloride, mebezoniom iodide, and embutramid (1 ml/kg, T61, Intervet Deutschland GmbH, Germany). Digestive tract samples of crop, jejunum, cecum, and colon were quantitatively analyzed, and organ samples of liver and spleen were qualitatively analyzed for the occurrence of both bacterial strains as described in Robé et al. [12]. In brief, the digestive tract content was weighed in a reaction tube into which PBS was added at the ratio of 1:2. A dilution series in PBS was performed, and appropriate dilutions were plated on the plate set as described above. For quantification, a minimum of two dilution levels were counted. In addition, to exclude the possibility of systemic spread, organ samples of liver and spleen were investigated qualitatively as described before [12].

Statistical analysis

Statistical analysis was carried out using SPSS Statistics 25 (IBM, New York, USA). Confidence intervals of proportions were calculated using the Clopper-Pearson method. For all analyses, the probability level to denote significance was set to 0.05.

The effects of the tested management measures on the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* during the trials were analyzed via a logistic mixed regression model. The variables 'group', 'sampling time', and 'animal' were included in the analysis for both *E. coli* strains. To account for the repeated measurement of the same individual, 'ID number' and 'sampling time' were included in the analysis as random effects. Using backward selection, the best fitting model from a full model, including 2- and 3-way interactions, was obtained. The model with the lowest AIC value and lowest number of included effects was chosen, with an AIC change of two or less considered as equal. It resulted in the model with all main factors and the interaction term 'animal and sampling time'. Residuals were inspected for normality and homoscedasticity for each sampling time point.

Mann-Whitney-U-Tests were performed to compare the level of colonization between the seeder- and sentinel-birds at necropsy. Kruskal-Wallis-Tests were performed to compare the final colonization of the digestive tracts of the tested groups to the control group at necropsy. Due to multiple comparisons in the Kruskal-Wallis-Test, the level of significance was set to 0.0167 (Bonferroni correction).

Results

Cloacal swabs

Colonization of broiler chickens with the ESBL- and pAmpC- *E. coli* strains was monitored via cloacal swabs throughout each trial, starting 24 h *p.i.* A strain-dependent course of colonization was evident in the investigated groups (Fig 2, S1 Fig and S1 Table). 'Group', 'animal', 'sampling time' as well as the interaction between 'animal and sampling time' were statistically significant (Table 1). Compared to the control group for the ESBL- *E. coli* strain, a reduced hazard of colonization was solely apparent under the reduction of stocking density to 25 kg/m^2, with a hazard ratio (HR) of 0.18 (95% CI: 0.12–0.28). An increased amount of litter in the pen led to a higher risk of colonization for both *E. coli* strains (ESBL- *E. coli* HR 2.38 (95% CI: 1.49–3.82); pAmpC- *E. coli* HR 4.58 (95% CI: 2.09–10.06)), while the use of an alternative breed showed no effect on the hazard of colonization of broiler chickens with both *E. coli*

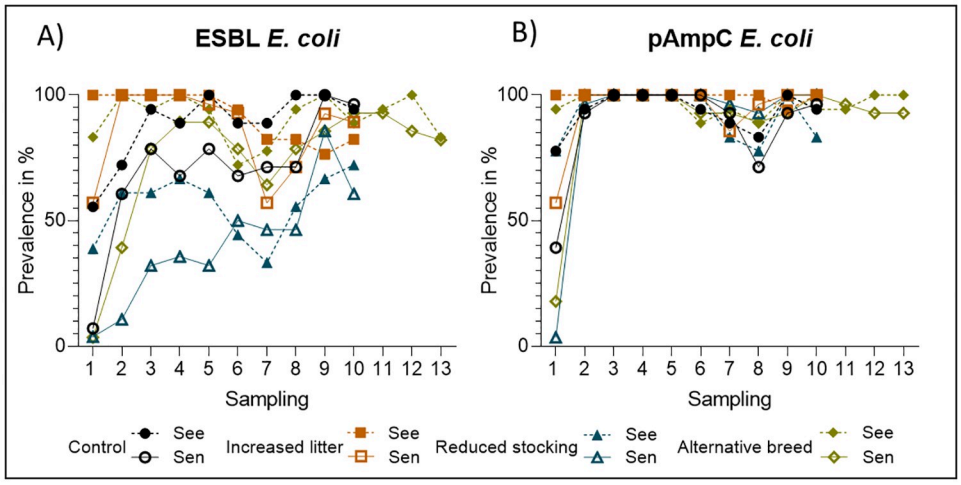

Fig 2. Prevalence of broiler chickens with (A) ESBL- and (B) pAmpC- producing *E. coli* throughout the different trials (control group, increased litter, reduced stocking density (reduced stocking) and alternative breed).
See = seeder-birds, Sen = sentinel-birds; sampling 1 = 24 h post-inoculation, 2 = 72 h post-inoculation, 3–5 = 2nd week of trial, 6–7 = 3rd week of trial, 8–9 = 4th week of trial, 10–11 = 5th week of trial, 12–13 = 6th week of trial.

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Table 1. Hazard ratios (HR) of broiler chickens' colonization with ESBL- and pAmpC- producing *E. coli* in the four investigated groups (control group, increased litter, reduced stocking density (reduced stocking) and alternative breed) adjusted for interaction 'animal and sampling time'.

Strain	Factor	*p*-value		HR (95% CI)
ESBL- *E. coli*	Group	< 0.001	Control (reference)	1
			Increased litter (3 kg/m^2)	2.38 (1.49–3.82)
			Reduced Stocking (25 kg/m^2)	0.18 (0.12–0.28)
			Alternative breed (Rowan x Ranger)	1.01 (0.66–1.56)
	Animal	< 0.001	Seeder (reference)	1
			Sentinel	0.11 (0.05–0.23)
	Sampling time	< 0.001	1 (reference)	1
			2	3.90 (1.68–9.10)
			3	6.46 (2.57–16.25)
			4	7.54 (2.91–19.54)
			5	6.49 (2.57–16.36)
			6	2.36 (1.06–5.26)
			7	1.82 (0.83–3.97)
			8	4.35 (1.83–10.34)
			9	5.58 (2.26–13.75)
			10	4.91 (2.03–11.87)
pAmpC- *E. coli*	Group	< 0.001	Control (reference)	1
			Increased litter (3 kg/m^2)	4.58 (2.09–10.06)
			Reduced Stocking (25 kg/m^2)	0.94 (0.50–1.78)
			Alternative breed (Rowan x Ranger)	1.25 (0.65–2.40)
	Animal	> 0.999	Seeder (reference)	not estimated
			Sentinel	
	Sampling time	< 0.001	1 (reference)	1
			2	10.56 (1.29–86.71)
			3	not estimated
			4	not estimated
			5	not estimated
			6	3.41 (0.86–13.48)
			7	1.61 (0.53–4.93)
			8	1.01 (0.36–2.80)
			9	10.83 (1.31–89.49)
			10	2.00 (0.62–6.49)

95% CI = 95% confidence interval; sampling time: 1 = 24 h *post inoculation*, 2 = 72 h *post inoculation*, 3–5 = 2nd week of trial, 6–7 = 3rd week of trial, 8–9 = 4th week of trial, 10 = 5th week of trial.

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strains (ESBL- *E. coli* HR 1.01 (95% CI: 0.66–1.56); pAmpC- *E. coli* HR 1.25 (95% CI: 0.65–2.40)).

For the ESBL- *E. coli*, the hazard rate of colonization was lower for sentinel-birds compared to seeder-birds with a hazard ratio of 0.11 (95% CI: 0.05–0.23). A reduced hazard for sentinel-birds could not be shown for the pAmpC- *E. coli*, while the interaction between 'animal and sampling time' was highly significant (Table 1).

Except the seventh sampling time point, the hazard rates of colonization for the ESBL- *E. coli* strain were higher at all sampling times after 24 h. Regarding the pAmpC- *E. coli* strain, the hazard rates of colonization were higher at all sampling times after 24 h. However,

statistical significance could only be shown for the second and ninth sampling time points due to strong interactions with the animal (Table 1).

Necropsy

At necropsy, respective contents of crop, jejunum, cecum, and colon were analyzed. As there was no significant difference in the level of colonization between the seeder- and sentinel-birds in any of the experimental groups (Mann-Whitney-U-Test, $p > 0.05$, S2 Table), each digestive tract sample per group was analyzed jointly for all investigated birds. None of the applied measures significantly reduced the bacterial counts of both the ESBL- and the pAmpC- *E. coli* strains in any of the digestive tract samples compared to the control group (Kruskal-Wallis-Test, Fig 3 and S2 Fig). We focused on the cecum samples for the following analyses as the results of all digestive tract samples correlated (Fig 3 and S2 Fig). By the reduction of the stocking density to 25 kg/m^2, a strain-dependent significant reduction of cecum colonization of the ESBL- *E. coli* (Kruskal-Wallis-Test, $p < 0.001$, Fig 3) with a considerably lower prevalence of 63% (95% CI: 48–77%) compared to the control group (93%; 95% CI: 82–99%) was observed (S1 Table). On the other hand, the bacterial counts for the pAmpC- *E. coli* in the group with the reduced stocking density were significantly higher (Kruskal-Wallis-Test, $p < 0.001$, Fig 3) compared to the control group, with almost all broiler chickens pAmpC-colonized (96%; 95% CI: 85–99%, S1 Table). A significant increase in the cecum colonization with both bacterial strains (Kruskal-Wallis-Test, $p < 0.001$, Fig 3) was evident for the experimental

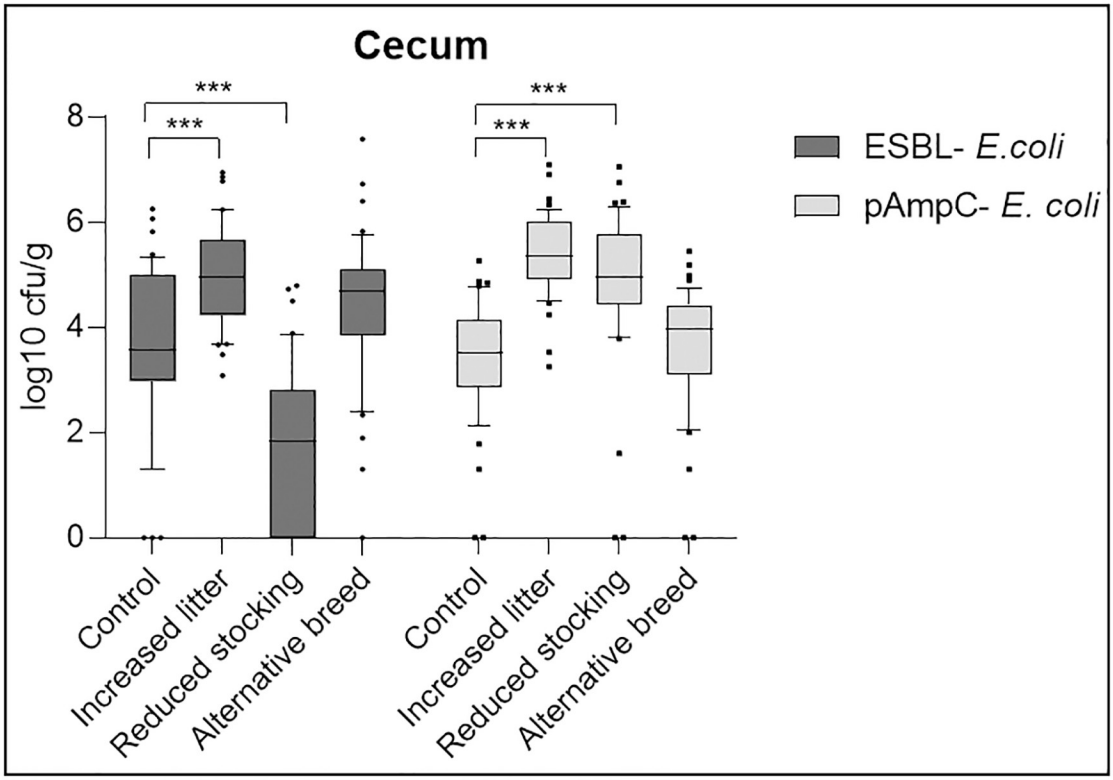

Fig 3. Cecum colonization of broiler chickens (log10 cfu/g) with ESBL- and pAmpC- producing *E. coli* in the four investigated groups (control group, increased litter, reduced stocking density (reduced stocking) and alternative breed) determined at necropsy. *** $p < 0.001$ (Kruskal-Wallis-Test).

