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Airborne emission of ESBL/AmpC-producing *E. coli* from organic fertilizers by tillage operations and wind erosion

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III: List of Abbreviations

AGI-30	all-glass-impinger 30
AIEC	adherent invasive *Escherichia coli*
AmpC	Ampicillinase C
APEC	avian pathogenic *Escherichia coli*
ARG	antibiotic resistance gene
BMEL	German Federal Ministry of Food and Agriculture
BRICS	Brazil, Russia, India, China, and South Africa
BSA	bovine serum albumin
CAZ	ceftazidime
cfu	colony-forming unit
CMY	cephamycinase
CTX	cefotaxime
CTX-M	*cefotaximase-München*
DAEC	diffusely adherent *Escherichia coli*
DNA	deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
E. coli	*Escherichia coli*
e.g	exempli gratia
EAEC	enteroaggregative *Escherichia coli*
EFSA	European Food Safety Authority
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ESBL	extended-spectrum-beta-lactamase
et al	et alii
ETEC	enterotoxigenic *Escherichia coli*
FEP	cefepime
FOX	cefoxitin
IPM	imipeneme
K. pneumoniae	*Klebsiella pneumoniae*
LOG	logarithm
Mbp	mega base pair
MLST	*multilocus sequence type*
MRSA	methicillin-resistant *Staphylococcus aureus*
OR	odds ratio

pAmpCs	plasmid-encoded Ampicillinases C
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PM	particulate matter
PMA	propidium monoazide
RH	relative humidity
SHV	*sulfhydryl reagent variable*
SNP	single-nucleotide-polymorphism
STEC	Shiga toxin-producing *Escherichia coli*
TEM	*Temoneira*
UPEC	uropathogenic *Escherichia coli*
USA	United States of America
VBNC	viable but non-culturable
WGS	whole-genome sequencing
ZALF	Leibniz Centre for Agricultural Landscape Research

1. Introduction: Antibiotic resistance as a major threat to public health

Antibiotic resistance is among the most urgent problems in the “One Health” perspective, as antibiotic-resistant bacteria and genetic elements mediating antibiotic resistance are interchangeable between humans, animals and the environment. In a current report, it was estimated, that by 2050 up to 10 million deaths per year could be attributed to antimicrobial resistance, or in other words: Every three seconds a person will die through an infection caused by antibiotic-resistant bacteria (JIM O’NEILL, 2016).

Aggravatingly, the development of new antibiotics is stagnating because it is economically unattractive (Ventola, 2015). Concerning the development of new antibiotic agents ESBL (extended-spectrum beta-lactamase) and AmpC (Ampicillinase C)- producing Enterobacteriaceae are of outstanding importance and categorized in the critical priority by the World Health Organization (WHO, 2017).

Because of the unavailability of novel antibiotics, a reduction in the use of the currently available antibiotic substances is crucial, as antibiotic overuse was suggested as one of the main elements promoting antibiotic resistance (Llor and Bjerrum, 2014). Prescribing veterinarians play a key role in the reduction of antibiotic usage in the livestock sector. In Germany, the amount (in metric tons) of antibiotic substances used in the livestock sector has decreased by 57% in the years 2011-2017 (BMEL, 2018). This looks like a great success at first glance but when evaluated critically, it is striking that in the same timespan the prescription of fluorochinolones, which are considered critically important for human medicine has increased by 20%. From a global perspective, the estimates on the development of antibiotic use in livestock are devastating. It was projected, that antimicrobial consumption in the livestock sector will rise by 67 % in the years 2010-2030 and double in the BRICS (Brazil, Russia, India, China, and South Africa) countries in the same time span (Van Boeckel et al., 2015).

Similarly to veterinary usage, a rise in antimicrobial consumption was observed in human medicine. It was estimated, that the volume of antibiotics sold in retail and hospital pharmacies increased by 36% between 2000 and 2010. Again, the countries mainly contributing to this rise are the BRICS countries with an increase of 76% (Van Boeckel et al., 2014).

In the environment, antibiotic resistance may be driven by antibiotics emitted to the environment in sub-therapeutic doses by e.g. municipal wastewater systems, pharmaceutical manufacturing effluents, aquaculture, or livestock facilities (Berendonk et al., 2015).

Besides antibiotic substances, antibiotic-resistant bacteria might be emitted directly to the environment and promote environmental antibiotic resistance by horizontal gene transfer to environmental bacteria, or proliferate under selective pressure to antimicrobial compounds (Berendonk et al., 2015).

Antibiotic-resistant bacteria including ESBL/AmpC-producing *Escherichia coli* (*E. coli)* can be emitted from livestock facilities to the environment in different ways. A spread through manure used for fertilization (Kyakuwaire et al., 2019; Xie et al., 2017), a spread through vectors, e.g. flies (Blaak et al., 2014), a waterborne (Blaak et al., 2015a) and an airborne emission (Gao et al., 2015b; von Salviati et al., 2015; Laube et al., 2014) have been described.

Concerning the airborne dissemination of antibiotic-resistant bacteria, there is still a knowledge gap in current research (Environment Agency, 2019). Hence, the investigations in the "SOARiAL" project (spread of antibiotic resistance in an agrarian landscape) funded by the Leibniz Association (grant number: SAW-2017-DSMZ-2) focused on the airborne environmental spread of antibiotic-resistant bacteria present in organic fertilizers.

Potential airborne transmission routes of antibiotic-resistant bacteria to the environment that have never been investigated before and were addressed in this project are the spread through agricultural land utilization and the spread by wind erosion affecting fertilized soil. The subproject carried out at the Institute for Animal Hygiene and Environmental Health (Freie Universität Berlin) concentrated on the airborne spread of ESBL/AmpC-producing *E. coli*.

1.1 *E. coli*

E. coli is a Gram-negative, facultative anaerobic rod-shaped bacterium with a length of up to six µm belonging to the family of Enterobacteriaceae. *E. coli* inhabits the gut of most vertebrates, where it is the predominant aerobic organism and lives in symbiosis with its host (Tenaillon et al., 2010). However, some *E. coli* strains are capable of causing severe intraintestinal or even extraintestinal diseases (Smith et al., 2007).

The six major pathotypes of intraintestinal pathogenic *E. coli* are enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC) including enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enterotoxigenic *E. coli* (ETEC), as well as a new pathotype, adherent invasive *E. coli* (AIEC) (Croxen et al., 2013). Enteropathogenic *E. coli* typically cause diarrheic symptoms but Shiga toxin-producing strains can also cause severe implications like the hemolytic uraemic syndrome, which can lead to renal failure caused by lysis of premature erythrocytes (Nguyen and Sperandio, 2012).

Extraintestinal pathogenic *E. coli* can affect the urinary tract, the central nervous system, the circulatory system and the respiratory tract (Russo and Johnson, 2003). Uropathogenic *E. coli* (UPEC) are the leading cause of community-acquired urinary tract infections with over 80% of the cases (Hilbert, 2011). In veterinary medicine *E. coli* is known to cause a variety of specific disease patterns like diarrhea in weaning piglets (Fairbrother et al., 2005), colisepticeamia in calves (Logan and Penhale, 1971) and lambs (Mason and Corbould, 1981), colibacillosis in poultry (Guabiraba and Schouler, 2015) and colimastitis in cows (Murinda et al., 2019).

1.2 Beta-lactamases

Beta lactamases are bacterial enzymes occurring in Enterobacteriaceae including *E. coli*, which cleave the beta-lactam ring of beta-lactam-antibiotics. A multitude of beta-lactamase enzymes have been described, which made a classification of these enzymes necessary.

The most commonly used classification systems for beta-lactamases are those introduced by Ambler (1980), based on structural similarities and by Bush et al. (1995), based on functional similarities. There is also a more recent classification by Bush and Jacoby (2010), which is a combination of both. However, these classification systems have a high level of complexity, which limits their comprehensibility and practical relevance (Giske et al., 2009). In brief, the structural classification by Ambler categorizes beta-lactamases in four groups, of which groups A, C and D belong to the serine-beta-lactamases and group B are the so-called metallo-beta-lactamases, which require a metal ion (usually zinc) as a cofactor. Penicillinases, which are able to hydrolyze penicillin, broad-spectrum beta-lactamases, which additionally hydrolyze early cephalosporins as well as extended-spectrum beta-lactamases, capable to hydrolyze extended-spectrum cephalosporins of the 3rd and 4th generation and monobactams are all belonging to the Ambler-class A. AmpC-beta-lactamases belong to the Ambler-class C.

1.3 Extended-spectrum beta-lactamases- and AmpC-Cephamycinases

Enterobacteriaceae can acquire the ability to produce ESBL and AmpC enzymes, which hydrolyze the beta-lactam ring of a variety of beta-lactam antibiotics including extended-spectrum cephalosporins of the 3rd, 4th generation, thus inactivating their antibiotic properties. Additionally, resistance to ceftaroline, which belongs to the 5th generation of cephalosporins was reported (Flamm et al., 2012). Frequently, ESBL-producing Enterobacteriaceae are multiresistant and show resistance to other antimicrobial classes, such as fluoroquinolones, aminoglycosides and trimethoprim-sulphamethoxazole (Jacoby and Munoz-Price, 2005). Notably, genes encoding the production of ESBL and AmpC enzymes can be found in both, commensal and pathogenic Enterobacteriaceae (Day et al., 2016). The occurrence of

resistance against these classes of antimicrobials severely limits therapeutic options in human (Remschmidt et al., 2017) and veterinary medicine (Idelevich et al., 2016).

The genetic information encoding for the production of these enzymes is mostly located on mobile genetic elements (Carattoli, 2011) and can therefore be transferred from commensal to pathogenic strains and vice versa. Even a transfer of these resistance genes between different bacterial species was reported (Pfeifer et al., 2010). ESBL enzymes are typically encoded on plasmids (Knothe et al., 1983), while AmpC-enzymes originally are intrinsic cephalosporinases encoded on the chromosomal DNA. However, a growing number of plasmid-encoded AmpCs – so-called pAmpCs- has been observed in recent years (Bajaj et al., 2016).

ESBL/AmpC-producing Enterobacteriaceae can be phenotypically differentiated through their hydrolysis profile. Different methods including disk-diffusion, ESBL etests and broth microdilution can be used for phenotypic differentiation. ESBL-producers are resistant to cefotaxime, variably resistant to ceftazidime and susceptible to cefoxitin. They are inhibited by the use of clavulanic acids, a beta-lactamase inhibitor. AmpC producers are susceptible to cefepime and resistant to cefotaxime, ceftriaxone and cefoxitin (EFSA, 2011). The hydrolytic scheme of ESBL/AmpC-producers is summarized in Table 1.

Table 1 Hydrolytic scheme of ESBL and AmpC enzymes (Adapted from EFSA, 2011)

ß-lactamase	Hydrolysis profile[1]				Inhibited by clavulanic acid
	CAZ/CTX	FOX	FEP	IPM	
ESBL	+	-	+	-	Yes
AmpC	+	+	-	-	No

[1] CAZ: ceftazidime; CTX: cefotaxime; FOX: cefoxitin; FEP: cefepime; IPM: imipenem

Genetically, ESBL-producing Enterobacteriaceae are most frequently belonging to the TEM, SHV and CTX-M gene families (Paterson and Bonomo, 2005). All members of the CTX-M gene family are ESBL-producers. The progenitors of the SHV ($bla_{SHV\text{-}1}$) and TEM ($bla_{TEM\text{-}1}$ and $bla_{TEM\text{-}2}$) gene families are so called-broad spectrum beta-lactamases, which are unable to hydrolyze 3rd, 4th and 5th generation cephalosporins. However, through amino acid substitutions on specific positions around the active site of the enzyme, most members of these gene families now have the capability of expressing the ESBL phenotype (Jacoby and Munoz-Price, 2005). Of note, the genetic diversity of ESBL genes is increasing rapidly and new ESBL variants are described frequently. For example over 170 genotypes belonging to the $bla_{ctx\text{-}}$ gene have been described to date (Ur Rahman et al., 2018). AmpC-beta lactamases are also encoded by genes belonging to different gene families, either named after antibiotics to which they mediate resistance, for example CMY (cephamicins) FOX (cefoxitin) MOX (moxalactam)

and LAT (latamoxef) or based on their first discovery e.g. MIR,DHA, BIL (Philippon et al., 2002).