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group with the measure of a tripled amount of litter in the pen. The alternative broiler breed Rowan x Ranger caused no effect on the colonization with both bacterial *E. coli* stains (Kruskal-Wallis-Test, $p > 0.05$, Fig 3). These results are correlating to the analyses of the cloacal swabs (except the increased pAmpC- *E. coli* colonization in the group with reduced stocking density).

In summary, none of the tested measures prevented the colonization of broiler chickens with both the ESBL- and the pAmpC- producing *E. coli* strains. There was a significant reduction of the prevalence and the bacterial counts of the ESBL- *E. coli* in the group with the reduced stocking density. Besides, we did not detect colonization of the liver or spleen with our bacterial strains in any of our experimental groups.

Discussion

In our experiments, none of the applied intervention measures reduced the broiler chickens' colonization with both the ESBL- and the pAmpC- producing *E. coli* strains. All measures were tested separately under controlled conditions in a setup close to the real farming conditions, according to Robé et al. [12]. To the best of our knowledge, only little information about trials having our tested management measures to reduce the broiler chickens' colonization with ESBL- and pAmpC- producing bacteria is available.

A study by Guardia et al. [24] demonstrated an effect of high stocking densities on the composition of commensal bacteria in the digestive tracts of young broilers. A decrease in the overall bacteria and *E. coli* in the chickens' ceca at the age of three weeks followed by a reduced effect at the age of six weeks, was shown. Also, reduced stocking densities have been shown to affect the colonization of different bacteria, including pathogens such as *Clostridium perfringens*, *Campylobacter* spp. and *Salmonella* spp. [25–27]. A reasonable explanation is that the reduced bacterial contamination per square meter of the litter caused the effect on colonization [24]. As the intestinal microbiota of broiler chickens is affected by the composition, type, and quality of the litter [28, 29], an improved litter quality through less bacterial contamination could also lead to a reduced load of ESBL- and pAmpC- producing bacteria, particularly at the beginning of the fattening period. However, our results did not prove a general positive effect of reduced stocking density on the ESBL- and pAmpC- colonization of broiler chickens. On the one hand, we showed a significant reduction of cecum colonization of the ESBL- *E. coli*. On the other hand, a significant increase of the pAmpC- *E. coli* colonization of the broiler chickens was evident. Even though the co-colonization of different ESBL- and pAmpC- producing bacteria represents the real scenario of colonization [17], there is a lack of broiler studies using this approach. Based on our results, we hypothesize that different intervention measures have strain-dependent effects on the ESBL- and pAmpC- colonization of broiler chickens. A study by Nuotio et al. [15] described a variation in the effect of a commercial Competitive Exclusion culture on the broiler colonization with three separately tested ESBL- and pAmpC- *E. coli* strains. Thus, a combination of different approaches might be needed to reduce the spread of these resistant bacteria in the broiler production chain [17, 30].

A similar approach of less contact to contaminated feces was taken for the experimental group with increased amount of litter in the pen. We hypothesized that the fecal droppings which harbor the resistant bacterial strains mix in the bedding material, for example, due to the movement of the chickens. Conversely, the cecum colonization of both the ESBL- and the pAmpC- *E. coli* strains was significantly higher at necropsy compared to the control group. We assume that an increased amount of litter leads to a more pronounced explorative behavior of the broiler chickens with a higher oral intake of litter and results in an intensified (re-)

colonization with the resistant bacteria [12]. The described quantities of bedding material used for broiler chickens range from 1 kg/m^2 [31], which reflects the commercial standard in Germany and was applied in our control group, up to 6 kg/m^2 [24] in some studies. However, because of practical reasons, a tripled amount of litter (3 kg/m^2) was used in our related experimental group. A study by Persoons et al. [32] showed an impact of different litter materials on the occurrence of ceftiofur-resistant *E. coli* in broilers, with a higher risk for straw compared to wood curls. An effect of the bedding material on the ESBL- and pAmpC- colonization cannot be ruled out in our study, as all trials were conducted using pelletized straw granule. The fine litter structure of straw granule could cause a higher oral intake of the bedding material compared to other materials and a higher oral intake could have led to a higher (re-) colonization of the broiler chickens in our trial. As we want to mimic real farming conditions, we used pelletized straw granule which is most frequently used as bedding material in broiler fattening farms in Germany. As fresh litter is known to carry enteral bacteria [33], the absence of ESBL- and pAmpC- producing bacteria was confirmed before the trials. In order to reflect real farming conditions to the best of our ability, we did not sterilize the litter. Hence, an interaction between the broiler chickens and the surrounding environment including the present bacterial spectrum was possible as usual in broiler production [34].

An impact of the broiler breed on the microbial composition and colonization of the gut with resistant or pathogen bacteria is discussed [32, 35–37]. A strong influence of the environment on the microbial composition with a minor impact of the used broiler breed was concluded by Richards et al. [38]. Contrastingly, Schokker et al. [39] drew a different conclusion with a major influence of broilers' genetic on microbial gut colonization. These diverse results of the two exemplary mentioned studies could also be caused by the different experimental designs. While Richards et al. [38] placed all breeds together in one pen, Schocker et al. [39] housed the investigated breeds simultaneously, but separately, under identical conditions for the chickens. Furthermore, Rychlik [40] demonstrated a highly variable microbiota development even in the ceca of chickens of the same line, which had the same background but were not kept simultaneously. A general statement on the impact of the breed on broiler chickens' gut colonization is not possible yet, due to the different housing conditions and a large variety of breeds used in various studies. In our trial, we placed the broiler breeds separately but under identical conditions. Investigating the broiler breeds Ross 308 and Rowan x Ranger, no significant difference in colonization with the ESBL- and the pAmpC- *E. coli* was apparent. Thus, our results support the findings of Richards et al. [38] even though we placed the two broiler breeds in separate pens.

Various scenarios can lead to ESBL- and pAmpC- colonization of broiler chickens, as reviewed by Dame-Korevaar et al. [11]. Transmission of antibiotic-resistant bacteria can take place before the arrival of the broiler chickens on the fattening farms [6] as well as after the placement of the chickens [7]. We investigated the impact of singularly applied management measures on the colonization of both the ESBL- and the pAmpC- inoculated seeder-birds and non-inoculated sentinel-birds. In our recently published broiler colonization model, a ratio of 1:5 ESBL- and pAmpC- inoculated seeder-birds to non-inoculated sentinel-birds on the third day of the trial led to the colonization of all seeder-birds 24 h *p.i.* and colonization of all sentinel-birds 72 h *p.i.* [12]. In contrast to our broiler colonization model, the resulting prevalence of the seeder- and sentinel-birds of our control group in the current study was lower, 24 h and 72 h *p.i.* The lower prevalence may be due to the differences in the group size, with 20 broiler chickens in the colonization model compared to 90 broiler chickens in the current study. A bigger group size results in a larger pen, which might have affected the group dynamics. Compared to the control group, the prevalence in the intervention groups might be due to an impact of the applied intervention measure itself or can partly be a result of the variability of

cloacal swab samples. As fermentation of the bacteria takes place in the ceca [41], and the cecal content is ejected only twice a day [40], the detection rate of our colonization strains might be affected by the chosen sampling type. Stanley et al. [42] pointed out the need of cloacal swab samples from the same birds for a repeated measurement in a trial but also showed quantitative differences between the analysis results of cloacal swab samples and cecal samples. However, statistical analyses of the swab samples revealed results comparable to the results for cecal samples. Additionally, there was a correlation among the analysis results of crop-, jejunum-, colon-, and the cecum samples per experimental group. As the contents of crop and jejunum simply reflect the recent uptake of the resistant bacteria from the surrounding, they are strongly influenced by the eating and drinking behavior of the broilers immediately before the necropsy [12]. Nevertheless, a reasonable explanation for the correlating results for the different digestive tract samples is that the uptake of fecal droppings caused an oral (re-)colonization of the broiler chickens. Consequently, cecum samples are needed in order to have the ESBL- and pAmpC- status of the broilers evaluated [40].

To summarize, the effects of the tested management measures, namely (I) an increased amount of litter in the pen, (II) the reduction of stocking density to 25 kg/m^2, and (III) the use of an alternative broiler breed, are negligible on the ESBL- and pAmpC- colonization of broiler chickens. Nevertheless, these results do not entirely exclude the possibility of management measures reducing the colonization of broiler chickens with ESBL- and pAmpC- producing bacteria. Particularly, microbiome-directed measures could represent a promising effect as they directly address the digestive tract. Probiotics such as commercial Competitive Exclusion cultures and phytobiotics already showed positive results of reducing the ESBL- and pAmpC- colonization of broiler chickens [15–18] and should be further investigated. In addition, a combination of promising measures is another approach which needs to be evaluated for a potential synergistic effect against the ESBL- and pAmpC- colonization of broiler chickens. Apart from the colonization of the broiler chickens, the external contamination with ESBL- and pAmpC- producing bacteria has to be considered as a source of contamination at slaughterhouse level. In terms of the consumer protection, investigations on the external contamination of broiler chickens are necessary.

Supporting information

S1 Fig. Prevalence of seeder-birds and sentinel-birds throughout all trials (control group, increased litter, reduced stocking density, and alternative breed) with (A) ESBL- and (B) pAmpC- producing *E. coli*. Samplings: 1 = 24 h post-inoculation, 2 = 72 h post-inoculation, 3–5 = 2nd week of trial, 6–7 = 3rd week of trial, 8–9 = 4th week of trial, 10 = 5th week of trial; Error bar = 95% Confidence interval.
(TIF)

S2 Fig. Colonization of broiler chickens (log10 cfu/g) with ESBL- and pAmpC- producing *E. coli* in (A) crop, (B) jejunum, and (C) colon in the four investigated groups (control group, increased litter, reduced stocking density (reduced stocking) and alternative breed) determined at necropsy. *** $p < 0.001$ (Kruskal-Wallis-Test).
(TIF)

S1 Table. Prevalence of ESBL- and pAmpC- producing *E. coli* of seeder-birds and sentinel-birds determined during the trial (cloacal swabs) and at necropsy of the four investigated groups (control group, increased litter, reduced stocking density (reduced stocking) and alternative breed). * Prevalence in percent (%); 10716 = ESBL- *E. coli*, 10717 = pAmpC- *E. coli*; See = seeder-birds, Sen = sentinel-birds; sampling: 1 = 24 h post-inoculation, 2 = 72 h

post-inoculation, 3–5 = 2nd week of trial, 6–7 = 3rd week of trial, 8–9 = 4th week of trial, 10–11 = 5th week of trial, 12–13 = 6th week of trial.
(DOCX)

S2 Table. Mean values and confidence intervals of ESBL- and pAmpC- producing *E. coli* of digestive tract samples (crop, jejunum, cecum, and colon) of the four investigated groups (control group, increased litter, reduced stocking density (reduced stocking) and alternative breed) determined at necropsy. 10716 = ESBL- *E. coli*, 10717 = pAmpC- *E. coli*; all data shown are log10 transformed (log10 cfu/g); ± CI = ± 95% Confidence interval.
(DOCX)

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4 Unpublished data

4.1 Intervention groups: Competitive Exclusion- culture, Competitive Exclusion-strain and drinking water acidification

Publication I covers the establishment of the broiler chickens colonization model using one ESBL- and one pAmpC- producing *E. coli* strain for the co-colonization of the animals. Following, publication II deals with the potential of three distinct intervention measures to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* throughout the fattening process using the established colonization model. The published intervention measures are (i) the reduction of the common stocking density from 39 kilograms per square meter (kg/m^2) to 25 kg/m^2, (ii) the use of an alternative, 'slow-growing' broiler breed (Rowan x Ranger) and (iii) the increase of the common litter quantity of one kg/m^2 to three kg/m^2. In addition to the published data on the groups mentioned above, three more intervention measures were investigated separately. In accordance with the groups i-iii, the additionally tested measures were investigated using the same study design, including the experimental setup, sample collection and –processing, and data analysis. The so far unpublished tested measures are:

1. The acidification of the drinking water with a commercially available product. The water supplement is based on organic acids and was administered at three different times throughout the trial as recommended by the manufacturer (day 1-7, day 15-19 and day 31-38).
2. The application of a Competitive Exclusion (CE)- strain, isolated by the Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig-University Giessen. 0.5 ml of the nonpathogenic Enterobacterales strain (IHIT36098) were orally administered once in a bacterial count of 10^8 cfu to each day-old broiler chicken right after hatch and before placing the animals into the experimental facilities.
3. The application of a complex, non-defined available CE- culture originated from specific pathogen-free chickens' cecum content. The culture was prepared following the manufacturer's specifications. Right after hatch and before placing the animals into the experimental facilities, 0.1 ml of the culture were orally administered to each day-old broiler chicken.

4.2 Results

4.2.1 Cloacal swabs

The broiler chickens' colonization was assessed via cloacal swabs throughout each trial, starting 24 h *p.i.* (*post inoculation*). The determined prevalence of the seeder- and sentinel-birds of all investigated groups is shown in Figure 3 and Supplement Table 1.

The two investigated ESBL- and pAmpC-producing *E. coli* strains showed correlating results with all investigated factors statistically significant, namely 'group', 'animal' and 'sampling time' and the interaction 'animal and sampling time' (Table 1). Compared to the control group, a reduced hazard ratio for both the ESBL- and pAmpC- *E. coli* strain was apparent after the application of the single CE-strain (ESBL- *E. coli* hazard ratio (HR) 0.02 (95% CI: 0.01-0.03); pAmpC- *E. coli* HR: 0.38 (95% CI: 0.20-0.70) and the application of the complex CE-culture (ESBL- *E. coli* hazard ratio (HR) 0.002 (95% CI: 0.001-0.006); pAmpC- *E. coli* HR 0.001 (95% CI: 0.001-0.003)). In contrast to the two CE-groups, the acidification of the drinking water led to a higher risk of colonization for both *E. coli* strains (ESBL- *E. coli* HR: 59,88 (95% CI: 14.20-252.57); pAmpC *E. coli* HR 8.02 (95% CI: 3.21-20.03)).

For both *E. coli* strains, the hazard of colonization was lower for sentinel-birds than seeder-birds with hazard ratios of 0.003 (95% CI: 0.000-0.017) for the ESBL- *E. coli* and 0.03 (95% CI: 0.01-0.09) for the pAmpC- *E. coli*.

At no sampling time, the hazard of colonization was lower than at the first sampling time (24 h *p.i.*). For the pAmpC- *E. coli*, the hazard rates of colonization were significantly higher starting from the fourth sampling time. For the ESBL- *E. coli*, significantly higher hazard rates were only apparent at sampling times three and nine due to strong interactions of the variables 'sampling time' and 'animal' in the logistic mixed regression model.

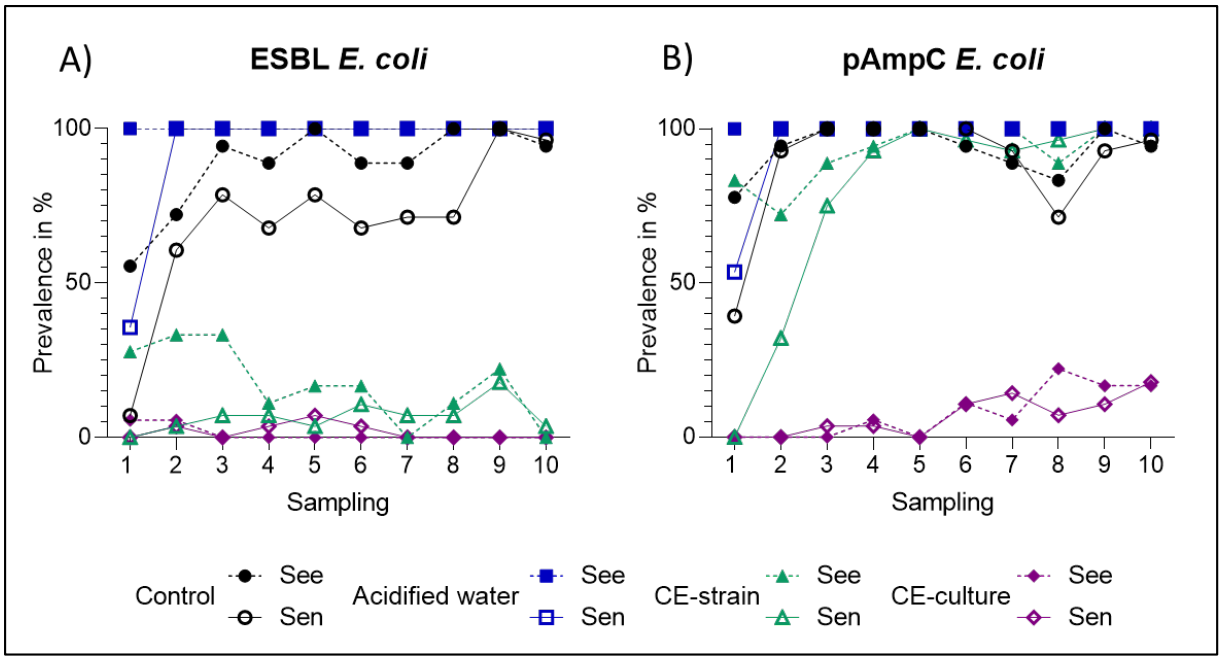

Figure 3. Prevalence of broiler chickens with (A) ESBL- and (B) pAmpC-producing *E. coli* throughout the different trials (Control group, Acidified drinking water (Acidified water), Competitive Exclusion- (CE-) strain, and CE-culture).

See = Seeder-birds, Sen = Sentinel-birds; sampling 1 = 24 h post inoculation, 2 = 72 h post inoculation, 3-5 = 2nd week of trial, 6-7 = 3rd week of trial, 8-9 = 4th week of trial, 10 = 5th week of trial.

Table 1. Hazard ratios of the broiler chickens' colonization with ESBL- and pAmpC-producing *E. coli* in the four investigated groups.

Factor		ESBL *E. coli*		pAmpC *E. coli*	
		p-value	HR (95% CI)	*p*-value	HR (95% CI)
Group	Control	≤ 0.001	1 (reference)	≤ 0.001	1 (reference)
	Acidified drinking water		59.88 (14.20-252.57)		8.02 (3.21-20.03)
	CE-strain		0.02 (0.011-0.03)		0.38 (0.20-0.70)
	CE-culture		0.002 (0.001-0.006)		0.001 (0.001-0.003)
Animal	Seeder	≤ 0.001	1 (reference)	≤ 0.05	1 (reference)
	Sentinel		0.003 (0.000-0.017)		0.03 (0.01-0.09)
Sampling	1	≤ 0.001	1 (reference)	≤ 0.001	1 (reference)
	2		2.52 (0.67-9.50)		1.20 (0.37-3.96)

3		4.71	(1.29-17.22)	3.33	(0.80-13.77)
4		1.59	(0.42-6.05)	7.27	(1.53-34.69)
5		3.13	(0.84-11.71)	7.23	(1.52-34.49)
6		2.01	(0.53-7.62)	10.94	(2.28-52.60)
7		1.00	(0.27-3.77)	4.75	(1.06-21.23)
8		2.52	(0.67-9.50)	4.77	(1.07-21.26)
9		3.86	(1.04-14.29)	7.19	(1.52-34.02)
10		1.26	(0.33-4.78)	16.08	(3.43-75.28)

95% CI = 95% confidence interval; HR = Hazard ratio; CE = Competitive Exclusion; sampling 1 = 24 h post inoculation, 2 = 72 h post inoculation, 3-5 = 2nd week of trial, 6-7 = 3rd week of trial, 8-9 = 4th week of trial, 10 = 5th week of trial

4.2.2 Necropsy

At necropsy, the content of crop, jejunum, cecum, colon, and organ samples from spleen and liver, were analyzed for the occurrence of the two investigated ESBL- and pAmpC- *E. coli* strains (Figure 4, Supplement Table 1 and 2). In principle, the detected quantities of the ESBL- and pAmpC- *E. coli* in the investigated intestinal samples of crop, jejunum, cecum, and colon per experimental group are correlating at necropsy (Figure 4). The seeder- and sentinel-birds were jointly analyzed as there was no significant difference in the colonization of the broiler chickens in any of the investigated intestinal samples (Mann-Whitney-U-Test, $p \geq 0.05$, Supplement Table 2). As the cecum is the reservoir for ESBL- and pAmpC- producing bacteria, we focused on the cecum samples to evaluate the colonization of the broiler chickens with the ESBL- and pAmpC- producing *E. coli*.

The administration of the CE-culture led to a significant reduction of the broiler chickens' colonization with both the ESBL- and pAmpC- *E. coli* strain (Kruskal-Wallis-Test, $p \leq 0.001$, Figure 4) along with a prevalence of almost zero in the cecum samples (ESBL- *E. coli* 0% (95% CI: 0-8%) and pAmpC- *E. coli* 4% (95% CI: 1-15%), Supplement Table 1). A significant reduction of colonization of the ESBL- *E. coli* was also apparent under the administration of the CE-strain (Kruskal-Wallis-Test, $p \leq 0.001$, Figure 4) with a detected prevalence of 2% (95% CI: 0-11%). No effects of the CE-strain on the colonization of the broiler chickens were shown for the pAmpC- *E. coli* strain with most birds colonized (96%, 95% CI: 85-99%). In contrast to the two CE-groups, the acidification of the drinking water led to a significant increase of the

colonization with both investigated ESBL- and pAmpC- *E. coli* strains (Kruskal-Wallis-Test, $p \leq 0.001$, Figure 4) and a prevalence of 100% (ESBL- *E. coli* 100% (95% CI: 92-100%) and pAmpC- *E. coli* 100% (95% CI: 92-100%), Supplement Table 1).

The results of the intestinal samples determined at necropsy are correlating with the results of the cloacal swab analyses (except a predicted lower colonization of the broiler chickens concerning the pAmpC- *E. coli* in the CE-strain group). In addition, no growth in any of the analyzed liver and spleen samples was apparent.