1.4 ESBL/AmpC-producing *E. coli* in animal husbandry and organic fertilizers

ESBL/AmpC-producing *E. coli* are frequently detected in different farm animals worldwide. *E. coli* is a commensal bacterium colonizing the gut, consequently, ESBL/AmpC-producing *E. coli* are excreted in the feces of farm animals if they are colonized with these bacteria. Subsequently, the feces of the farm animals are used as organic fertilizer. Only studies investigating the prevalence of ESBL/AmpC-producing *E. coli* in livestock animals that did not show clinical symptoms are included in the following overview.

The highest prevalence of ESBL/AmpC-producing *E. coli* among livestock in Germany was reported for broiler chickens, with a prevalence of up to 100% on farm level (Friese et al., 2013; Laube et al., 2013). Accordingly, a prevalence of 100% was reported for broiler farms in studies conducted in the Netherlands (Blaak et al., 2015; Dierikx et al., 2013). Interestingly, in broiler chickens the ESBL/AmpC prevalence seems to increase over the course of a fattening period. In a study conducted in the Netherlands, four fattening flocks were sampled on different points in time. The prevalence of ESBL/AmpC-positive fattening chickens increased within the first week of the fattening period from 0–24% to 96–100% independent of the use of antibiotics and stayed at 100% until slaughter (Dierikx et al., 2013b). Similarly, an increasing prevalence during the fattening period was shown in a German study, where seven broiler flocks were sampled on different time points. For all flocks combined 0.7 % (2/280) of cloacal swabs taken at day 1 of the fattening period were ESBL/AmpC-positive, whereas 32.5 % (91/280) of cloacal swabs taken at the end of the fattening period were ESBL/AmpC positive (Daehre et al., 2017).

For fattening pigs, similarly to broiler chickens, high rates of ESBL/AmpC-positive farms were reported in Germany. Hering et al. (2014) detected cefotaxime resistant *E. coli* in 85% of the investigated farms (41/48). Lower rates were reported by von Salviati et al. (2014) with 43.8% ESBL/AmpC-positive fattening farms (7/16) and Dohmen et al. (2015) with 52.9% positive farms (18/34).

ESBL/AmpC-producing *E. coli* were also detected in other production branches of pig and chicken farming and other livestock species, mostly to a lesser extent. A summary of farm-level prevalence is depicted in Table 2.

Table 2 Farm level prevalence of ESBL/AmpC-producing *E. coli* in different livestock farming types

farm type	country	Investigated farms	ESBL/AmpC prevalence	reference
broiler parent farms	Germany	16	35.5%	Projahn et al., 2017
laying hen farms	The Netherlands	5	100%	Blaak et al., 2015a
turkey rearing farms	United Kingdom	337	6.2%	Randall et al., 2011
turkeys fattening farm	Czech Republic	40	20%	Dolejska et al., 2011
turkey fattening farms	Germany	48	60.4%	Friese et al., 2019
duck fattening farms	South Korea	85	4.7%	Na et al., 2019
pig breeding farms	Germany	12	33%	Friese et al., 2013
dairy farms	The Netherlands	100	41%	Gonggrijp et al., 2016
dairy farms	United Kingdom	48	35.4%	Snow et al., 2012
dairy farms (organic)	The Netherlands	90	13%	Santman-Berends et al., 2017
beef cattle farms	Germany	15	73.3%	Schmid et al., 2013
beef cattle farms (extensive)	USA	17	64.7%	Lee et al., 2020

For further livestock species data on the prevalence of ESBL/AmpC *E. coli* is scarce. ESBL/AmpC-producing *E.* coli have been detected in rabbit farms in Spain, but no prevalence was reported in this study (Blanc et al., 2006). No ESBL/AmpC-producing *E. coli* were detected in rabbits sampled in Tunisia (Ben Sallem et al., 2012). In sheep, no ESBL/AmpC-producing *E. coli* were detected in extensively kept sheep in a study from Tunisia (Ben Sallem et al., 2012). In contrast, in a study recently performed in Saudi Arabia, ESBL-producing *E. coli* were detected in the feces of 41.3% of healthy sheep and 43.3% of healthy goats (Shabana and Al-Enazi, 2020). To date, no study investigated the prevalence of ESBL/AmpC *E. coli* in geese or pigeons.

Concerning horses, a study was recently carried out in the Netherlands. In this study, 362 fecal samples submitted by 281 horse owners were analyzed and ESBL-producing *E. coli* were detected in 39 (11%) of the samples (Hordijk et al., 2020). A higher prevalence of ESBL-producing *E. coli* in horses was reported in a French study, with 29% ESBL-positive healthy horses (de Lagarde et al., 2019).

Of note, in some European countries like Finland, a substantially lower prevalence of ESBL-producing *E. coli* in livestock was reported. In a Finish study 25% of broiler, 3% of pig, 2% of

cattle farms and 0% of turkey farms were ESBL-positive. It was assumed, that this low prevalence is attributed to a prudent use of antibiotics in Finland (Päivärinta et al., 2016).

1.5 Genetic background of ESBL/AmpC-producing *E. coli* in animal husbandry

According to the EFSA report on ESBL/AmpC-producing *E. coli* in livestock published in 2011, the predominant ESBL gene families detected in livestock are $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} the predominant AmpC-family is bla_{CMY}. The genes most commonly associated with resistance in animal husbandry are $bla_{CTX\text{-}M1}$, which is the most commonly identified ESBL genotype, $bla_{CTX\text{-}M14}$, $bla_{TEM\text{-}52}$ and $bla_{SHV\text{-}12}$. The most common AmpC-genotype is $bla_{CMY\text{-}2}$ (EFSA, 2011).

To date, this situation has barely changed, as more recently published reviews concluded that the aforementioned ESBL/AmpC-genotypes are still the most prevalent in animal husbandry. Interestingly, these genotypes are predominant in different livestock species, which might indicate that they have selective advantages compared to other ESBL/AmpC-genotypes.

Concerning the occurrence of ESBL *E. coli* in poultry, Saliu et al. (2017) concluded, that $bla_{CTX\text{-}M1}$, $bla_{SHV\text{-}12}$ and $bla_{TEM\text{-}52}$ are the most frequently detected genotypes.

Concerning cattle, a recently published review by Dantas Palmeira and Ferreira (2020) came to the conclusion, that the most frequently detected ESBL types in cattle belong to the $bla_{CTX\text{-}M1}$ group with the highest prevalence for $bla_{CTX\text{-}M1}$, $bla_{CTX\text{-}M14}$ and $bla_{CTX\text{-}M15}$. ESBL-producing *E. coli* of the bla_{SHV} and bla_{TEM} genotypes have also been detected in cattle worldwide with the highest occurence of $bla_{SHV\text{-}12}$ and $bla_{TEM\text{-}52}$, respectively. This study did not review the prevalence of AmpC-beta-lactamases, however, AmpC-producing *E. coli* of the $bla_{CMY\text{-}2}$ subtype have been detected in beef and dairy cattle in various studies (Lee et al., 2020; Manga et al., 2019; Gonggrijp et al., 2016).

No recent review concerning the genetic background of the predominant ESBL/AmpC-genotypes is available for pigs. However, a recently conducted study investigated the prevalence of ESBL/AmpC genotypes in 200 *E. coli* Isolates taken from pigs in spain in the years 1998 to 2018. In this study, it was concluded, that the most prevalent ESBL genotypes identified in pigs are $bla_{CTX\text{-}M1}$, $bla_{CTX\text{-}M14}$ and $bla_{CTX\text{-}M32}$. The sole AmpC-Genotype detected in the study was $bla_{CMY\text{-}2}$ (Aguirre et al., 2020).

1.6 ESBL/AmpC-producing *E. coli* in the environment

ESBL/AmpC-producing *E. coli* have been detected in different matrices in the environment. The occurrence of ESBL/AmpC-*E. coli* in the environment is frequently linked to the occurrence of these bacteria in animal husbandry.

One of the most obvious transmission paths of ESBL/AmpC *E.* coli from livestock facilities to the environment, which has been investigated in several studies, is the application of ESBL/AmpC-positive organic fertilizers to arable land. ESBL/AmpC *E. coli* have been detected in soil enriched with organic fertilizers from different livestock species. ESBL-producing *E. coli* were detected in soil fertilized with pig manure in studies carried out in Germany (Friese et al., 2013) and China (Gao et al., 2015a). In another Chinese study ESBL-producing *E. coli* were detected in soil fertilized with pig and broiler manure (Zheng et al., 2017). In a French study, ESBL-producing *E. coli* were detected in soil fertilized with cow manure, even up to one year after fertilizer application (Hartmann et al., 2012). In a recent study ESBL/AmpC-producing *E. coli* were detected in 20.1% (16/77) of soil samples taken at extensively managed beef cattle farms (Lee et al., 2020). Fertilizer management can play a critical role in preventing the spread of ESBL/AmpC-producing *E. coli* in the environment through organic fertilizers.

Another possible transmission path of ESBL/AmpC-producing *E.coli* from livestock facilities to the environment that has been proven relevant is via the airborne route. ESBL/AmpC -producing *E. coli* were detected in air samples taken in the vicinity of pig (von Salviati et al., 2015; Gao et al., 2015b) and broiler (Laube et al., 2014) barns. In the studies by von Salviati, Laube et al., ESBL/AmpC-producing *E. coli* deposited to ground surfaces were detected in up to 500 m distance downwind from the livestock facilities. Besides the emission from livestock, an airborne emission of ESBL-producing *E. coli* to the environment has also been shown from municipal sewage in two studies (Korzeniewska and Harnisz, 2013; Teixeira et al., 2016).

Apart from airborne emission, waterborne emission seems to play an important role in the environmental dissemination of ESBL/AmpC -producing *E. coli*. Several studies detected ESBL/AmpC-producing *E. coli* in surface water in Germany (Falgenhauer et al., 2019), Norway (Jørgensen et al., 2017), the Netherlands (Blaak et al., 2015b) and the UK (Leonard et al., 2018). In a Chinese study, the detection of ESBL-producing *E. coli* in surface water was attributed to a nearby chicken farm. In this study, ESBL-producing *E. coli* were detected in 25.9 % (7/27) of the water samples taken 10 m downstream and 3.7 % (1/27) of the samples taken 100 m downstream. Five ESBL-producing isolates from the surface water showed >90% similarity with isolates from feces from the nearby chicken farm, which might indicate a waterborne emission (Gao et al., 2014). Blaak et al. (2015a) detected ESBL-producing *E. coli* in 91% of water samples taken from surface water adjacent to broiler stables while or shortly after cleaning between production rounds.

In a Swedish study, ESBL-producing *E. coli* have not only be detected in surface water, but also in 17% (16/96) of feces samples taken from seagulls (Atterby et al., 2017), indicating a waterborne spread of ESBL-producing *E. coli* to wildlife.

Besides gulls, ESBL-producing *E. coli* have also been detected in a variety of other wild animals species e.g. birds of prey in Germany and Mongolia (Guenther et al., 2012), vampire bats in Peru (Benavides et al., 2018), urban brown rats in Austria (Desvars-Larrive et al., 2019) and with a low prevalence of 0.9% positive samples (1/108) in wild boars in Italy (Bonardi et al., 2018).

Additionally, flies could play a role in the environmental dissemination of ESBL/AmpC-producing *E. coli* as living vectors. Flies have been identified as carriers of ESBL/AmpC *E. coli* in studies carried out in rural (von Salviati et al., 2015; Blaak et al., 2014) and urban areas (Wetzker et al., 2019).

1.7 Potential Transmission of ESBL/AmpC-producing *E. coli* from animal husbandry to humans in the "One Health" perspective

The transmission of ESBL/AmpC-producing *E. coli* from animals to humans and vice versa has been hypothesized for more than a decade (Smet et al., 2010). A certain proof of an animal to human transmission of ESBL/AmpC *E. coli* is missing because finding identical resistance traits in different places does not necessarily prove a causal relationship (Madec et al., 2017), however, several studies came to the conclusion that an animal to human transmission seems likely. In a recently performed study, a close genetic relationship of isolates from human, livestock and food isolates for AmpC-(Pietsch et al., 2018) producing *E. coli* was shown. Additionally, it was demonstrated that the STs (sequence types) 10 and 410, which are common in animals are occurring in hospital settings (Pietsch et al., 2017).