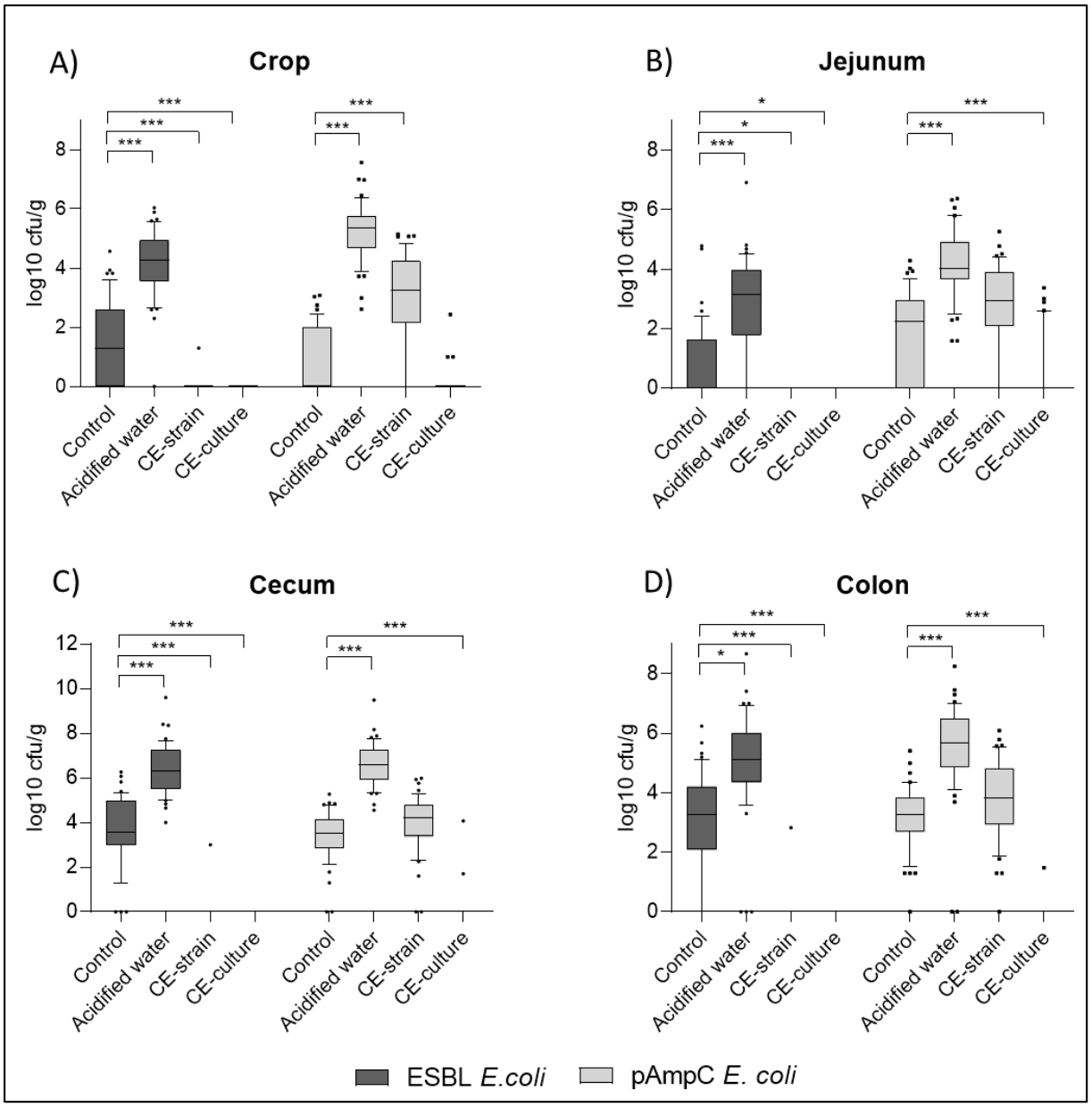

Figure 4. Broiler chickens' colonization of A) crop, B) jejunum, C) cecum, and D) colon (log10 cfu/g) with ESBL- and pAmpC-producing *E. coli* in the four investigated groups (Control group, Acidified drinking water (Acidified water), Competitive Exclusion- (CE-) strain, and CE-culture) attained at necropsy. * $p \leq 0.05$, *** $p \leq 0.001$, not significant results are not highlighted (Kruskal-Wallis-Test)

5 Discussion

Antimicrobial-resistant bacteria can cause difficulties in treating bacterial infections in animals and humans and might worsen the prognosis of an illness. Additionally, the colonization of broiler chickens with antimicrobial-resistant bacteria like ESBL- and pAmpC- producers poses a risk for transmitting these bacteria to humans. As the number of infections with antimicrobial-resistant bacteria is rising in both human and veterinary medicine, this study aimed to investigate potential intervention measures to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing bacteria in the course of the fattening period. The reduction of antimicrobial-resistant bacteria at this step of the broiler production chain is crucial for reducing the contamination of the broiler chickens. A reduced load of resistant bacteria during the fattening period can decrease the transmission to the broiler chickens' surrounding environment and carcasses at the slaughterhouse level, which reduces the load of ESBL- and pAmpC- producing bacteria on the sellable products.

To investigate potential intervention measures, we established a broiler chicken colonization model. We determined the minimal bacterial count for the colonization of broiler chickens to elucidate the transmission dynamics of ESBL- and pAmpC- producing bacteria within a broiler flock. Following, distinct hygiene- and management measures were analyzed on their potential to lower the ESBL- and pAmpC- prevalence of broiler chickens in the course of the fattening period.

5.1 Broiler chicken colonization model for ESBL- and pAmpC- producing *E. coli*

We aimed to establish a broiler chicken colonization model close to real farming conditions to comply with commercial broiler production conditions and reflect the ESBL- and pAmpC- transmission dynamics in broiler fattening flocks. Several factors need to be considered for a practically relevant setup, such as the bacterial inoculation and the broiler chickens' keeping conditions, including the feed, water, and litter. For the bacterial inoculation, the day of inoculation, the route of administration, the growth phase and the bacterial counts of the administered bacterial strains, as well as the number of inoculated broiler chickens (inoculation ratio) are of crucial importance. The broiler chickens were kept conventionally with a stocking density of 39 kg/m^2, no enrichment, fresh litter once at the beginning of the trail, and conventional feed and water *ad libitum*. No antimicrobial agents were administered to the broilers in any of the trials.

5.1.1 Determination of the minimal bacterial count

To determine the bacterial count for the subsequent seeder-bird trials, we decided to co-inoculate all broiler chickens with the two ESBL- and pAmpC- *E. coli* strains at the third day of trial (corresponding to the third day of life). In separate trials, the bacterial strains were orally administered into the crop using end log phase bacterial counts of 10^1 to 10^4 cfu. Already a bacterial count of 10^1 cfu led to the colonization of all broiler chickens in the trail 72 h *p.i.* All other tested bacterial counts of 10^2, 10^3, and 10^4 cfu caused the colonization of all broiler chickens 24 h *p.i.* (except one animal in the trial using 10^2 cfu). The bacterial count of 10^2 cfu ESBL- and pAmpC- *E. coli* was determined as the minimal bacterial count necessary for the colonization within 24 hours.

The bacterial counts of ESBL-producing *E. coli* used for the colonization experiments in animal trials are usually high and range between 10^5 - 10^8 cfu for day-old broiler chickens [172,178,181,182]. Day-old broiler chickens are not exposed to such high loads of resistant *E. coli* in real livestock farming. The bacterial burden in broiler production is minimized due to a continuous cleaning and disinfection regime at every level of the production chain. Still, residual bacteria can be detected after cleaning and disinfection [197,198], including ESBL- and pAmpC- producing bacteria [18,22,199]. High bacterial counts are used to ensure the colonization of all broiler chickens in a trial for a quantitative evaluation of the investigated measure (e.g. effects of probiotics). These trials are designed to prove the applied concept, wherefore the stable colonization of the tested animals is necessary to measure the efficacy of a specific product or method. To mimic real farming conditions, we aimed to determine the minimal bacterial count necessary to colonize broiler chickens with ESBL- and pAmpC-producing *E. coli*. Our results highlight that already an oral uptake of a very low bacterial count of 10^1 cfu ESBL- and pAmpC- producing *E. coli* leads to the colonization of broiler chickens without applying any antimicrobial selection pressure. Furthermore, we demonstrated an equivalent quantitative cecum colonization independent of the investigated bacterial counts after two weeks of trial. Independent of our study, concordant results for ESBL- and pAmpC-producing *E. coli* were obtained by another research group [200]. In the study by Dame-Korevaar et al. [200], no significant differences in the excretion of the bacterial strains were apparent 32 h *p.i.* using bacterial counts of 10^1 to 10^5 cfu ESBL- and pAmpC- producing *E. coli*. Interestingly, a colonization study by Nair et al. [201] shows a bacterial count as low as 10^2 cfu to be sufficient to colonize turkeys with *Salmonella enterica*, but lower bacterial counts were not tested. The low bacterial count necessary for the colonization is a reasonable explanation for the frequent detection of ESBL- and pAmpC- producing bacteria in broiler chickens. The oral uptake of bacteria out of the surrounding environment can lead to a colonization and multiplication of the bacteria in the broilers ceca. A spread of resistant bacteria

is plausible by a subsequent shedding of the bacteria and the possibility of horizontal gene transfers within a flock.

Antimicrobial treatments are assumed to be a driving factor for the selection of resistant bacteria and the colonization of broiler chickens with ESBL- and pAmpC- producing bacteria [18,189,202]. The antibiotic is supplied in the feed or water to all broiler chickens to treat bacterial infections in a flock causing a selection of resistant bacteria in both diseased and healthy animals [130]. A growing number of studies report transmission and persistence of ESBL- and pAmpC- producing bacteria in the broiler production chain without any selection pressure by antimicrobial treatments [142,203,204]. Furthermore, the colonization of wild birds with ESBL- and pAmpC- producing bacteria is frequently confirmed, which have never been faced with antibiotics [76,123,151]. Our results support the assumption that the occurrence of ESBL- and pAmpC- producing bacteria in broiler chickens is not necessarily linked to antibiotic treatment, showing the colonization of the broiler chickens with bacterial counts as low as 10^1 cfu.

A carriage of different or multiple *E. coli* strains represents the broilers' gut's physiological conditions [172]. It was already demonstrated that the colonization capabilities vary between different *E. coli* strains and the impact of applied intervention measures is affected by the investigated *E. coli* strain [176,182]. Still, all conducted studies on the ESBL- and pAmpC-colonization of broiler chickens are applying an inoculation with only a single bacterial *E. coli* strain per trial. We used a co-colonization with two *E. coli* strains previously isolated from broiler chickens which represent commonly detected commensal strains with one ESBL-producing (CTX-M-15, ST 410, phylogenetic group B1) and one pAmpC- producing (CMY-2, ST 10, phylogenetic group A) strain [144]. Using a co-colonization, we showed significant differences in the colonization of the two *E. coli* stains while applying distinct intervention measures in separate trials (see below).