In contrast, in another recently performed study, a molecular relatedness of ESBL/AmpC-producing *E. coli* from humans, animals, food and the environment could not be proven and distinguishable ESBL/AmpC *E. coli* transmission cycles in different hosts were suspected (Dorado-García et al., 2018).

One possible transmission route of ESBL/AmpC-producing *E. coli* between farm animals and humans is by direct contact. Farmers have contact with livestock daily, therefore several studies investigating the colonization of farmers with ESBL/AmpC-producing *E. coli* were carried out. To draw conclusions, whether a professional group is at a higher risk to be colonized with ESBL/AmpC-producing *E. coli*, knowledge of the ESBL/AmpC-colonization in the general population is necessary. For Germany, it was estimated that 3.5 - 6.8 % of the general population is colonized with ESBL/AmpC-producing *E. coli* (Idelevich et al., 2016). In an English study, of 2430 human fecal samples analyzed for *bla*$_{CTX-M}$ - *E. coli,* 7.3% were positive (McNulty et al., 2018)

The highest colonization rates with ESBL/AmpC-producing *E. coli* among farmers were detected in broiler farmers in two studies carried out in the Netherlands. Dierikx et al. (2013) reported an ESBL/AmpC-colonization rate of 33% for broiler farmers (6/18). Huijbers et al. (2014) reported a similar proportion of 27.3% ESBL/AmpC-colonized broiler farmers (15/55). In the study by Huijbers et al., the ESBL/AmpC-colonization of the partners and family of the broiler farmers was additionally investigated. Of these persons, which had contact with the animals to a lesser extent, 14% were ESBL/AmpC positive (12/86). In this study, five pairs of human–broiler isolates showed identical genes, plasmid families and *E. coli* sequence types, therefore a clonal transmission between humans and chickens was assumed.

In a study carried out on pig and cattle farms in Germany, 6.3% of pig farmers (2/32) and 12.5% (3/24) of cattle farmers were colonized with ESBL-producing *E. coli.* For one cattle farm, an identical MLST sequence type and $bla_{\text{CTX-M}}$ allele could be identified in ESBL *E. coli* isolates from cattle and humans, indicating a zoonotic transfer (Dahms et al., 2015). In another German study, a similar rate of colonization in pig farmers of 6.0% was determined (5/84). In this study, it was possible to identify clonally identical isolates from the farm environment and humans in one case (Fischer et al., 2017).

Besides direct contact, ESBL/AmpC-producing *E. coli* might be transferred to farm personnel working in the barns via the airborne route. However, the relevance of this potential transmission path is still unclear. Dohmen et al. (2017b) quantified the presence of $bla_{\text{CTX-M}}$ group 1 genes in dust, pig feces and stool samples from 131 pig farmers, family members and employees working on pig farms. Human prevalence of $bla_{\text{CTX-M}}$ group 1 carriage was 3.6%. It was detected in dust on 26% of the farms and in pigs on 35% of the farms. No positive correlation in the odds ratio (OR) for an increased ESBL-colonization in humans was found for ESBL-positive farms. In a German study, *E. coli* were detected in only 7% (8/114) of nasal swabs taken from pig farmers and no ESBL-producing *E. coli* were detected in the swab samples, suggesting that in contrast to MRSA, the nares are no reservoir for ESBL *E.coli* (Fischer et al., 2016). A different approach to investigate transmission scenarios of ESBL *E. coli* to humans in turkey production was used in an Italian study: A semi-quantitative Failure Modes and Effect Analysis was conducted with detailed information on turkey farming phases, working practices and all current data from the scientific literature on risk factors and prevalence of ESBL- (and MRSA) colonization in turkey farmers. The authors came to the conclusion, that ESBL-colonization of farmers working on turkey farms is associated with direct contact and the oral-fecal route. Tipping over turkeys turned on their back, and the individual administration of therapies were identified as working practices associated with ESBL-colonization. Regarding MRSA- colonization analyzed in the same study, exposure to dust by milling and removal of litter was identified as a risk factor, but no correlation was found between

ESBL *E.coli* carriage and this working practices, indicating that airborne colonization through ESBL *E. coli* seems unlikely (Franceschini et al., 2019).

Persons working in slaughterhouses likewise have close contact with livestock animals and carcasses. In a study conducted in a pig slaughterhouse in the Netherlands, the prevalence of ESBL-carriage in slaughterhouse workers was 4.8% (16/334), which is not higher than the ESBL-colonization in the general population. However, a higher prevalence of ESBL-carriage of 33% was observed for workers who removed the lungs, heart, liver and tongue (Dohmen et al., 2017a)

Veterinarians are another professional group, for which an increased rate of ESBL/AmpC-producing *E. coli* colonization could be suspected because of their close contact with animals. The only study investigating this issue so far was carried out in Finland. In this study, only 3.0% of veterinarians (9/297) carried ESBL-producing *E. coli* (Verkola et al., 2019). The transferability of the results of this study to the situation in other countries is questionable, because of the low prevalence of ESBL-producing *E. coli* in livestock in Finland (Päivärinta et al., 2016).

Because of the high prevalence of ESBL/AmpC *E.* coli in livestock facilities, it was assumed that living in rural areas might be associated with a higher risk to be colonized with ESBL/AmpC *E. coli* compared to living in urban areas. However, living in livestock dense areas does not seem to be a risk factor for ESBL/AmpC-carriage: Wielders et al. (2017) analyzed 2432 fecal samples and corresponding questionnaires from adult humans and detected ESBL/AmpC-producing *E. coli* in 4.5% of the fecal samples from people living in livestock dense areas. This ESBL/AmpC-colonization rate is similar to the general population, it was therefore concluded that living in close proximity to livestock is not a risk factor for an increased carriage of ESBL/AmpC-producing *E. coli.*

Another potential way of transmission of ESBL/AmpC-producing *E. coli* from livestock to humans that has been in the focus of research is via the food chain. ESBL/AmpC-producing *E. coli* are frequently detected in retail meat in Germany and other European countries. Typically the highest prevalence is reported for poultry meat.

Kola et al. (2012) detected ESBL-producing *E. coli* in 43.9% of 175 retail chicken meat samples and did not observe differences in the ESBL prevalence between organic and conventional chicken samples. In a recently published German study, 2,256 food samples taken in 2012 and 2013 were analyzed for the presence of ESBL/AmpC-producing *E. coli.* The prevalence was highest in chicken meat with 74.9 % (149/199), followed by turkey meat with 40.1 % (91/227). The prevalence reported for pork and beef was considerably lower with 12.7% (36/283) and 4.2% (12/284), respectively. In this study, ESBL/AmpC-producing *E. coli* were

detected in only 0.3% (1/399) of vegetable samples analyzed (Kaesbohrer et al., 2019). In an English study, 397 raw meat and 400 fruit and vegetable samples were analyzed for ESBL-producing *E. coli.* ESBL *E. coli* were detected in 65.4% of the chicken samples, 2.5% of the pork samples and 1.9% of the beef samples after enrichment, but in none of the fruit or vegetable samples (Randall et al., 2017). Despite the high prevalence of ESBL *E. coli* reported in chicken meat, a recently published study from the Netherlands observed a decreasing prevalence of ESBL contamination in retail chicken meat from 68.3% in 2014 to 44.6% in 2015, which was assumed to be correlated with prudent use of antibiotics in broiler farming in the latter year (Huizinga et al., 2019).

The relevance of transmission of ESBL/AmpC-producing *E. coli* to humans via the food chain is controversial. Similar plasmids, genes and *E. coli* strains were detected in patients, poultry and retail chicken meat (Leverstein-van Hall et al., 2011). However, de Been et al. (2014) used whole-genome sequencing (WGS) to analyze human and poultry- associated ESBL *E. coli* strains that had previously been considered identical based on traditional typing methods and found a considerable heterogeneity between human and poultry-associated isolates with at least 1263 single-nucleotide polymorphisms (SNPs) per Mbp core genome between the isolates. In contrast, in the same study strains from humans and pigs only differed by 1.8 SNPs per Mbp core genome. It was therefore assumed, that for the transmission of ESBL strains from poultry to humans, clonal transmission is neglectable, but instead, resistance genes are mainly disseminated in animals and humans via plasmids.

In a German study, it was shown, that frequent consumption of pork (three or more meals per week) is positively correlated with ESBL *E. coli* colonization (OR=3.5), but no positive correlation between ESBL colonization and the consumption of poultry was detected (Leistner et al., 2013). Denkel et al. (2016) explained this deviation by assuming, that especially the consumption of raw meat products contaminated with ESBL/AmpC *E. coli* might be an important source of transmission because plasmids are likely destroyed by cooking. To their knowledge, no dish in the European culture contains raw poultry but several dishes contain raw pork.

Crops and vegetables may get contaminated with ESBL/AmpC-producing *E. coli* by the application of ESBL/AmpC containing organic fertilizers (Hartmann et al., 2012). But as previously mentioned, the prevalence of ESBL/AmpC *E. coli* detected in vegetables is typically very low in Europe (Kaesbohrer et al., 2019; Randall et al., 2017; van Hoek et al., 2015). However, a considerably higher prevalence was reported for vegetables imported from developing countries, with ESBL-producing Enterobacteriaceae detected in 25.4% (43/169) of the vegetable samples (Zurfluh et al., 2015). Despite the generally low prevalence of ESBL/AmpC-producing *E. coli* in vegetables compared to meat products, surprisingly in a

recent study it was found, that the OR for ESBL *E. coli* or *K. pneumoniae* carriage was 2.2 for vegetarians and 1.6 for pescatarians when compared with non-vegetarians (Meijs et al., 2020).

The aforementioned contamination of soil, crops and vegetables is one possible scenario, where an environmental contamination with livestock-associated ESBL/AmpC *E. coli* could pose a health hazard for humans, in this case via the food chain. Furthermore, if the environment is contaminated with livestock-associated ESBL/AmpC *E. coli* a transmission to humans via the waterborne or airborne route is also imaginable.

Several studies suspected, that colonization of humans with ESBL/AmpC *E. coli* via the waterborne route is possible. In a Swedish and a Norwegian study, ESBL-producing *E. coli* with similar characteristics were found in Isolates originating from surface water and humans (Atterby et al., 2017; Jørgensen et al., 2017). Leonard et al. (2018) found a significantly increased OR of 4.1 for surfers to be colonized with ESBL *E. coli* compared to non- surfers (p= 0.046). However, for humans in order to get colonized with ESBL *E. coli* originating from livestock via the waterborne route, contamination of recreational waters with these resistant bacteria is a prerequisite. For Germany, contamination of recreational water with livestock-associated ESBL-producing *E. coli* originating from organic fertilizers seems unlikely, because the contamination of ground- and surface water through the application of organic fertilizers has to be avoided according to the German fertilizing ordinance (Düngeverordnung; § 3 (1)). However, the situation might be different in other parts of the world. A waterborne spread of ESBL *E. coli* from a chicken farm to surface water was suspected in a Chinese study (Gao et al., 2014).

In contrast to the waterborne route, indications for an airborne environmental transmission of livestock-associated ESBL/AmpC-producing *E. coli* to humans are to date still lacking in research.

1.8 Livestock-associated bioaerosols as carriers for antibiotic resistance

Livestock associated dust has the capability of forming bioaerosols and originates mainly from feed, skin, feathers, bedding material, and feces (Carpenter, 1986). Bioaerosols may carry substances or microorganisms, which can lead to mechanical, infectious, immunosuppressive, allergic, or toxic health implications (Hartung and Saleh, 2007). However, in order to cause such health hazards in humans and farm animals, aerosolized particles have to access the respiratory tract. The smaller particles are, the deeper they can be deposited in the respiratory tract. Particles with a diameter of 7 µm or less are considered alveoli-accessible (Vincent and Mark, 1981). A systematic review came to the conclusion that particles smaller than 3 µm have an increased probability to deposit deep in the lungs (Williams et al., 2011).