We inoculated the broiler chickens on the third day of trial. In this way, an interaction between the broiler chickens and the surrounding bacterial spectrum was possible before the inoculation with the ESBL- and pAmpC- producing *E. coli* strains [205]. Additionally, field studies have already demonstrated that day-old broiler chickens are colonized with ESBL- and pAmpC- producing *E. coli* [18,22,27]. Thereby, the detected prevalence in day-old broiler chickens varies between the conducted studies. The different study designs might cause a diverse prevalence. In two studies that used the same study design, the detection frequencies vary between 0% [22] and 95% [27]. Both studies investigated seven broiler flocks using 20 cloacal swabs [27] or 40 cloacal swabs [22] per sampling and used a pre-enrichment in Luria Bertani Broth (LB) to determine the ESBL- and pAmpC- prevalence. Interestingly, the

differences between the two studies are in the chosen sampling time points. While Daehre et al. [22] sampled right after placing the broiler chickens into the stable, Laube et al. [27] sampled within the first two days of life. However, it has to be mentioned that the overall prevalence of the seven investigated flocks was higher in the study by Laube et al. [27]. Consequently, an inoculation on the third day of trial was chosen to stick to real farming conditions.

We inoculated our broiler chickens with a crop needle to ensure the uptake of the bacterial strains. Other application methods for intestinal *E. coli* colonization appear to be closer to real farming conditions as an application into the beak [174] or a treatment of the feeders [206]. Still, the determination of a minimal bacterial count requires a safe method of inoculation. Another prerequisite to determine the minimal bacterial count is the absence of other ESBL- and pAmpC-producers, as they could compete with the inoculated bacterial strains and distort the results. To ensure the absence of ESBL- and pAmpC- producing bacteria, the entire experimental setup, including litter and feed as well as the broiler chickens, were tested beforehand, and the absence was confirmed before each trial. We did not treat the feed, water, and litter other than usual in the broiler production. No reduction of germs (e.g. irradiation) was applied to create a setup close to real farming conditions.

Even in the absence of antimicrobial selection pressure, a horizontal gene transfer of ESBL- or pAmpC- resistance genes has to be considered since the coding genes are frequently located on plasmids [207,208]. Genetic exchange between the two colonization strains or the colonization strain(s) and other bacterial strains resident in the broiler chickens' gut (including *E. coli*) is plausible in our trials. The acquisition of the resistance determinants that we used to screen for the colonization strains could lead to a misinterpretation of our data due to recombinant strains from horizontal gene transfer events. To minimize the probability of false-positive isolates, we used various mechanisms to verify the numbers and identity of the bacterial colonization strains. First, antibiotic supplemented chromogenic agar plates were used to detect the *E. coli* colonization strains and check the colony morphologies. In addition, an agar plate without antibiotic supplementation was used to quantify the total *E. coli* count. The sum of the quantified ESBL- and pAmpC- strains corresponded to the total *E. coli* counts in the separate trials using bacterial counts of 10^1 to 10^4 cfu. Second, colonies grown on the selective agar plates extracted from cecal samples were checked for their phenotypic antimicrobial resistance patterns using VITEK 2. The resistance profile of the randomly selected colonies corresponded to the resistance profiles of the inoculation strains. Third, the statistical analysis revealed an equivalent cecum colonization between the four conducted trials to determine the minimal bacterial count for a broiler chickens' colonization. An equal horizontal gene transfer to other *E. coli* strains harboring identical antimicrobial resistance patterns seems to be of low probability. Still, an exchange of mobile genetic elements carrying

resistance mechanisms can not be excluded entirely in our experiments but one should keep in mind that an exchange represents the dynamics affecting the colonization of broiler chickens with antimicrobial-resistant bacteria under real farming conditions [118,209].

5.1.2 Seeder-bird model

The seeder-bird method is used to approximate animal models to real farming conditions and described for different enteral bacteria [172,178–180,210–212]. In a seeder-bird trial, only a part of the broilers is inoculated with the bacterial suspension (seeder-birds), while the other broilers remain untreated (sentinel-birds). The non-inoculated sentinel-birds mirror the route of natural colonization with the bacteria by the oral uptake of the contaminated material out of the surrounding environment [212,213]. We demonstrated that an oral uptake of ESBL- and pAmpC- producing *E. coli* by the sentinel-birds leads to a comparable colonization of all broiler chickens (n = 20) after 35 days of trial while inoculating one-fifth of the broilers (n = 4) with 10^2 cfu of the bacterial strains at the third day of trial. Our results underline the importance of the oral bacterial uptake for the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli*.

Various inoculation ratios for enteral bacteria in seeder-bird trials are described in the literature ranging from 1:2 to 1:20 [172,178,180,210,211,214–216]. We decided to inoculate one-fifth of the broiler chickens (1:5) as similar ratios are more frequently applied. In many seeder-bird studies, the seeder-birds are kept separately after inoculation to verify the colonization with the inoculated strains. We decided to keep the seeder-birds and sentinel-birds together right from the beginning of the trail to mimic the course of a natural infection. Perhaps the inoculation of a seeder-bird failed in our experimental setup, and the colonization took place due to the uptake out of the housing environment. Still, these dynamics also affect the colonization under real farming conditions as the differentiation of colonized and non-colonized broilers is not possible in livestock farming.

The bacterial counts obtained from the seeder-bird trial are lower than the colonization trials using 10^2 cfu ESBL-/pAmpC- *E. coli*. The lower bacterial counts are apparent in all investigated sample types of crop, jejunum, cecum, and colon attained at necropsy. The discrepancies in the experimental designs might cause different bacterial counts. All broiler chickens were inoculated with the ESBL- and pAmpC- producing *E. coli* strains for the trials determining the minimal bacterial count. The initial lower rate of inoculated broiler chickens in the seeder-bird trial might have caused less shedding of the bacterial strains and led to a lower oral uptake vice versa. Moreover, a study by Cox et al. [212] points out that the bacterial challenge of the pen mates in seeder-bird models is difficult to estimate because it is unknown how much the

birds take up contaminated material. Another reasonable explanation for the discrepancy in the bacterial counts is the difference in the durations of the trials, with 14 days of the colonization trial to 35 days of the seeder-bird trial. As *E. coli* is an early gut colonizer, it can already be detected within the first day after hatch in the broiler chickens microbiota [217]. While aging, the broiler chickens' microbial flora composition changes, and the complexity of the detected bacterial genera increases [218]. The Enterobacteriaceae content decreases over time with a shift in the predominantly Gram-negative composition to a more Gram-positive composition of the microbiota [219]. The changes in the microbial composition could cause a competition of the inoculated bacterial *E. coli* strains with other strains resident in the gut and could lead to a decrease of the ESBL- and pAmpC- colonization of the broiler chickens in the seeder-bird trial. Changes of the cecal flora of Ross hybrids were described by Lu et al. [220] up to an age of 49 days (= end of the trial). Still, it has to be mentioned that the composition of the broiler chickens' gut microbiota in our trials and livestock farming is artificial and does not reflect the microbiome of a mature chicken [221,222].

5.1.3 Sampling strategy

We used cloacal swabs and a direct processing of the samples without an enrichment step to determine the colonization status of the broiler chickens during the trials. The sampling strategy could affect the detection frequency of the ESBL- and pAmpC- producing *E. coli* strains in the colonization trials and the seeder-bird model trial. Our detection limit is fairly high, as the investigated amount of fecal content is limited using cloacal swab sampling. Additionally, the detection in fecal samples is also dependent on the cecal excretion of the bacterial strains [221,223]. Cecal sampling is a safer method to ensure bacterial detection as the cecum has the greatest bacterial diversity in the broiler chickens' gut [205,222] and is the reservoir for ESBL- and pAmpC- producing bacteria [16,222]. Cloacal swab sampling is widely used as cecal samples require a necropsy and do not allow a repeated measurement of the same broiler chicken [224,225]. Two studies investigated the comparability of cloacal swabs and cecal samples for broiler chickens. Both the ‚Cobb 500'-study by Stanley et al. [224] and the ‚Ross 308'-study by Andreani et al. [225] demonstrated a good comparability of the results from cloacal swab and cecal samples. Andreani et al. [225] limit the results to abundant taxa, as less abundant taxa from the cecum can not be detected in cloacal samples. As a limitation, only 19 to 31 day old broilers were investigated in the studies. Still, a very early cloacal detection of ESBL- and pAmpC- producing *E. coli* in day-old broiler chickens only nine hours after inoculation with 10^2 cfu was shown for Ross 308 by Dame-Korevaar et al. [200]. Consequently, a cloacal swab sampling was used to allow a repeated measurement of the same broiler. Cecal samples were obtained at necropsy to ensure a reliable quantification of

the bacterial strains. In addition, due to the high detection limit, we decided for the bacterial count of 10^2 cfu of the bacterial strains even if one animal was not tested positive for the ESBL- and pAmpC- *E. coli* strains 24 h *p.i.*

5.2 Intervention measures against an ESBL- and pAmpC- colonization of broilers

There is a need for intervention as ESBL- and pAmpC- producing bacteria are frequently detected in broiler chickens and transmission to humans via direct contact, or the consumption of contaminated meat is assumed. High prevalence and various transmission routes for ESBL- and pAmpC- producing bacteria are described as mentioned above, with only 10^1 to 10^2 cfu of the resistant bacteria necessary for colonization of broiler chickens. Various studies investigated diverse measures like feed additives, alternative medical treatments to antibiotic administration, or different broiler management systems. The comparability of different studies is limited as they use various study designs and frequently combine distinct measures within one trial. We aimed to separately investigate distinct intervention measures on their potential to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli*. Using the seeder-bird colonization model, we aimed to emulate real farming conditions while testing the measures under controlled conditions at the same time. We modified the broiler chickens conventional conditions (used broiler breed, different amount of bedding material, and a reduced stocking density) as one approach and used probiotics and the acidification of the drinking water to investigate their impact on the broiler chickens' colonization with ESBL- and pAmpC- producing *E. coli* as another approach.

5.2.1 Broiler breed

From the current literature, no reliable conclusion can be drawn between the broiler chickens' breed and the colonization of broiler chickens with antimicrobial-resistant bacteria. Some studies conclude an impact of the genetic background [189,226], whereas other studies point out the importance of environmental factors affecting the broiler chickens' colonization [227–229]. The different results might be caused by the diverse experimental setups used in these studies. Are the tested breeds obtained from the same hatchery, are the broiler chickens housed together in one experimental room or separate rooms, and are the broiler chickens reared under identical conditions? All these factors can influence the outcome of a study. A study by Shokker et al. [226] drew the conclusion of a major impact of the used broiler breed on the microbial gut colonization while housing the broiler breeds separately but simultaneously under identical conditions. By contrast, a study by Richards et al. [227] concluded a strong impact of the environment on the microbial composition of the broiler chickens' gut with a minor impact of the genetic background by housing the different breeds

together in one pen. An impact of the environmental factors on the broiler chickens' colonization is supported by a retrospective comparison of 17 different control groups using the same broiler breed obtained from the same hatchery and housed in the same experimental facilities by Rychlik [221] as different microbial cecum compositions were shown for the analyzed groups. We obtained the broiler chickens' eggs for Ross 308 and Rowan x Ranger from different hatcheries and housed the broiler chickens in separate rooms but under identical conditions. No dependency of the broiler chickens' ESBL- and pAmpC- colonization on the breed was found as the bacterial counts determined at necropsy showed no significant differences. Our results are in line with Richards et al. [227], assuming a major impact of the environment on the broiler chickens' gut colonization, even though we did not house the broiler breeds together in one pen.

5.2.2 Litter quantity

One kilogram of litter per square meter is a common quantity used for bedding material in German broiler production [230]. We compared the commercial standard to a tripled amount of litter (three kg/m^2) to investigate a potential impact of an increased amount of litter on the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli*. Studies investigating the microbial composition of the broiler chickens' gut are using various amounts of litter with up to six kg/m^2 [231]. Higher litter quantities than a tripled amount of litter could cause different dynamics but were not applied due to the practical irrelevance in German broiler production. As the broiler chickens constantly ingest the litter, the quality and composition affect the broiler chickens' intestinal microbiota [232,233] and might influence the detection of the bacterial strains. We hypothesized that the fecal droppings might mix in the larger amount of litter due to the movement of the broiler chickens, thereby lowering the "concentration" of droppings in the litter. But, in contrast to our assumption, the tripling of the litter led to higher colonization of the broiler chickens with both the ESBL- and pAmpC-producing *E. coli* strains in our trial. An increased amount of litter might cause an intensified explorative behavior of the broiler chickens with a more frequent ingestion, particularly at the beginning of the fattening period.

An impact of the type of bedding material on the microbial composition of broiler chickens is controversially discussed [233–235] and can not be excluded by our study as only pelletized straw granule was investigated. For the colonization with ceftiofur-resistant *E. coli*, Persoons et al. [189] concluded a higher risk when broilers are kept on straw compared to wood curls. As we only investigated pelletized straw granule, no statement on the impact of the type of bedding material on the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* is possible in our trials. The fine structure of pelletized straw granule could lead to a higher

ingestion of litter compared to more rough-textured bedding materials and causing a higher (re-) colonization with the bacterial strains.

5.2.3 Stocking density

Stocking densities are controversially discussed to influence the performance and welfare of broiler chickens [236–240] as well as the microbial composition of broiler chickens' gut [231,241–244]. A study by Guardia et al. [231] demonstrated an effect of high stocking densities on the composition of the microbial flora of young broiler chickens. An overall decrease in the commensal bacteria and the *E. coli* population in cecal samples was observed for three-week-old broiler chickens while comparing stocking densities of 12 to 17 birds/m^2. Contrastingly, for Ross hybrids at the age of four weeks and six weeks, no impact of the stocking densities of 12 to 16 birds/m^2 on the microbial cecum composition including *E. coli* was concluded by Li et al. [241]. Other studies concluded an effect of different stocking densities on the detection of various pathogenic bacteria [242–244]. We hypothesized an effect of the stocking density on the colonization of broiler chickens with ESBL- and pAmpC-producing *E. coli*, as the lower number of broiler chickens per square meter might lead to lower contamination of the surrounding environment, thus, leading to less contact with the resistant bacterial strains. A subsequent experimental slaughter of 40 of our sentinel-birds was conducted and confirmed a significantly lower detection of the ESBL- and pAmpC- producing *E. coli* for the broiler chickens' carcasses together with less dirty and less wet feathers of the broiler chickens upon arrival at the slaughtering facilities [245]. However, our data are heterogeneous, with a significant decrease in the cecum colonization of the ESBL- *E. coli* and a significant increase in the cecum colonization of the pAmpC- *E. coli*. Our results support the assumption that a combination of different intervention measures is necessary to counter the colonization of broiler chickens with different ESBL- and pAmpC- producing bacteria [172,246].

5.2.4 Drinking water acidification

Since the ban of antibiotic growth promoters in the European Union in 2006, organic acids have been investigated for their potential as "natural growth promoters" [247]. Thus, organic acids are widely used in broiler production as feed additives to enhance the performance of broiler chickens and to influence the bacterial colonization of the broiler chickens' gastrointestinal tract [248–250]. For a reliable reduction of specific bacteria, a high efficacy level of formulations *in vitro* has to be confirmed *in vivo* as the complex interactions of the gastrointestinal tract can only be poorly mirrored in laboratory experiments [251]. In our broiler chicken trial, applying a commercially available formulation for drinking water acidification based on organic acids led to significantly higher colonization rates of the broiler chickens' gut

with the two investigated ESBL- and pAmpC- producing *E. coli* strains. To the best of our knowledge, a significant increase of the *E. coli* counts in acidification experiments *in vivo* was not shown before for broiler chickens [195,248,252–255]. Still, the level of comparability for the conducted studies is low as they use various study designs. One important factor is that most studies are based on a 'natural colonization' in feeding experiments rather than a bacterial challenge to evaluate the effect of organic acids on the broiler chickens' gut colonization with different bacteria [248,252–254]. Two recent studies used an oral *E. coli* challenge of Ross hybrids to evaluate the effect of the applied organic acids [195,255]. Both studies used high bacterial counts for colonization by inoculating every second broiler with 10^6 cfu *E. coli* [195] or every broiler with 10^8 cfu *E. coli* [255]. Both studies used a mixture of formic and propionic acids, while acetic acid was included in the formulation used by Roth et al. [195]. No significant differences in the cecal *E. coli* count after 38 days of trial were shown by Roth et al., even if the bacterial count for inoculation and the inoculation ratio was lower in the study and the concentrations of the administered organic acids were higher (0.2%) than in the study by Emami et al. [255]. In contrast, Emami et al. [255] showed significant reductions in cecal *E. coli* colonization after 35 days of trial by using different concentrations of formic and propionic acids ranging from 0.07% to 0.4%. The contrasting results of the two exemplary named studies highlight the complexity of *in vivo* studies. Other factors might influence the colonization dynamics in the trials as the husbandry conditions, the day of the oral *E. coli* challenge, or the applied *E. coli* strain.

E. coli can withstand acid stress due to multiple acid resistance and acid tolerance systems [166]. Without growth, *E. coli* can survive for several hours at pH 2.0 [164]. In combination with the low bacterial count of 10^2 cfu, *E. coli* is able to passage the acid environment of the stomach (pH 1.5-2.5) [163,165]. In the small intestine, *E. coli* encounters a less acid environment of pH 4.0-6.0. The ability to survive the stomach's highly acidic environment and the quick growth in moderate acidic environments leads to the successful colonization of *E. coli* [166]. Interestingly, acid-tolerant *E. coli* strains are described, able to survive in different acidic environments like soil, farm water, or meat with several cross-protective benefits like enhanced antimicrobial resistance, biofilm formation, or pathogenic adhesion [256].

Besides the type of acid, the concentration of the acid, the period of application, and the applied form of the acid formulation might be an crucial influencing factor for the efficacy of an acid formulation. Formic acid, propionic acid, acetic acid, and citric acid are frequently used in broiler chicken production. These acids vary in their chemical characteristics, such as the solubility in water and their acid dissociation constants (pKa) [249]. Most acids have a pKa value between three and five, with lower pKa values describing stronger acids. As high acid concentrations might affect the palatability and thus affecting the feed intake and the

performance of the broiler chickens, the applied acid concentrations have to be considered [249]. When administered to the broiler chickens, a positive effect of a discontinuous administration was shown by Hamid et al. [248]. Moreover, it was demonstrated that microencapsulation can protect the acids from modifications in the stomach with a slow release in the intestine, thus, leading to stronger effects when the acid is applied in the broilers' feed [253,257]. In our trial, an acid formulation without microencapsulation with a low pKa value was administered discontinuously to the drinking water of the broiler chickens, leading to higher colonization rates of the investigated ESBL- and pAmpC- *E. coli* strains. A possible explanation for the higher colonization in our trial might be a higher oral uptake of the *E. coli* strains out of the environment due to an appetite stimulation of the broiler chickens and/or a low efficacy of the applied product (e.g. caused by a low acid concentration in the administered drinking water). Another explanation might be a survival of the ESBL- and pAmpC- producing *E. coli* strains in the acidic environment due to an enhanced stress tolerance as described above.

5.2.5 Competitive Exclusion

CE is based on an early application of living bacteria to broiler chickens to protect them from colonization with other bacteria [190,258]. Different CE-products consisting of a single strain or a mixed culture of different strains are available for poultry [259]. Because many of the complex CE-cultures are of undefined composition as they are derived from specific-pathogen-free chickens, they are not approved by the European Union [192]. The impact of complex CE-cultures on the infection of broiler chickens with different pathogenic bacteria was investigated with promising results [177,180,260,261]. In recent years more studies focused on the effects of complex CE-cultures on the colonization of broiler chickens with ESBL- and pAmpC-producing bacteria. Several factors need to be considered to evaluate the different projects. Some studies used high bacterial counts of 10^5-10^6 cfu ESBL- and pAmpC- producing *E. coli* to colonize the broiler chickens in the trial [172,176,178]. The high bacterial counts do not reflect the exposure of broiler chickens under real farming conditions as the bacterial counts are much lower [190]. All studies showed a reduction of the ESBL- and pAmpC- colonization of the broiler chickens but not a protection from colonization what might be caused by the high bacterial counts used for the initial ESBL- and pAmpC- *E. coli* inoculation. Besides the applied bacterial counts, the ratio of ESBL-/pAmpC- inoculated to non-inoculated broiler chickens affects the colonization with the resistant bacteria. An inoculation of every broiler chicken [176,182] or low ratios of 1:2 [172,190] do not reflect the colonization frequencies of day-old broiler chickens in livestock farming as the load of ESBL- and pAmpC- producing bacteria is minimized due to a strict cleaning and disinfection regime [197]. Consequently, low bacterial

counts in combination with a high inoculation ratio should be preferred for the broiler chickens' colonization with ESBL- and pAmpC- producing *E. coli* to evaluate the efficacy of CE- cultures. Studies using higher bacterial counts with the inoculation of all broiler chickens can be seen as 'proof of principle' studies and are important for the overall evaluation of the performance of CE- products.

No studies for ESBL- and pAmpC- producing bacteria are available which investigated the effect of CE- cultures consisting of a single strain. A reasonable explanation would be that the results of complex CE-cultures are expected to be of greater importance as a single strain does not reflect the complex microbial composition of a broiler chickens' gut. We investigated a complex CE-culture on the one hand and a single CE-strain on the other hand on their potential to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli*. Using a co-colonization with 10^2 cfu of one ESBL- and one pAmpC- producing *E. coli* strain (inoculation ratio 1:5), we were able to investigate strain-dependent effects of the applied CEs. The complex CE-culture led to a significant reduction in the colonization of both the ESBL- and pAmpC- *E. coli* with a prevalence close to zero percent, whereas the single CE-strain reduced the ESBL- *E. coli* close to zero percent but had almost no impact on the colonization of the pAmpC- *E. coli* strain. The limited effect of the CE-strain supports the assumption of complex interactions in the gastrointestinal tract, which affect the microbial colonization of the broiler chickens.

The inoculation of broiler chickens with only one ESBL- or pAmpC- producing *E. coli* strain is another weakness of CE- studies. Methner et al. [178] and Nuotio et al. [176] investigated the effects of complex CE-cultures on the colonization of different ESBL- and pAmpC- producing *E. coli* strains. Both studies showed strain-dependent results but investigated every bacterial strain separately. The individual trials do not reflect the complex scenario affecting the colonization of broiler chickens in livestock [172,259] as different bacterial strains might affect or outcompete each other (see above).

We inoculated the broiler chickens directly after the hatch into the crop to ensure the uptake of the CE- suspension. The individual application is not possible under real farming conditions. A practical approach of CE- administration was carried out by Dame-Korevaar et al. [190,262] as they supplied the CE- culture to newly hatched chickens for seven or 14 days in the drinking water. Still, whether a prolonged application has a beneficial effect on the colonization of broiler chickens compared to a single application and whether an interference with other procedures (e.g. vaccination) exists needs further investigations.