Dust from animal facilities is an important carrier of bacteria, fungi and viruses (Hartung and Saleh, 2007). The majority of bacteria detected in bioaerosols is Gram-positive, only a small proportion is Gram-negative (Zucker et al., 2000). Bakutis et al. (2004) reported a proportion of 2.6% Gram-negative bacteria of the total airborne bacteria in poultry houses. The reason for the low abundance of Gram-negative bacteria in bioaerosols is probably attributed to their thinner cell wall compared to Gram-positive bacteria, which makes them more susceptible to environmental stress (Zhao et al., 2014). Despite the low tenacity of Gram-negative bacteria in the aerosolized state, they seem to have a considerably increased tenacity when adhering to dust. It was reported, that *E. coli* survived in dust samples for over 20 years (Schulz et al., 2016). O'Brien et al. (2016) extracted genomic DNA from settled dust and air samples in poultry houses. They used high throughput genomic sequencing to analyze the composition of dust and air samples and concluded, that poultry dust is mainly composed of bacteria (64–67%) with only a small quantity of avian, human and feed DNA (< 2% of total reads). Staphylococci, salinicocci and lactobacilli were the most abundant bacterial genera detected in the samples and aerosolized dust showed little variation between the samples compared to settled dust.

Bioaerosols might contribute to the spread of antibiotic resistance in livestock facilities and in the environment, as they might act as carriers for either residues of antimicrobial substances, viable antibiotic-resistant bacteria or antibiotic resistance genes (ARG) encoded on mobile genetic elements like plasmids or integrons.

Antimicrobial residues have been detected in dust and air samples in various studies. Hamscher et al. (2003) detected antibiotic residues in 90% of analyzed dust samples. Tylosin, tetracyclines, sulfamethazine and chloramphenicol have been detected in total amounts up to 12.5 mg/kg dust. In a recently published study, fluoroquinolone residues were detected in 47% (49/125) of dust samples from pig, poultry and cattle barns (Schulz et al., 2019). A study conducted by M. W. Murphy et al. (2007) investigated the concentration of tylosin in air samples and detected an average tylosin concentration of 18 ng/m^3 in the inhalable and 3 ng/m^3 in the respirable fraction of the air samples.

Viable airborne antibiotic-resistant bacteria have been detected in various studies inside livestock facilities and their vicinity. Brooks et al. (2010) took air samples inside and in the proximity of broiler barns at different stages of the fattening period. Bacteria were non-selectively cultured from the air samples and bacterial isolates were screened for resistance to 12 antibiotic classes. A cyclical increase in bacterial concentrations in the air samples of over 2 LOG_{10} was observed in the progress of the fattening cycle. Staphylococci were estimated to account for over 90% of cultured aerobic bacteria. Resistance to more than four antibiotic classes was rarely observed in the isolates; however, an upward trend concerning overall resistance was observed as the flock cycle progressed. Although in this study, high

levels of cultivable, antibiotic-resistant bacteria were detected inside the chicken house, bacterial levels in the outdoor samples were much lower: An average of 4.0 x 10^6 cfu/m³ cultivable bacteria was detected in air samples taken inside the barn compared to 6.7 x 10^3 cfu/m³ outside the barns.

Of note, most studies that investigated the presence of airborne livestock-associated antibiotic-resistant bacteria focused on the detection of specific bacteria, mainly MRSA and ESBL/AmpC-producing Enterobacteriaceae (Environment Agency, 2019). MRSA have been detected in air samples taken in pig barns and the proximity of the barns (Schmithausen et al., 2015; Schulz et al., 2012). Schulz et al. (2012) detected MRSA in air samples taken in a distance of up to 150 m downwind from the pig barn. As mentioned previously, ESBL-producing Enterobacteriaceae were detected in pig and broiler barns and the vicinity of these livestock facilities. Concerning pig farms, Von Salviati et al. (2015) detected ESBL/AmpC-producing *E. coli* in 9% (6/63) of indoor air samples and 5% (2/36) of outdoor samples. Gao et al. (2015b) detected ESBL *E. coli* in 25% of air samples (3/12) taken inside pig barns and 15% (3/20) of air samples taken outside. Concerning broiler farms, ESBL/AmpC-producing *E. coli* were detected in 16% (10/63) of the air samples taken inside the barns and 7.5% (3/40) of ambient air samples by Laube et al. (2014). Blaak et al. (2015a) reported 7.7% ESBL *E. coli* positive air samples taken inside broiler barns, however, no ESBL-producing *E. coli* were detected in air samples taken in the vicinity of the barns in this study. To date, no ESBL/AmpC -producing *E. coli* have been detected in air samples taken on or in the vicinity of cattle farms. However, Navajas-Benito et al. (2017) detected a multidrug-resistant *E. coli* strain in air samples taken inside and outside a cattle farm.

Besides the airborne emission of viable, antibiotic-resistant bacteria from livestock facilities to the environment, an airborne spread of livestock-associated antibiotic resistance genes was reported. Gao et al. (2018) took 29 up- and downwind air samples from four composting plants, and detected 22 subtypes of ARGs in the air samples. De Rooij et al. (2019) analyzed air samples taken in different distances from livestock facilities for the *tet*W and *mec*A resistance genes and reported a spatial association between the detection of the resistance genes and the distance from the livestock facilities.

1.9 Wind erosion as a potential source for an airborne environmental spread of antibiotic-resistant bacteria from fertilized soil

Wind erosion is occurring, when wind speeds exceed 6 m/s over dry soil (Zobeck and Van Pelt, 2015). There are three modes of wind erosion: Surface creep, saltation and suspension. Surface creep affects large particles or soil aggregates over 500 µm, which move by rolling

along the soil surface. Saltation affects particles of about 70 to 500 µm and results in particles bouncing along the surface typically under a meter in height. Suspension affects the smallest fraction of particles below 70 µm, which are typically aerosolized for a period of time, which is strongly related to particle size (TSOAR and PYE, 1987). Saltation accounts for the majority of total particles carried in the wind (50–70%), followed by suspension (30–40%) and surface creep (5–25%) (Acosta-Martínez et al., 2015)

Research on bioaerosol formation caused by wind erosion has very rarely been conducted. Jones and Harrison (2004) stated, that bacteria may be aerosolized from the soil by wind erosion and that a quarter of the total airborne particulate may consist of biological material in the form of pollen, fungal spores, bacteria, viruses, or fragments of plant and animal matter. Agricultural systems can significantly contribute to atmospheric dust loading and aerosolization of soil microorganisms (Acosta-Martínez et al., 2015). Gardner et al. (2012) used a portable field wind tunnel to generate aerosols from organic-rich soils and subsequently analyzed the bacterial diversity via pyrosequencing. They concluded that Bacteroidetes are associated with dust, whereas Proteobacteria were associated with coarser wind-eroded sediment, which suggests that particle size influences the phyla of bacteria that are released by wind erosion. Schlatter et al. (2018) used high-throughput DNA sequencing to investigate the impact of biosolid-amendment on the transport of dust-associated fungal and bacterial communities by simulating wind events and concluded that dust from biosolid-amended fields did not harbor a greater abundance of potentially pathogenic taxa except for Clostridiaceae, which were enriched after amendment.

Likewise, knowledge of the bioaerosol emission potential of organic fertilizers is scarce. Only one study investigated this issue under experimental conditions to date. In the study by Chien et al. (2011) chicken and pig feces were exposed to an airflow of 0.01 m/sec in a test chamber. It was concluded, that the bacterial count (in cfu) released by chicken feces was approximately 1 order of magnitude higher compared to pig feces. Over 10^4 cfu were released from 1g chicken feces per hour and approximately 80% of the generated bioaerosol belonged to the respirable fraction.

As mentioned previously, it was shown that antibiotic-resistant bacteria including MRSA and ESBL-producing Enterobacteriaceae were detected in fertilized soils (Zheng et al., 2017; Gao et al., 2015a; Friese et al., 2013; Hartmann et al., 2012). If this fertilized soil is affected by wind erosion, this could lead to bioaerosol formation and might enable an airborne spread of resistant bacteria to the environment. To date, this study is the first to investigate wind-driven emission of antibiotic-resistant, livestock-associated bacteria from fertilized agricultural soil. Additional research in this field is warranted.

2. Outline of the Study

The project “SOARiAL” (spread of antibiotic resistance in an agrarian landscape) was carried out between April 2017 and September 2020 and was funded by the Leibniz Association (grant number: SAW-2017-DSMZ-2).

2.1 Objective of the study

The main research question of the “SOARiAL” collaborative joint project was to elucidate, to what extent antibiotic-resistant bacteria are dispersed with soil dust from fertilized agricultural fields.

The specific objectives that were addressed in the subproject carried out at the Institute for Animal Hygiene and Environmental Health (Freie Universität Berlin) included:

- Isolation and quantification of viable ESBL/AmpC-producing Enterobacteriaceae from organic fertilizers, soil and dust and Molecular characterization of the bacterial isolates.
- Assessment of the potential of ESBL/AmpC -producing *E. coli* for atmospheric release and estimation of the bioaerosol emission potential of soil fertilized with chicken litter in dependence of the wind velocity.
- The comparison of two air samplers (AGI-30 and Coriolis µ) regarding their biological collection efficiency at different wind velocities
- Evaluation of fertilizer management for reducing emissions.
- The tenacity of poultry- associated ESBL/AmpC-producing *E. coli* strains and the laboratory strain *E. coli* K12 in the aerosolized state under different conditions.

To address the specific objectives and questions, four experimental series were carried out in the course of the project.

2.2 Experimental series carried out to investigate the potential of an airborne environmental spread of ESBL/AmpC *E. coli* through agricultural land utilization and wind erosion

A summary of the experimental series carried out in this study including the investigated objectives is depicted in Figure 1.

1. Field trials

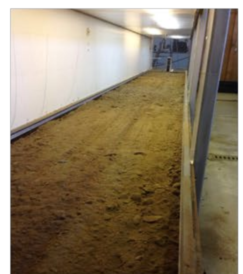
Investigation of an airborne spread of ESBL/AmpC- producing *E. coli* during tillage

2. Litter storage trials

Investigation of the tenacity of ESBL- producing *E. coli* in stored chicken litter

3. Wind tunnel trials

Investigation of an airborne environmental spread of ESBL/AmpC- producing *E. coli* by wind erosion



4. Aerosol chamber trials

Investigation of the tenacity of ESBL/AmpC- producing *E. coli* in the airborne state

Figure 1: Experimental series carried out during the SOARiAL- project

At the beginning of the study, two field trials with chicken litter were carried out. One field trial with pig slurry was carried out at a later stage of the study. The aim of the field trials was to quantify the airborne spread of ESBL/AmpC-producing *E. coli* from organic fertilizers to the environment during agricultural land utilization and through subsequent wind erosion.

For the first field trial, carried out at a trial plot near Potsdam, chicken litter was pretreated in four different ways: fresh, stored, composted and dried. The litter was subsequently applied to the field and incorporated the following day. Air samples were taken at different distances from the emission source during manure application and incorporation. Additionally, samples from the differently treated chicken litter and fertilized and non-fertilized soil from the trial plots were taken for microbiological analysis and the field surface was sampled with boot swabs before

and after litter application and incorporation. All samples were analyzed for ESBL/AmpC-producing and non-resistant *E. coli.* For the air samples, the total viable count was additionally determined. For the second field trial with chicken litter, carried out at a trial plot in Friedrichshof, the sampling scheme and microbiological analyses were carried out accordingly. However, only fresh chicken litter was used in this trial and broiler barns were screened for the presence of ESBL/AmpC *E. coli* in advance to identify chicken litter with a high concentration of ESBL/AmpC-producing *E. coli*. The third field trial with ESBL-positive untreated pig slurry in Paulinenaue was carried out accordingly.

The second experimental series carried out in this study were the short-term litter storage trials. In these trials, the aim was to assess, whether a five day storage period is sufficient to inactivate ESBL-producing *E. coli* in anaerobically stored chicken litter.

Two litter storage trials, one in winter and one in summer were carried out. For both trials, ESBL-positive chicken litter was removed from a broiler barn and stored behind the barn on a concrete surface for five days. Triplicates of surface and deep litter samples were taken from the litter in close sampling intervals at the beginning of the trials and daily in the last three days. All samples were qualitatively and quantitatively analyzed for ESBL-producing and non-resistant *E. coli* and enterococci. Additionally, the pH, moisture and temperature of all litter samples were determined.