6 Conclusion

We established a broiler chicken colonization model close to real farming conditions and determined the minimal bacterial count necessary to colonize day-old broiler chickens with ESBL- and pAmpC- producing *E. coli*. Subsequently, distinct intervention measures were investigated on their potential to reduce the colonization of broiler chickens with ESBL- and pAmpC-producing bacteria throughout the fattening process. The fact that already a bacterial count as low as 10^1 cfu (by inoculating every broiler chicken) to 10^2 cfu (by inoculating one-fifth of the broiler chickens in a seeder-bird model) of ESBL- and pAmpC- producing *E. coli* strains was sufficient for the colonization of all day-old broiler chickens in a trial is a reasonable explanation for the high detection frequencies of ESBL- and pAmpC- producing bacteria in the broiler production chain. The resistant bacteria are shed by colonized broiler chickens and can be easily transmitted to other broiler chickens. Therefore, an intervention should be implemented as early as possible to protect the broiler chickens from colonization with ESBL- and pAmpC- producing bacteria rather than an attempt to reduce an already existing colonization. A promising approach is the application of a complex CE-culture, and partly the application of a single strain CE-culture, whereas other separately applied management measures were not capable of reducing the broiler chickens' colonization with both the ESBL- and pAmpC- producing *E. coli* strains. It needs further investigations whether a combination of different measures can contribute to a reduced colonization of broiler chickens with ESBL- and pAmpC- producing bacteria.

7 Summary

Management measures to reduce the prevalence of broiler chickens with ESBL-/pAmpC- producing enterobacteria

Extended-spectrum beta-lactamase (ESBL) and plasmid-mediated AmpC beta-lactamase (pAmpC) producing bacteria are frequently detected in the broiler production chain. Investigations revealed a high prevalence of ESBL- and pAmpC- producing bacteria throughout the broiler fattening process up to the slaughterhouse level and consumer goods and revealed several transmission routes of these antibiotic-resistant bacteria. Insights into the colonization dynamics of broiler chickens and possible intervention measures are needed as the transmission of ESBL- and pAmpC- producing bacteria to humans via close contact with livestock or the consumption of contaminated meat is assumed. This thesis aimed to determine the minimal bacterial count necessary to colonize broiler chickens with ESBL- and pAmpC-producing *Escherichia coli* (*E. coli*) and to establish a broiler chicken colonization model (seeder-bird) close to real farming conditions without applying any antimicrobial selection pressure. Subsequently, we aimed to evaluate distinct intervention measures on their potential to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* throughout the fattening process.

For the determination of the minimal bacterial count, ESBL- and pAmpC- negative day-old broiler chickens (Ross 308) were kept under conventional conditions and were orally co-inoculated on day three of the trial with 10^4,10^3, $10^{2,}$ or 10^1 colony forming units (cfu) of one ESBL- (CTX-M-15) and one pAmpC- (CMY-2) producing *E. coli* strain in separate trials. All investigated bacterial counts led to the colonization of all broiler chickens in the trials, with all broiler chickens tested positive after 24 h (10^4-10^2 cfu) or 72 h (10^1 cfu) *post inoculation* (*p.i.*) up to the end of each trial. At necropsy (14 d *p.i.*), the cecal colonization with the ESBL- and pAmpC- producing *E. coli* strains of all investigated bacterial counts showed equivalence in the statistical analysis. To assure stable colonization, the bacterial count of 10^2 cfu ESBL- and pAmpC- producing *E. coli* was chosen to establish the seeder-bird model. An inoculation of one-fifth of the day-old broiler chickens with 10^2 cfu *E. coli* led to the colonization of all inoculated broiler chickens (seeder-birds) after 24 h *p.i.* and all non-inoculated broiler chickens (sentinel-birds) after 72 h *p.i.* up to the end of the trial. At necropsy (35 d *p.i.*) no significant differences in the cecal colonization with the ESBL- and pAmpC producing *E. coli* strains was apparent between the seeder-birds or sentinel-birds.

Distinct intervention measures were subsequently investigated on their potential to reduce the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* throughout the fattening process using the established seeder-bird model. Applying a complex, non-defined

Competitive Exclusion-culture led to a significant reduction of both the ESBL- and pAmpC-producing *E. coli* strain in our trial. Applying a single CE-strain or reducing the stocking density to 25 kg/m2 led to a strain-dependent reduction of the ESBL- *E. coli* strain but had no impact on the colonization with the pAmpC- *E. coli* strain. No effect on the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* was evident for the use of an alternative, slower-growing broiler breed (Rowan x Ranger). A negative effect on the colonization of broiler chickens with ESBL- and pAmpC- producing *E. coli* was shown for the application of a tripled amount of litter in the pen and the acidification of the drinking water with a commercially available product.

The minimal bacterial counts of 10^1 to 10^2 cfu ESBL- and pAmpC- producing *E. coli* highlight the need for effective intervention measures to reduce the broiler chickens' colonization with ESBL- and pAmpC- producing bacteria including improved biosecurity- and disinfection measures. A promising approach is the application of a Competitive Exclusion-culture, whereas other separately applied management measures were not capable of reducing the broiler chickens' colonization with both the ESBL- and pAmpC- producing *E. coli* strains. It needs further investigations whether a combination of different measures can contribute to a reduced colonization of the broiler chickens' with ESBL- and pAmpC- producing bacteria.

8 Zusammenfassung

Managementmaßnahmen zur Senkung der Prävalenz ESBL-/ pAmpC- bildender Enterobakterien beim Masthähnchen

Extended-Spektrum Beta-Laktamase (ESBL) und plasmid-vermittelte AmpC Beta-Laktamase (pAmpC) produzierende Bakterien können häufig in der Masthähnchenproduktionskette nachgewiesen werden. Untersuchungen zeigten hohe Prävalenzen und identifizierten verschiedenste Transmissionswege sowohl im Mastprozess und auf Schlachthofebene als auch auf Hähnchenfleisch-Produkten. Da ein Übertrag auf den Menschen durch einen engen Kontakt zu Nutztieren oder durch den Verzehr von kontaminiertem Fleisch angenommen wird, sind Erkenntnisse über die Kolonisierungsdynamiken von Masthähnchen und über mögliche Interventionsmaßnahmen erforderlich. Die vorliegende Dissertationsschrift hatte zum Ziel, die geringste erforderliche Bakterienzahl an ESBL- und pAmpC- produzierenden *Escherichia coli* (*E. coli*) für eine Kolonisierung von Masthähnchen zu ermitteln und ein praxisnahes Kolonisierungsmodell (Seeder-Bird) zu entwickeln, ohne dabei einen antimikrobiellen Selektionsdruck auszuüben. Ein weiteres Ziel der vorliegenden Arbeit war die Evaluation verschiedener Interventionsmaßnahmen, um die Kolonisierung von Masthähnchen mit ESBL- und pAmpC- produzierenden *E. coli* während des Mastprozesses zu reduzieren.

Für die Ermittlung der geringsten erforderlichen Bakterienzahl wurden ESBL- und pAmpC-negative Eintagsküken (Ross 308) konventionell aufgestallt und in separaten Versuchen jeweils am dritten Versuchstag mit einem ESBL- (CTX-M-15) und einem pAmpC- (CMY-2) produzierenden *E. coli* Stamm bivalent kolonisiert. Alle untersuchten Keimzahlen von 10^4,10^3, 10^2 und 10^1 koloniebildenden Einheiten (KbE) führten zu einer Kolonisierung aller Masthähnchen in den einzelnen Versuchen. Eine Kolonisierung aller Tiere war mittels Kloakentupfer 24 h (10^4-10^2 KbE *E. coli*) oder 72 h (10^1 KbE *E. coli*) nach Inokulation bis zum Versuchsende nachweisbar. Die am Versuchsende durchgeführte Sektion (14 d nach Inokulation) zeigte eine äquivalente zäkale Kolonisierung der ESBL- und pAmpC-produzierenden *E. coli* Stämme in allen Versuchsgruppen. Für eine sichere experimentelle Kolonisierung der Masthähnchen wurde bei der Etablierung des Seeder-Bird Kolonisierungsmodells eine Bakterienzahl von 10^2 KbE ESBL- und pAmpC- produzierende *E. coli* genutzt. Im Seeder-Bird Modell führte eine Inokulation von einem Fünftel der Eintagsküken mit 10^2 KbE *E. coli* nach 24 h zu einer Kolonisierung aller inokulierten Tiere (Seeder-Birds) und nach 72 h zu einer Kolonisierung aller nicht inokulierten Tiere (Sentinel-Birds). Im Folgenden war die Kolonisierung über den gesamten Versuchszeitraum nachweisbar. Zu Versuchsende (35 d nach Inokulation) war kein statistisch signifikanter Unterschied der

zäkalen Kolonisierung von Seeder-Birds und Sentinel-Birds mit den ESBL- und pAmpC-produzierenden *E. coli* Stämmen nachweisbar.

Das etablierte Seeder-Bird Modell wurden nachfolgend genutzt, um unterschiedliche Interventionsmaßnahmen auf ihr Potenzial einer Reduktion der ESBL- und pAmpC-Kolonisierung von Masthähnchen während des Mastprozessen hin zu untersuchen. Eine signifikante Reduktion beider eingesetzter *E. coli* Stämme war unter Anwendung einer nicht definierten, komplexen Competitive Exclusion-Kultur ersichtlich. Eine stammabhängige Reduktion des ESBL- *E. coli* Stammes, ohne einen Effekt auf die Kolonisierung der Masthähnchen mit dem pAmpC- *E. coli* Stamm, war sowohl unter der Applikation eines einzelnen CE-Stammes als auch bei der Reduktion der Besatzdichte auf 25 kg/m^2 nachweisbar. Keinen Effekt auf die Kolonisierung von Masthähnchen mit ESBL- und pAmpC-produzierenden *E. coli* zeigte sich beim Einsatz einer alternativen, langsam-wachsenden Rasse (Rowan x Ranger). Eine stärkere Kolonisierung von Masthähnchen mit ESBL- und pAmpC- produzierenden *E. coli* lag bei der Verdreifachung der Einstreumenge im Stall und bei der Ansäuerung des Tränkwassers mit einem kommerziell erwerblichen Produkt vor.

Die sehr geringe Keimzahl von 10^1-10^2 KbE ESBL- und pAmpC- produzierender *E. coli* zeigt die Notwendigkeit von effektiven Interventionsmaßnahmen zur Reduktion der Kolonisierung von Masthähnchen mit ESBL- und pAmpC- produzierenden Bakterien, einschließlich verbesserter Biosicherheits- und Desinfektionsmaßnahmen. Ein vielversprechender Ansatz ist die Applikation von Competitive Exklusion-Kulturen. Alle anderen einzeln untersuchten Maßnahmen konnten die Kolonisierung mit jeweils beiden eingesetzten ESBL- und pAmpC-produzierenden *E. coli* Stämmen nicht reduzieren. Weitere Untersuchungen sind in diesem Kontext notwendig, um den Einfluss einer Kombination von Maßnahmen auf die Reduktion der ESBL- und pAmpC- Kolonisierung von Masthähnchen zu evaluieren.

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10 Appendix

Supplement Table 1. Prevalence of ESBL- and pAmpC-producing *E. coli* of seeder- and sentinel-birds attained in the ongoing trial and at necropsy of the four investigated groups (Control group, Acidified drinking water (Acidified water), Competitive Exclusion- (CE-) strain, and CE-culture).