The third experimental series carried out were the wind erosion trials. These trials were carried out in a wind tunnel under standardized conditions at the Leibniz Centre for Agricultural Landscape Research (ZALF). The aim was to assess, whether there is an airborne emission of ESBL/AmpC-producing *E.coli* from fertilized soil by wind erosion.

For the three wind erosion trials, ESBL/AmpC-positive chicken litter was mixed with sandy soil and exposed to different wind speeds in the wind tunnel. Air samples were taken at the terminus of the wind tunnel with the all-glass-impinger-30 (AGI-30) and Coriolis µ air samplers. Additionally, soil and litter samples and samples of the soil-litter mixture were taken. All samples were analyzed for ESBL/AmpC-producing and non-resistant *E. coli*. For the air samples, the total viable count was additionally determined.

The fourth experimental series carried out in the project were the aerosol chamber trials. In the aerosol chamber trials, the aim was to gain knowledge on the tenacity of ESBL/AmpC-producing, poultry-associated *E. coli* strains and the laboratory strain *E. coli* K12 in the aerosolized state under different conditions. The *E. coli* strains were aerosolized in the aerosol chamber at different relative humidities (RH; 30 %, 50 % and 70 %) and with and without the addition of organic substances (10 g/l BSA and yeast extract). Air samples were taken for 30

min with three AGI-30 impingers. The air samples were subsequently analyzed for the quantity of *E. coli*.

3. Publications

3.1 Publication I

Siller, Paul; Daehre, Katrin; Thiel, Nadine; Nübel, Ulrich; Roesler, Uwe (2020): **Impact of short-term storage on the quantity of extended-spectrum beta-lactamase-producing Escherichia coli in broiler litter under practical conditions**. In: Poultry Science Volume 99, p. 2125-2135. DOI: https://doi.org/10.1016/j.psj.2019.11.043

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Impact of short-term storage on the quantity of extended-spectrum beta-lactamase–producing *Escherichia coli* in broiler litter under practical conditions

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ABSTRACT Applying broiler litter containing extended-spectrum beta-lactamase (**ESBL**)–producing *Escherichia coli* (***E. coli***) to arable land poses a potential risk for humans to get colonized by contact with contaminated soil or vegetables. Therefore, an inactivation of these bacteria before land application of litter is crucial. We performed 2 short-term litter storage trials (one in summer and winter, respectively), each covering a time span of 5 D to investigate the effectiveness of this method for inactivation of ESBL-producing *E. coli* in chicken litter. Surface and deep litter samples were taken from a stacked, ESBL-positive chicken litter heap in triplicates in close sampling intervals at the beginning and daily for the last 3 D of the experiments. Samples were analyzed quantitatively and qualitatively for ESBL-producing *E. coli*, total *E. coli*, and enterococci. Selected isolates were further characterized by whole-genome sequencing (**WGS**). In the depth of the heap ESBL-producing *E. coli* were detected quantitatively until 72 h and qualitatively until the end of the trial in winter. In summer detection was possible quantitatively up to 36 h and qualitatively until 72 h. For surface litter samples a qualitative detection of ESBL-producing *E. coli* was possible in all samples taken in both trials. In the deep samples a significant decrease in the bacterial counts of over 2 Log_{10} was observed for total *E. coli* in the winter and for total *E. coli* and enterococci in the summer. Genetic differences of the isolates analyzed by WGS did not correlate with survival advantage. In conclusion, short-term storage of chicken litter stacked in heaps is a useful tool for the reduction of bacterial counts including ESBL-producing *E. coli*. However, incomplete inactivation was observed at the surface of the heap and at low ambient temperatures. Therefore, an extension of the storage period in winter as well as turning of the heap to provide aerobic composting conditions should be considered if working and storage capacities are available on the farms.

Key words: antibiotic resistance, ESBL, *E. coli*, broiler litter, environment

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INTRODUCTION

Extended-spectrum beta-lactamases (**ESBL**) are enzymes occurring in Enterobacteriaceae. Their ability to hydrolyze the β-lactam ring of a variety of β-lactam antibiotics including extended-spectrum cephalosporins of the third and fourth generation leads to an inactivation of antibiotic properties. Cephalosporins of the third and fourth generation have a broad-spectrum activity against Gram-positive and Gram-negative bacteria and are often used for the treatment of infections in intensive care units. The emergence of resistance against these drugs limits therapeutic options (Remschmidt et al., 2017).

ESBL-producing *Escherichia coli* (***E. coli***) are commonly found in broiler production with a prevalence of up to 100% in fattening farms (Dierikx et al., 2010, 2013; Laube et al., 2013; Blaak et al., 2015; Hering et al., 2016; Daehre et al., 2018). Furthermore, ESBL-producing *E. coli* have been detected in the vicinity of broiler barns, and an airborne and waterborne

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dissemination have been described (Laube et al., 2014; Blaak et al., 2015). In Germany, 600 million broiler chickens were slaughtered in 2017 (Statistisches Bundesamt, 2017) contributing to the 1.1 million metric tons of poultry litter that are spread to arable land in Germany annually (Statistisches Bundesamt, 2016). This presents a possible important emission source of resistant bacteria from the barns to the environment.

Blaak et al. (2015) reported that ESBL-producing *E. coli* were found in the soil at a distance of 1-5 m of litter storage areas with up to 2.0×10^4 cfu/kg. Additionally, it was shown that ESBL-producing *E. coli* can be transferred from animal husbandry to soil and are able to survive on the fields for at least 1 y (Hartmann et al., 2012). A cross-transmission of ESBL-producing *E. coli* and mobile genetic elements encoding for the production of ESBL between animals, including chickens, humans, and the environment is hypothesized (Leverstein-van Hall et al., 2011; Huijbers et al., 2014). The spread of litter containing ESBL-producing Enterobacteriaceae to the environment poses a potential risk for humans to be colonized with these bacteria after contact with contaminated soils or via contaminated vegetables. Hence, inactivation of these resistant bacteria before land application is crucial.

Storing the litter in piles after removal from the barns could be a useful and cost efficient tool for the reduction of resistant bacteria in litter. Studies that investigated the reduction of nonresistant *E. coli* in chicken litter by storage (anaerobic conditions) and composting (active aeration) under practical conditions were performed previously (Erickson et al., 2010; Wilkinson et al., 2011). Considering practicability and economic sustainability short-term storage of litter presents the most advantageous method of litter treatment. The decline of ESBL-producing *E. coli* in chicken litter under field conditions has not been investigated so far. For a more detailed assessment of bacterial inactivation in short-term chicken litter storage, concentrations of nonresistant *E. coli* and enterococci were additionally monitored in this study. Enterococci are approved gram-positive indicator microorganisms present in feces and have a higher tenacity compared to *E. coli.* Two short-term storage trials each covering a time span of 5 D were performed. One trial was performed in the summer (summer trial) and one in the winter (winter trial) to explore climatic influences on the decline of these bacteria.

MATERIALS AND METHODS

Experimental Design

An initial screening of 40 different barns of a large broiler farm in Germany was performed to select barns with high quantities of ESBL-producing *E. coli* in the litter. Screening consisted of one boot swab and one composite litter sample per barn and was done for both the summer trial and the winter trial. The winter trial was carried out in early March, and the summer trial in early June. The time span between the screening and the litter storage trial was 2 wk for the winter trial and 1 wk for the summer trial.

In all barns, 1.5 kg/m^2 of wood pellets were used as bedding material. The litter (approximately 15 metric tons for each trial) was removed from the barns with a front-end loader and piled up on a concrete surface behind the barn directly after the chickens were housed out.

Storing the litter behind the barns for several days is not unusual in broiler production. Although on the farm where the trials were performed, the litter is removed as fast as possible if working capacities are available. Under suitable conditions, it is used directly for fertilization or otherwise transported to further storage areas.

The first samples were taken immediately after the litter heap was stacked. For both trials, the litter heaps were sampled at 6 points in time: 12 h, 24 h, 36 h, 48 h, 72 h, and 96 h after storage begin. We expected a faster reduction of the bacterial counts in the summer because of the higher ambient temperatures. Hence, in the summer trial, additional samplings were performed at 1 h, 3 h, and 6 h after the begin of storage. At each point in time, 3 surface and 3 deep samples were taken from the litter heap.

For the surface litter samples, approximately 50 g of litter from the heap's surface were collected in sterile 120 mL specimen containers (VWR, Radnor, PA)

For the deep litter samples, custom-built steel sample containers were used. These containers are cylindrical, 9.9 cm long, have a diameter of 4.4 cm, and drill holes with a diameter of 7 mm, ensuring the same environmental conditions in the sampling container and the surrounding litter heap. These sterilized containers were filled with litter from the heap and were placed in the litter heap at a depth of 50 to 55 cm at the start of the experiment. Wires were attached to the containers allowing quick retraction and sample collection at each point in time.

The ALMEMO 2490 device (AHLBORN, ZA9020-FS and FH A696-GF1 Holzkirchen, Germany) was used to record temperature and moisture at each sampling spot immediately after sampling.

The weather data for the trial periods were obtained from the closest weather station located approximately 20 km from the sampling site (Archive of the German Meteorological Office)

pH Value Analysis

The pH value was measured for all litter samples. Samples were diluted with purified water at a ratio of 1:10 and homogenized for 30 s with a vortex mixer. The pH value was measured with the handheld measurement instrument AL10 (AQUALYTIC, Dortmund, Germany).

Microbiological Analyses

All boot swabs and litter samples from the screenings were analyzed quantitatively and qualitatively for ESBL-producing *E. coli.* Litter samples from the litter

storage trials were analyzed quantitatively and qualitatively for total *E. coli* and ESBL-producing *E. coli*. Additionally, enterococci were analyzed quantitatively.

All litter samples were mixed with Luria/Miller-broth (**LB**) (Roth, Karlsruhe, Germany) in stomacher bags at a ratio of 1:10. Boot swabs were put in stomacher bags, and 200 ml of LB medium was added. The samples were homogenized using a Stomacher 400 Circulator (Seward Limited, West Sussex, UK) for 2 min at 200 rpm.

For the quantitative analyses aliquots of the suspensions were taken, and triplicates of 100 μL were streaked on specific agar plates after serial dilution. For *E. coli,* MacConkey agar No. 3 (Oxoid, Wesel, Germany) was used. To detect ESBL-producing *E. coli*, 1 mg/L cefotaxime (AppliChem, Darmstadt, Germany) was added as suggested by the EFSA (2011). For enterococci, we used Bile Aesculin Azide agar (Merck, Darmstadt, Germany). We did not set a minimum number of cfu per plate for the evaluation of the undiluted samples, resulting in a quantitative detection limit of 3.3×10^1 cfu/g of litter.

For qualitative testing, the homogenized samples were incubated in LB medium for 20 to 24 h at 37°C. Subsequently, 10 μL were streaked on MacConkey agar with and without the addition of cefotaxime, respectively, with an inoculation loop.

MALDI-TOF Mass Spectrometry (MALDI Microflex LT and Biotyper database, Bruker Daltonics, Bremen, Germany) was used for species confirmation of colonies which were phenotypically suspected to be *E. coli* or enterococci.

Real-Time PCR and Sanger Sequencing

Real-time qPCR as described by Roschanski et al. (2014) was used to detect the most important beta-lactamase genes bla_{SHV}, bla_{TEM}, $bla_{\mathrm{CTX\text{-}M}}$, and the CIT-type AmpC $bla_{\mathrm{CMY\text{-}2}}$ in isolates of all samples.

For both trials, the ESBL-gene of 8 isolates were sequenced by Sanger sequencing to identify the present ESBL-variants. Two isolates from the litter and one isolate from a surface and deep litter sample for day 1, 3, and 5 were chosen for sequencing for each trial, respectively. All isolates that showed a bla_{TEM} resistance gene in addition to the predominant resistance gene were also chosen for sequencing.

The DNA was isolated, and PCR was performed as published previously by Projahn et al. (2017). The purified PCR products were sent to LGC Genomics (Berlin, Germany) who provided the sequences. Nucleotide sequences were analyzed using DNASTAR Lasergene (Madison, WI) and compared with the reference sequences of GenBank (https://www.ncbi.nlm.nih.gov/genbank/) according to the accession numbers of the lahey database (https://www.lahey.org/studies/).

Phylotyping

All isolates were analyzed for their phylogenetic group as published by Clermont et al. (2013) with modified PCR conditions according to Projahn et al. (2017). Isolates that could not be assigned to a phylogroup because of unspecific band patterns were declared as a combined phylogroup.

Whole Genome Sequencing

Forty-four ESBL-producing *E. coli* isolates were selected for whole genome sequencing and recultivated on Brain-Heart-Infusion agar (Roth, Karlsruhe, Germany). DNA was isolated using the Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany), and sequencing libraries were prepared using the Nextera XT protocol with modifications (Baym et al., 2015; Steglich et al., 2018). The libraries were sequenced on a NextSeq machine with a NextSeq 500/550 mid output v2 kit (Illumina, San Diego) to ≥ 50-fold sequencing coverage.

Short-read sequencing data were uploaded to the online platform Enterobase (https://enterobase.warwick.ac.uk), where they were assembled. Resulting contigs were quality-controlled and subjected to classification by 7-gene multilocus sequence typing (**MLST**), core-genome multilocus sequence typing (**cgMLST**), and cgMLST-based hierarchical clustering. Clusters at the level HC1100 (Hierarchical Cluster 1,100, that is, chains of genomes differing pairwise by maximally 1,100 cgMLST alleles) represent major genetic populations within the species *E. coli*, largely congruent with sequence-type complexes based on legacy 7-gene MLST (Zhou et al., 2019). In addition, EnteroBase used genome sequence information to predict phylogroups according to Clermont typing (Zhou et al., 2019) based on algorithms by Beghain et al. (2018) and Waters et al. (2018).

Genome sequences were screened for antibiotic resistance genes by using the tools Resfinder (Zankari et al., 2012), AMRFinder (Feldgarden et al., 2019), and CARD (Jia et al., 2017), as implemented in ABRicate (https://github.com/tseemann/abricate). Genome sequencing data were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under study number PRJEB34161.

Statistical Analysis

The software SPSS, version 25, (SPSS, Inc., Chicago, IL) was used for statistical analysis. The data on microbial counts had no normal distribution. We used log transformation to achieve log normal distribution, and geometric means were calculated as proposed by Bland and Altman (1996). The upper and lower 95% confidence intervals were calculated. The winter trial and summer trial were analyzed separately.

RESULTS

Environmental Conditions During the Litter Storage Trials Conducted in Summer and Winter

The relevant weather data for the period of both trials are summarized in Table 1.

Table 1. Environmental conditions during the short-term litter storage trials as provided by the weather station closest to the trial site (German meteorological office).

Trial	Day	Daily air temperature (°C) Minimum	Maximum	Mean	RH (%)[1]	Sunshine (h)	Rainfall (mm)
Winter	1	−13.1 °C	−2.0 °C	−7.2 °C	58.8%	9.5 h	0.0 mm
	2	−12.8 °C	−0.6 °C	−6.1 °C	56.5%	9.4 h	0.0 mm
	3	−7.7 °C	7.4 °C	−0.5 °C	54.7%	5.1 h	1.1 mm
	4	−3.8 °C	11.9 °C	4.4 °C	74.5%	7.9 h	0.0 mm
	5	−5.5 °C	5.2 °C	1.0 °C	82.6%	0.9 h	6.9 mm
Summer	1	13.6 °C	28.8 °C	21.6 °C	75.6%	10.0 h	20.4 mm
	2	13.6 °C	30.4 °C	23.5 °C	69.9%	12.3 h	0.0 mm
	3	14.9 °C	27.0 °C	21.6 °C	78.8%	6.5 h	0.2 mm
	4	14.4 °C	21.5 °C	19.2 °C	86.1%	0.3 h	0.1 mm
	5	13.7 °C	25.2 °C	20.5 °C	76.0%	5.4 h	0.0 mm

[1]Relative humidity.

Temperature, Moisture, and pH Value of the Litter

In both trials, the temperature of the litter increased continuously at a depth of 50 to 55 cm compared with the surface of the litter heap. In the winter trial, 50.4°C were reached at the end of the trial period (96 h). In the summer trial, temperatures over 50°C were already reached after 36 h, and the maximum temperature measured was 58.5°C at the end of the trial (96 h). The temperature in the surface samples was lower for both trials ranging from 16.8°C to 24.2°C in the winter trial and 29.3°C to 42.7°C in the summer trial.

In the winter trial, moisture of the deep samples ranged from 16.2 to 23.2%. In the summer trial, moisture of the deep samples increased from approximately 9.0% at the beginning of the experiment to 27.9% at the end of the experiment. Surface samples from both trials showed lower moisture levels with values ranging from 5.8 to 8.6% in the winter trial and 5.9 to 8.8% in the summer trial. Temperature and moisture development for both trials is shown in Figure 1.

The pH values measured in the chicken litter directly after the removal from the barn was 8.0 in the winter trial and 8.1 in the summer trial.

For both trials, the pH value increased in the surface samples over 5 consecutive day up to a maximum pH value of 8.8 in the winter trial and 8.6 in the summer trial. In the deep samples, the pH value decreased in both trials to a minimum of 5.6 in the winter trial and 6.5 in the summer trial. The mean pH values for both trials are depicted in Figure 2.

Microbiological Status of the Barns in the Initial Screenings

For both trials, the barn with the highest concentration of ESBL-producing *E. coli* in the litter samples of the initial screenings was chosen for the litter storage trial. The bacterial counts of the investigated microorganisms in the barns are shown in Table 2 for the boot swabs and composite litter samples.

Quantitative and Qualitative Detection of ESBL-Producing E. coli in the Litter

Surface litter samples were firstly taken immediately after the litter was removed from the barns (0 h). The mean number of ESBL-producing *E. coli* per g of litter was 5.2×10^4 cfu in the winter trial and 9.5×10^2 cfu in the summer trial.

In the winter trial, the mean number of ESBL-producing *E. coli* was 1.3×10^4 cfu/g of litter at the 12 h sampling point for the surface samples, decreased until 36 h and increased again to 5×10^3 cfu/g after

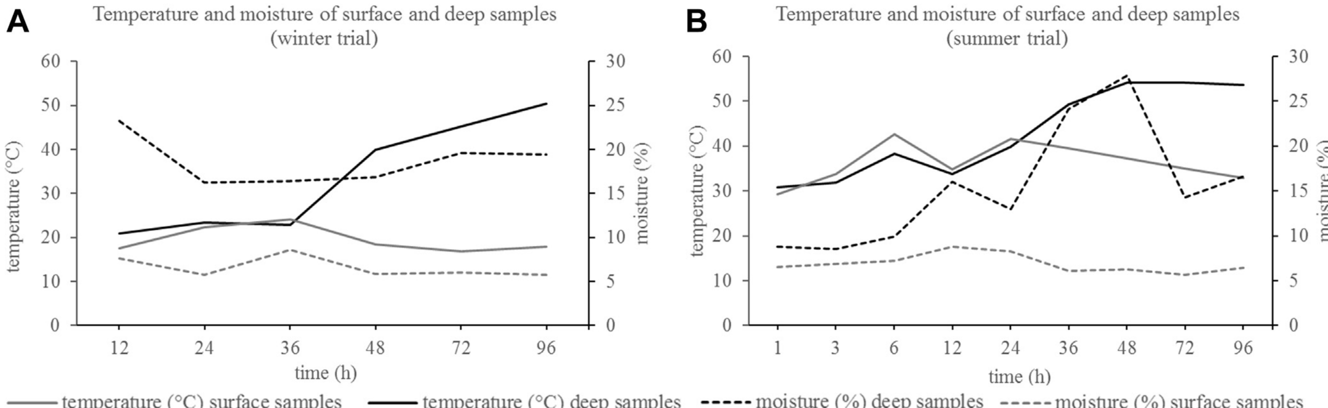

Figure 1. Development of mean sample temperatures and moistures in the winter trial (A) and summer trial (B).

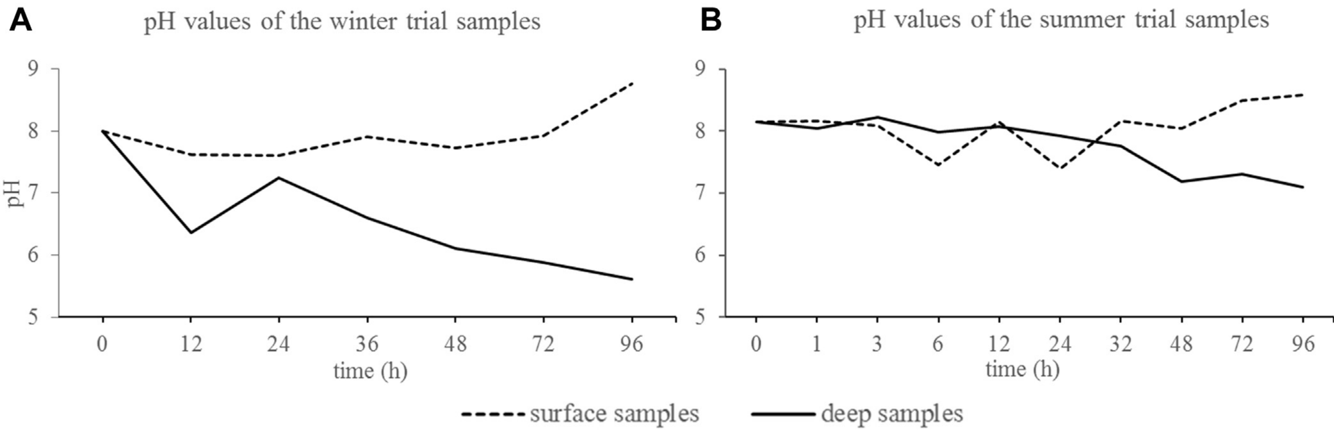

Figure 2. Mean sample pH values in the winter trial (A) and summer trial (B) for each point in time.

72 h. At the end of the trial (96 h), ESBL-producing *E. coli* were not quantitatively detectable.

The deep litter samples in the winter trial showed an ESBL-producing E. coli concentration of 3.6×10^3 cfu/g at 12 h and gradually decreased in the following measurements. For the 72 h and 96 h samples, ESBL-producing *E. coli* could not be detected quantitatively. A qualitative detection was possible for 33% (n = 1/3) of the samples after 72 h and for 66% (n = 2/3) of the samples after 96 h.

In the summer trial, the number of ESBL–producing *E. coli* in the surface samples decreased slightly in the first 12 hours (mean at 1 h = 2×10^2 cfu/g, mean at 12 h = 9.3×10^1 cfu/g). At 24 h and 72 h, the concentration of ESBL-producing *E. coli* was higher than the initial count with up to 6.7×10^3 cfu/g. At 36 h, the number of ESBL-producing *E. coli* was below the detection limit.

ESBL-producing *E. coli* were not quantitatively detectable in the deep samples of the summer trial for the 6 h point in time. Additionally, after 36 h for the last 4 points in time (36 h, 48 h, 72 h, and 96 h), ESBL-producing *E. coli* were constantly under the detection limit. The qualitative analysis was only positive for 67% (n = 2/3) of the samples after 48 h and for 0% (n = 0/3) after 72 h and 96 h, respectively.

A qualitative detection of ESBL-producing *E. coli* was possible for all surface samples in both trials. The data on ESBL-producing *E. coli* are shown in Figure 3.

Quantitative and Qualitative Detection of Total E. coli in the Litter

In the litter samples taken directly after the litter was removed from the barns (0 h), the mean number of *E. coli* per g of litter was 5.6×10^6 cfu for the winter trial and 5.8×10^4 cfu for the summer trial.

In the winter trial, the number of *E. coli* dropped in both, the surface and the deep samples. After 96 h, the mean amount of *E. coli* was 1.1×10^4 cfu/g of litter in the surface and 1.1×10^3 cfu/g of litter in the deep samples. Qualitative *E. coli* detection was possible in 66% (n = 2/3) of the deep samples at 72 h and 96 h.

In the summer trial, on the other hand, the mean number of *E. coli* in surface litter samples increased from 5.8×10^4 cfu/g of litter at 0 h to a maximum of 2.8×10^6 cfu/g at 72 h and 2.4×10^6 cfu/g at 96 h. Deep litter samples showed a constant decrease in *E. coli* concentrations with a drop below the detection limit at 72 h. A qualitative detection was possible for 67% (n = 2/3) of samples at 48 h and for 0% (n = 0/3) of samples at 72 h and 96 h. The data for total *E. coli* are shown in Figure 4.