Group	**Control group**				**Acidified water**				**CE-strain**				**CE-culture**			
Strain	10716		10717		10716		10717		10716		10717		10716		10717	
Animal	See*	Sen*	See*	Sen*	See*	Sen*	See*	Sen*	See*	Sen*	See*	Sen*	See*	Sen*	See*	Sen*
1	56	7	78	39	100	36	100	54	28	0	83	0	6	0	0	0
2	72	61	94	93	100	100	100	100	33	4	72	32	6	4	0	0
3	94	79	100	100	100	100	100	100	33	7	89	75	0	0	0	4
4	89	68	100	100	100	100	100	100	11	7	94	93	0	4	6	4
5	100	79	100	100	100	100	100	100	17	4	100	100	0	7	0	0
6	89	68	94	100	100	100	100	100	17	11	100	96	0	4	11	11
7	89	71	89	93	100	100	100	100	0	7	100	93	0	0	6	14
8	100	71	83	71	100	100	100	100	11	7	89	96	0	0	22	7
9	100	100	100	93	100	100	100	100	22	18	100	100	0	0	17	11
10	94	96	94	96	100	100	100	100	0	4	100	100	0	0	17	18
Necropsy																
Crop (See+Sen)	56	50 (52)	39	50 (46)	100	96 (98)	100	100 (100)	0	4 (2)	83	89 (87)	0	0 (0)	11	4 (7)
Jejunum (See+Sen)	33	29 (30)	72	75 (74)	72	89 (83)	100	100 (100)	0	0 (0)	78	86 (83)	0	0 (0)	17	14 (15)
Cecum (See+Sen)	94	93 (93)	94	96 (96)	100	100 (100)	100	100 (100)	0	4 (2)	100	93 (96)	0	0 (0)	6	4 (4)
Colon (See+Sen)	78	89 (85)	100	96 (98)	94	93 (93)	94	96 (96)	0	4 (2)	100	96 (98)	0	0 (0)	6	0 (2)

* Prevalence in percent (%); 10716 = ESBL *E. coli*, 10717 = pAmpC *E. coli*; See = Seeder-birds, Sen = Sentinel-birds; sampling 1 = 24 h post inoculation, 2 = 72 h post inoculation, 3-5 = 2nd week of trial, 6-7 = 3rd week of trial, 8-9 = 4th week of trial, 10 = 5th week of trial

Supplement Table 2. Mean values and confidence intervals of ESBL- and pAmpC-producing *E. coli* of digestive tract samples (crop, jejunum, cecum, and colon) of the four investigated groups (Control group, Acidified drinking water (Acidified water), Competitive Exclusion- (CE-) strain, and CE-culture) attained at necropsy.

Sample	**Crop**				**Jejunum**				**Cecum**				**Colon**			
Strain	10716		10717		10716		10717		10716		10717		10716		10717	
	Mean	± CI	Mean	± CI	Mean	± CI	Mean	± CI	Mean	± CI	Mean	± CI	Mean	± CI	Mean	± CI
Control group																
Seeder-birds	1.18	0.66 1.71	0.78	0.03 1.04	0.80	0.20 1.39	1.88	1.28 2.48	3.70	3.00 4.32	3.51	2.95 4.07	2.84	2.00 3.68	3.41	2.89 3.93
Sentinel-birds	1.50	0.89 2.10	1.03	0.62 1.43	0.67	0.23 1.12	2.00	1.49 2.51	3.57	2.98 4.16	3.35	2.97 3.74	3.07	2.50 3.63	3.03	2.68 3.37
Acidified water																
Seeder-birds	4.34	3.82 4.85	5.31	4.82 5.80	2.49	1.70 3.27	4.25	3.83 4.66	6.30	5.81 6.79	6.50	6.12 6.88	5.53	4.67 6.38	5.79	4.99 6.58
Sentinel-birds	4.02	3.62 4.42	5.13	4.77 5.48	2.94	2.36 3.52	4.14	3.68 4.59	6.55	6.11 6.98	6.65	6.27 7.02	4.66	4.11 5.21	5.26	4.76 5.76
CE-strain																
Seeder-birds	0.00	0.00 0.00	2.99	2.28 3.70	0.00	0.00 0.00	2.60	1.87 3.32	0.00	0.00 0.00	4.37	4.74 3.99	0.00	0.00 0.00	4.10	3.60 4.61
Sentinel-birds	0.05	0.00 0.14	2.99	2.43 3.54	0.00	0.00 0.00	2.78	2.27 3.30	0.11	0.00 0.31	3.68	3.15 4.22	0.10	0.00 0.30	3.56	3.03 4.08
CE-culture																
Seeder-birds	0.00	0.00 0.00	0.19	0.00 0.46	0.00	0.00 0.00	0.50	0.00 1.02	0.00	0.00 0.00	0.09	0.00 0.27	0.00	0.00 0.00	0.08	0.00 0.24
Sentinel-birds	0.00	0.00 0.00	0.04	0.00 0.10	0.00	0.00 0.00	0.31	0.02 0.61	0.00	0.00 0.00	0.15	0.00 0.43	0.00	0.00 0.00	0.00	0.00 0.00

10716 = ESBL *E. coli*, 10717 = pAmpC *E. coli*; all data shown are log10 transformed (log10 cfu/g); ± CI = ± 95% confidence interval

11 List of publications

11.1 Articles

Roedel, A.; Vincze, S.; Projahn, M.; Roesler, U.; Robé, C.; Hammerl, J. A.; Noll, M.; Al Dahouk, S.; Dieckmann, R. (2021): Genetic but no Phenotypic Associations between Biocide Tolerance and Antibiotic Resistance in *Escherichia coli* from German Broiler Fattening Farms. Microorganisms 9 (3), S. 651. DOI: 10.3390/microorganisms9030651.

Projahn, M.; Sachsenroeder, J.; Correia-Carreira, G.; Becker, E.; Martin, A.; Thomas, C.; Hobe, C.; Reich, F.; Robé, C.; Roesler, U.; Kaesbohrer, A.; Bandick, N. (2021): Impact of On-Farm Interventions against CTX-Resistant *Escherichia coli* on the Contamination of Carcasses before and during an Experimental Slaughter. Antibiotics (Basel) 10 (3). DOI: 10.3390/antibiotics10030228.

Robé, C.; Daehre, K.; Merle, R.; Friese, A.; Guenther, S.; Roesler, U. (2021): Impact of different management measures on the colonization of broiler chickens with ESBL- and pAmpC-producing *Escherichia coli* in an experimental seeder-bird model. PLoS ONE 16 (1), e0245224. DOI: 10.1371/journal.pone.0245224.

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. Front. Microbiol.; 10, S. Article 2124, DOI: 10.3389/fmicb.2019.02124.

11.2 Conference contributions

Talk: Robé, C.; Daehre, K.; Merle, R.; Fiedler, S.; Ewers, C.; Guenther, S.; Roesler, U. (2020): Entwicklung stufenübergreifender Reduktionsmaßnahmen gegen ESBL-/pAmpC-bildende Enterobakterien bei Masthühnern. 98. und 99. Fachgespräch über Geflügelkrankheiten, Online Seminar, 12.-14.11.20

Talk: Robé, C.; Daehre, K.; Merle, R.; Fiedler, S.; Ewers, C.; Guenther, S.; Roesler, U. (2019): Impact of hygiene- and management measures on the ESBL- and AmpC- colonization of broiler chickens. International Symposium on Zoonoses Research 2019, Berlin, 16.-18.10.19

Talk: Robé, C.; Dähre, K.; Merle, R.; Fiedler, S.; Ewers, C.; Günther, S.; Rösler, U. (2019): Einfluss von Hygiene- und Managementmaßnahmen auf die ESBL- und AmpC- Kolonisierung von Masthähnchen. 4. Tagung der DVG Fachgruppe Umwelt- und Tierhygiene, Stuttgart, 16.-17.09.19

Talk: Robé, C.; Daehre, K.; Fiedler, S.; Ewers, C.; Guenther, S.; Roesler, U. (2019): Strain-dependent reduction of broiler colonisation with ESBL-/ AmpC-producing *E. coli* using alternative hygiene- and management measures. XIX ISAH Congress 2019, Breslau, 10.-12.09.19

Talk: Robé, C.; Daehre, K.; Merle, R.; Fiedler, S.; Ewers, C.; Guenther, S.; Roesler, U. (2019): Evaluierung von Managementmaßnahmen zur Reduktion ESBL-/ AmpC- bildender Enterobakterien in der Hähnchenmast. Abschluss-Symposium des Verbundforschungsvorhabens EsRAM, Berlin, 13.06.19

Talk: Robé, C.; Dähre, K.; Blasse, A.; Friese, A.; Roesler, U. (2018): Evaluierung unspezifischer Managementmaßnahmen zur Reduktion ESBL-bildender Enterobakterian in der Masthähnchenproduktion. Symposium Antibiotikaresistenz in der Lebensmittelkette, Berlin, 08.11.18

Poster: Robé, C.; Daehre, K.; Guenther, S.; Roesler, U. (2018): Non- specific hygiene- and management interventions do not reduce broiler colonization with ESBL-/ AmpC- producing *E. coli*. National Symposium on Zoonoses Research 2018, Berlin, 17.-19.10.18

Talk: Robé, C.; Blasse, A.; Rösler, U.; Günther, S. (2018): Experimentelle Kolonisierung von Masthähnchen mit ESBL-/ AmpC- produzierenden *Escherichia coli* im Seeder- Bird Modell. Tagung der DVG- Fachgruppe Bakteriologie und Mykologie, Hannover, 30.05.-01.06.18

Talk: Robé, C.; Blasse, A.; Rösler, U.; Günther, S. (2017): ESBL-/ AmpC- produzierende *Escherichia coli* beim Masthähnchen: Experimentelle Kolonisierung im Seeder-bird Modell. 3. Tagung der DVG Fachgruppe Umwelt- und Tierhygiene, Hannover, 30.11.-01.12.17

Talk: Robé, C.; Blasse, A.; Dähre, K.; Rösler, U.; Günther, S. (2017): Colonisation of broiler chickens with ESBL-/ AmpC- producing *E.coli* using a seeder- bird model and detection of in vivo transformants. National Symposium on Zoonoses Research 2017, Berlin, 12.-13.10.17

Talk: Dähre, K.; Robé, C.; Blasse, A.; Rösler, U.; Günther, S. (2017): ESBL-/AmpC-producing Enterobacteriaceae in broiler fattening farms after cleaning and disinfection – identification of critical control points. National Symposium on Zoonoses Research 2017, Berlin, 12.-13.10.17

Talk: Robé, C.; Daehre, K.; Blasse, A.; Roesler, U.; Guenther, S. (2017): Seeder- bird- model: Colonisation of broiler chickens with ESBL-/ AmpC- producing *E. coli*. 10. Doktorandensymposium und DRS Präsentationsseminar, Berlin, 22.09.17

Talk: Blasse, A; Robé, C.; Friese, A.; Roesler, U. (2017): Do ESBL-/AmpC-producing Enterobacteriaceae survive disinfection measures in broiler farms? XVIII ISAH Congress 2017, Mazatlan, Sinaloa, Mexico, 19.03.-23.03.17

Talk: Robé, C.; Blasse, A.; Friese, A.; Rösler, U. (2016): Identification of critical points during cleaning and disinfection to reduce ESBL-/ AmpC-producing Enterobacteriaceae at broiler farms. 9. Doktorandensymposium und DRS Präsentationsseminar, Berlin, 16.09.16

Poster: Robé, C.; Blasse, A.; Friese, A.; Rösler, U. (2016): Identification of critical steps and control points during cleaning and disinfection to reduce the spread of ESBL-/AmpC-producing Enterobacteriaceae. Tagung der DVG-Fachgruppe “Bakteriologie und Mykologie”, Jena, 31.08.-02.09.16

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14 Interessenskonflikte

Es besteht kein Interessenskonflikt durch die finanzielle Unterstützung der Arbeit.

15 Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 26.01.2022

Caroline Robé