Quantitative Detection of Enterococci in the Litter

The number of enterococci for the 0 h point in time was 4.8×10^7 cfu/g of litter for the winter trial and 5.5×10^6 cfu/g for the summer trial, respectively.

In the winter trial, the quantity of enterococci was comparatively stable for both, surface and deep samples, ranging from 3.2×10^6 to 5.6×10^7 cfu/g in all samples taken.

In the summer trial, the quantity of enterococci increased in surface samples in the sampling period, reaching a maximum of 2.7×10^8 cfu/g after 48 h. The quantity at the end of the sampling period (96 h) was 1.6×10^8 cfu/g of litter.

Table 2. Bacterial counts for the initial screening of the barns in cfu/boot swab and cfu/g of litter.

Trial	Type of sample	ESBL-producing *E. coli* in cfu/boot swab or cfu/g	Total *E. coli* in cfu/boot swab or cfu/g	Enterococci in cfu/boot swab or cfu/g
Winter	Boot swab	1.1×10^6 cfu/boot swab	2.3×10^7 cfu/boot swab	2.7×10^8 cfu/boot swab
	Litter sample	3.9×10^4 cfu/g	4×10^5 cfu/g	2.9×10^7 cfu/g
Summer	Boot swab	3.6×10^6 cfu/boot swab	3.6×10^8 cfu/boot swab	5.9×10^8 cfu/boot swab
	Litter sample	6.3×10^5 cfu/g	1.7×10^7 cfu/g	7.2×10^7 cfu/g

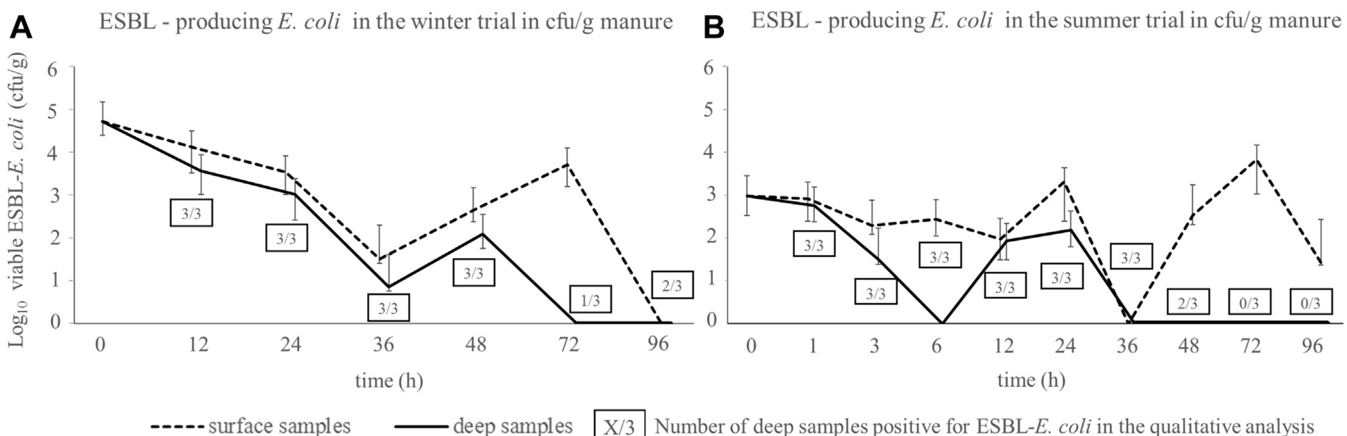

Figure 3. Results of the quantitative and qualitative analysis of ESBL-producing *E. coli* in cfu/g of litter for the winter trial samples (A) and the summer trial samples (B). The geometric mean of 3 samples is shown for each point in time. The error bars indicate the upper and lower 95% confidence intervals. The graphs were shifted to improve the visibility of the error bars. ESBL, extended-spectrum beta-lactamase.

In contrast, in the summer trial, deep litter samples showed a significant decrease in enterococci concentration. A mean of 1.3×10^4 and 3.4×10^4 cfu/g were detected after 72 h and 96 h, respectively. The data for enterococci are shown in Figure 4.

Molecular Characterization of ESBL-Producing E. coli

Phylogroups and Sequence Types In the winter trial, the phylogenetic group was determined for 48 ESBL-producing *E. coli* isolates using classical gel-based PCR. Forty-seven isolates were allocated to phylogroup F, and one isolate was allocated to phylogroup A/C.

In the summer trial, the number of ESBL-producing *E. coli* isolates available for phylogenetic analysis was 54 of which 17 isolates belonged to phylogroup B1. One isolate belonged to the groups A and F, respectively, and 35 isolates were allocated to the combined phylogroup D/E.

In Table 3, a comparison of the Clermont phylogroups determined by PCR and the by whole genome sequenced isolates is shown. It also provides information on the 7-gene MLST, cgMLST, and HC1100 clustering from the Enterobase analyses and the predicted O and H antigens for all isolates.

Resistance Genes Real time qPCR revealed that all isolates from the winter trial (n = 48) harbored a resistance gene belonging to the bla_{SHV} gene family. The resistance gene was sequenced in 8 isolates, identifying it as bla_{SHV-12} in all chosen isolates. In the summer trial, all isolates (n = 54) harbored a resistance gene belonging to the bla_{CTX-M} gene family. All 8 bla_{CTX-M} genes sequenced were identified as $bla_{CTX-M-1}$.

One winter trial isolate and 4 summer trial isolates showed an additional bla_{TEM} gene. All 5 bla_{TEM} genes were identified as broad spectrum beta-lactamase resistance gene bla_{TEM-1}.

For all genome-sequenced isolates from the winter trial (n = 19), genome sequencing confirmed the presence of bla_{SHV-12}. In addition, genome sequencing detected the plasmid-mediated quinolone-resistance

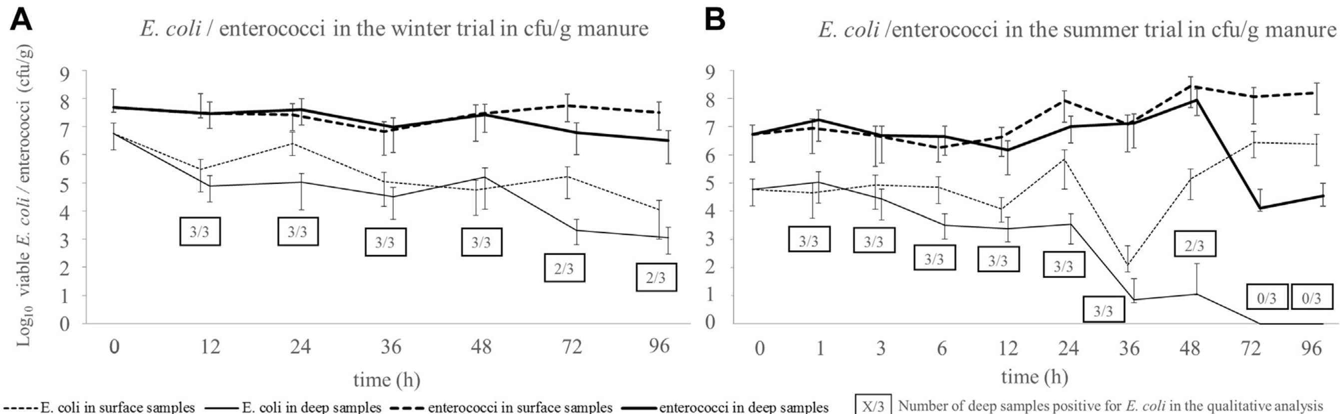

Figure 4. Results of the quantitative and qualitative analysis of total *E. coli* in cfu/g of litter, and the quantitative analysis of enterococci in cfu/g of litter for the winter trial samples (A) and the summer trial samples (B). The geometric mean of 3 samples is shown for each point in time. The error bars indicate the upper and lower 95% confidence intervals. The graphs were shifted to improve the visibility of the error bars.

Table 3. Phylogroups determined with gel-based PCR and phylogroups and sequence types determined via Enterobase.

Sample ID	Trial	Timepoint (h)	Sampling site	Phylogroup FU[1]	Phylogroup Enterobase	ST 7 gene MLST[2]	ST cGMLST[3]	HC1100 cgST[4]	H-Antigen	O-Antigen
7-1EP06	Summer	0 h	Barn	D/E	D	2,309	86,589	5,033	H6	O15
7-1EP05	Summer	0 h	Barn	D/E	D	2,309	86,626	5,033	H6	O15
7-1MP03	Summer	0 h	Surface	D/E	D	2,309	86,616	5,033	H6	O15
7-1MP05	Summer	1 h	Depth	D/E	D	2,309	86,618	5,033	H6	O15
7-1MP11	Summer	3 h	Depth	B1	B1	162	86,592	138	H10	O9
7-1MP10	Summer	3 h	Surface	D/E	D	2,309	86,619	5,033	H6	O15
7-1MP16	Summer	6 h	Surface	B1	B1	1,304	86,593	152	H7	O91
7-1MP17	Summer	6 h	Depth	D/E	D	2,309	86,608	5,033	H6	O15
7-1MP23	Summer	12 h	Depth	B1	D	2,309	86,590	5,033	H6	O15
7-1MP22	Summer	12 h	Surface	B1	B1	162	86,622	138	H10	O88
7-1MP31	Summer	24 h	Depth	D/E	D	2,309	86,580	5,033	H6	O15
7-1MP39	Summer	36 h	Depth	D/E	D	2,309	86,577	5,033	H6	O15
7-1MP34	Summer	36 h	Surface	B1	B1	1,304	86,593	152	H7	O91
7-1MP38	Summer	36 h	Surface	D/E	D	2,309	86,614	5,033	H6	O15
7-1MP35	Summer	36 h	Depth	D/E	D	2,309	86,620	5,033	H6	O15
7-1MP37	Summer	36 h	Depth	D/E	D	2,309	86,617	5,033	H6	O15
7-1MP41	Summer	48 h	Depth	D/E	D	2,309	86,580	5,033	H6	O15
7-1MP40	Summer	48 h	Surface	D/E	D	2,309	86,601	5,033	H6	O15
7-1MP42	Summer	48 h	Surface	D/E	D	2,309	86,623	5,033	H6	O15
7-1MP43	Summer	48 h	Depth	D/E	D	2,309	86,623	5,033	H6	O15
7-1MP44	Summer	48 h	Surface	D/E	D	2,309	86,580	5,033	H6	O15
7-1MP46	Summer	72 h	Surface	B1	B1	162	86,615	138	H10	O88
7-1MP48	Summer	72 h	Surface	D/E	D	2,309	86,625	5,033	H6	O15
7-1MP56	Summer	96 h	Surface	B1	B1	1,304	86,593	152	H7	O91
7-1MP54	Summer	96 h	Surface	D/E	D	2,309	86,624	5,033	H6	O15
5-2EP02	Winter	0 h	Barn	F	F	117	86,591	50	H4	O8
5-2EP01	Winter	0 h	Barn	F	F	117	86,578	50	H4	O8
5-2MP32	Winter	24 h	Surface	A/C	A	10	86,613	13	H48	O12
5-2MP33	Winter	24 h	Depth	F	F	117	86,579	50	H4	O8
5-2MP35	Winter	36 h	Depth	F	F	117	86,599	50	H4	O8
5-2MP34	Winter	36 h	Surface	F	F	117	86,653	50	H4	O8
5-2MP41	Winter	48 h	Depth	F	F	117	86,647	50	H4	O8
5-2MP43	Winter	48 h	Depth	F	F	117	86,600	50	H4	O8
5-2MP44	Winter	48 h	Surface	F	F	117	86,621	50	H4	O8
5-2MP42	Winter	48 h	Surface	F	F	117	86,632	50	H4	O8
5-2MP49	Winter	72 h	Depth	F	F	117	86,578	50	H4	O8
5-2MP50	Winter	72 h	Surface	F	F	117	86,578	50	H4	O8
5-2MP48	Winter	72 h	Surface	F	F	117	86,578	50	H4	O8
5-2MP46	Winter	72 h	Surface	F	F	117	86,578	50	H4	O8
5-2MP55	Winter	96 h	Depth	F	F	117	86,578	50	H4	O8
5-2MP54	Winter	96 h	Surface	F	F	117	86,627	50	H4	O8
5-2MP57	Winter	96 h	Depth	F	F	117	86,579	50	H4	O8
5-2MP56	Winter	96 h	Surface	F	F	117	86,578	50	H4	O8
5-2MP52	Winter	96 h	Surface	F	F	117	86,612	50	H4	O8

[1]Freie Universität Berlin.
[2]Sequence type 7 gene multilocus sequence type.
[3]Sequence type core genome multilocus sequence type.
[4]Hierarchical cluster 1,100 core genome sequence type.

determinant *qnrS1* and *mdfA* genes in all 19 isolates, and the broad-spectrum beta-lactamase resistance gene $bla_{\text{TEM-1}}$ and the oxytetracycline resistance determinant Tet 34 in one isolate.

In the summer trial, the ESBL-resistance gene $bla_{\text{CTX-M-1}}$ was confirmed or found in all sequenced isolates (n = 25). In addition, the *mdfA* gene was detected in all isolates, and Tet 34 was detected in 84% (n = 21/25) of the isolates. $bla_{\text{TEM-1}}$ was detected in 4 isolates and the *sulII* gene in 3 isolates.

Genome-Based Phylogeny

Phylogenetic trees were calculated in Enterobase and can be found in the Supplemental Figures 1 and 2.

While there are genomic differences between isolates from the summer and winter experiments, isolates within each experiment showed little variation with the summer experiment appearing to be slightly more diverse.

Within the 2 main clusters (one for each season), the different sampling sites are distributed equally as well as the time points for sampling. The genetic differences between the 2 experiments is also reflected in the distribution of resistance genes.

DISCUSSION

The aim of our study was to evaluate whether short-term litter storage provides sufficient inactivation of ESBL-producing *E. coli* present in chicken litter under field conditions in winter and summer.

The most important findings of the study concerning the amount of cultivable ESBL-producing *E. coli* were that regardless of the season, the inactivation occurs faster in a depth of about 50 cm compared with the

surface of the litter pile. Additionally, a seasonal influence concerning the required time span and the effectiveness of the inactivation of ESBL-producing *E. coli* was shown. Extended-spectrum beta-lactamases–producing *E. coli* were not detected quantitatively in the deep samples in the winter trial after 72 h and already after 36 h in the summer trial.

Initial concentrations of nonresistant *E. coli* in the chicken litter were significantly higher than ESBL-producing *E. coli* for both trials. The proportion of the ESBL-producing subpopulation on the total amount of *E. coli* was 0.94% in the winter trial and 1.64% in the summer trial for the samples taken at storage begin. A similar proportion of 1.1% ESBL-producing isolates was recently reported by Friese et al. (2019) for turkey-rearing flocks. This is a possible explanation for the extended time in which nonresistant *E. coli* were detected in the litter compared with the ESBL-producing subpopulation. In the study performed by Erickson et al. (2010), nonresistant *E. coli* naturally occurring in the litter were not detected quantitatively and qualitatively in surface and deep samples after 4 D in static piles of chicken litter in summer, fall, and winter.

Abiotic Factors Influencing Microbial Counts in the Litter Storage Trials

Litter piles are microbiologically highly heterogeneous and local conditions are influenced by a variety of biological, physical, and chemical factors. In our study, we explored the influence of temperature, pH value, and moisture content of the chicken litter on the bacterial cell count.

In the study performed by Erickson et al. (2010) in the USA, temperatures within static piles of chicken litter were measured at different intervals. The highest mean temperatures reported in a depth of 30 cm from the heaps surface were 54.4°C in the summer after 4 D of storage and 51.8°C in the winter after 3 D of storage. This is in accordance with our findings where a maximum temperature of 58.5°C was measured in the summer and 50.4°C in the winter. Temperatures over 65°C were reported by Wilkinson et al. (2011) for static piles of poultry litter in the first weeks of aging.

In a recently performed laboratory scale anaerobic digestion experiment by Thomas et al. (2019), ESBL-/AmpC-producing *E. coli* were added in a concentration of over 10^7 cfu/ml to a mix of chicken litter and an inoculum from a biogas plant. They showed that at a constant temperature of 55°C, ESBL-producing *E. coli* were quantitatively undetectable by direct count after 2 h of incubation. In our summer trial, temperatures reached levels constantly above 53°C at 48 h. In the subsequent samples (72 h and 96 h), we did not detect ESBL-producing *E. coli* and nonresistant *E. coli* quantitatively and qualitatively. We therefore assume that a temperature of 53°C is sufficient for inactivation of all ESBL-producing and nonresistant *E. coli* under practical conditions of an anaerobic litter storage.

A laboratory scale study by Laport et al. (2003) showed that a 2 h incubation period at 55°C will result in >90% reduction of *Enterococcus faecium*. Accordingly, at the end of our summer trial enterococci concentrations had decreased by > 99% compared with the initial concentrations in the deep samples.

In the winter trial, a temperature of over 50°C was observed in the deep samples for the last point in time after 96 h only. Extended-spectrum beta-lactamases–producing *E. coli* were qualitative, and nonresistant *E. coli* were quantitatively detectable until the end of the trial. The number of enterococci was stable in both the surface and deep samples in the winter trial, ranging from 3.2×10^6 to 5.6×10^7 cfu/g in all samples. Therefore, we assume that the increase in temperature to a maximum of 50.4°C in the winter trial was insufficient for a distinct reduction of the monitored bacteria in chicken litter.

A further factor, which might influence the bacterial counts in the chicken litter, is the pH value. The pH value of the litter at the beginning of the experiment (0 h) was in the alkaline range, a frequent finding in chicken litter (Huang et al., 2017). The pH value drop in the depth of the litter heap was presumably because of anaerobic fermentation and formation of organic acids like propionic acid, butyric acid, and acetic acid (Cornell Waste Management Institute, 1996). The minimum pH value of 5.6 measured in the winter trial and 6.5 in the summer trial is not sufficient to inactivate *E. coli*. It was shown that *E. coli* has a high probability of surviving pH values of 1.5 to 4.0 (Takumi et al., 2000).

Insights From Whole Genome Sequencing

Whole genome sequencing revealed that the analyzed isolates for both trials harbored additional resistance genes besides ESBL resistance genes. Identified genes included *qnrS1*, which may mediate resistance to quinolones (Cerquetti et al., 2009), *mdfA,* which substantially increases resistance to amphoteric lipophilic compounds (e.g., ethidium bromide, benzalkonium, and tetracycline) (Edgar and Bibi, 1997) and the Tet 34 and *sulII* resistance genes, which mediate for resistance against oxytetracycline (Nonaka and Suzuki, 2002) and sulfonamides (Radstrom and Swedberg, 1988).

The results also indicate that one strain of ESBL-producing *E. coli* was predominant in the chicken barns for each of the trials. This finding is especially prominent in the winter trial, where 94.7% (n = 18/19) of the isolates belonged to the same 7 gene MLST (ST117) and HC1100cgST (ST50).

Results of the phylogenetic analyses suggest that genetic differences do not equip the isolates of a certain cluster with a survival advantage.

Advantages and Limitations of the Study

In our study, we did not artificially add ESBL-producing *E. coli* to the litter. Instead, chicken litter naturally contaminated with these resistant bacteria

was used, mirroring true field conditions. Hutchison et al. (2005) pointed out that this is crucial because bacteria are already adapted to their environment, and therefore, bacterial stress is minimized (Wesche et al., 2009). A further advantage over lab-scale studies is that the litter heaps were exposed to environmental conditions which are typical for the winter and summer season in central Europe. For on-farm waste management, similar environmental conditions are likely to appear; therefore, better transferability of the results can be expected when compared with laboratory studies.

A limitation of the study is that the measurements of temperature, humidity, and the litter samples taken for the microbiological analyses cannot cover the conditions in the entire storage mass. It was shown previously that because of increased deposition of fecal droppings on the litter surface, the number of coliform bacteria is significantly higher in the top layer of a chicken litter bed compared with the bottom layers (Barker et al., 2010). Using a front-end loader to remove and stack the litter at the end of the fattening period will not assure complete mixing of the litter, resulting in an inhomogeneous distribution of coliform bacteria. This might explain inconsistencies we saw in the course of the bacterial counts for some points in time. However, through our sampling scheme that covered both surface and deep samples with 3 samples evenly distributed over the litter heap for each point in time, we achieved representative results.

Evaluation of Short-Term Litter Storage as an On-Farm Strategy to Prevent the Spread of EBSL-Producing E. coli *to the Environment*

It was shown by Merchant et al. (2012) that resistant *E. coli* were detectable in soil fertilized with chicken litter for at least 7 mo. In that study performed in Canada of 295 *E. coli* isolated from soil, 139 carried either a bla_{SHV}, bla_{TEM}, or $bla_{CMY\text{-}2}$ resistance gene. This highlights the importance of sufficient inactivation of resistant *E. coli* in litter before land application.

Very low quantities of resistant microorganisms might be able to horizontally transfer mobile genetic elements to microorganisms in the environment, thus potentially contributing to a spread of antibiotic resistance. As a result, the qualitative detection of ESBL-producing *E. coli* in litter is of particular importance. A recent study by Pornsukarom and Thakur (2017) demonstrated that the application of manure containing Enterobacteriaceae which carry plasmids mediating for antibiotic resistance enriches the environmental resistome. Our results indicate that a storage period of 5 D is sufficient to reduce the amount of ESBL-producing *E. coli* in the depth of a chicken litter heap in the summer below the detection limit. For very low ambient temperatures, as present in our winter trial, an extension of the storage period should be considered because we observed an incomplete inactivation of EBSL-producing *E. coli* in a 5-day storage period.

Even if cultivation-based methods are unable to detect resistant bacteria, a transfer of plasmids carrying resistance genes might occur. In a study performed by Le Devendec et al. (2016), chicken manure was stored for 6 wk. After this time span, *E. coli* were not detected in the manure by cultivation without enrichment. Plasmid capture assays with the stored chicken manure revealed an uptake of plasmids encoding resistance to sulfonamides, aminoglycosides, and streptomycin in recipient strains. This indicates that even if there are no cultivable bacteria left in the litter, the possibility of spread of resistance cannot be discounted because viable plasmids could still be present. In contrast, Guan et al. (2007) stated that composting of chicken manure at high temperatures could help prevent the spread of antibiotic-resistant genes via plasmids in the environment. In their study, neither viable *E. coli* nor their plasmids could be detected in compost microcosms, which reached temperatures of over 50°C.

The outer edges of litter piles may present a reservoir for bacteria, and turning the litter pile may therefore lead to a recontamination of the interior parts (Pereira-Neto et al., 1986). This is in accordance with our observation in the summer trial, where the number of *E. coli* and enterococci significantly increased on the surface of the litter heap.

The increased quantity of these bacteria over the course of the trial may be caused by beneficial environmental factors such as rainfall, which influences moisture levels in litter piles and can promote regrowth of enteric bacteria (Gibbs et al., 1997). In the summer trial of our study, rainfall at the end of the first trial day led to increased moisture in the litter heap. Corresponding temperatures on the surface of the litter pile ranged from 29.2°C to 42.7°C, which are known to be sufficiently high for bacterial regrowth (Kumar and Libchaber, 2013).

The survival time of *E. coli* in manure is significantly longer under anaerobic than under aerobic conditions (Semenov et al., 2011). Additionally, it was shown in the trial by Wilkinson et al. (2011) that temperatures in composted chicken litter piles are higher than in stored piles. As previous research and this study indicate a faster inactivation of ESBL-producing *E. coli* can be achieved at higher temperatures. It appears therefore that composting litter under aerobic conditions could lead to a faster inactivation of ESBL-producing *E. coli* compared with storing it in anaerobic conditions. However, increased working and litter-storage capacities are required and not available on all farms.

In conclusion, short-term litter storage is a useful, easily realizable tool leading to an effective reduction of the amount of ESBL-producing *E. coli* in chicken litter. However, we did not observe a complete inactivation of ESBL-producing *E. coli* in the depth of the heap in the winter and on the surface of the heap for both trials. An extension of the storage period for low ambient temperatures and stirring the pile one time or composting the litter instead of storing it could increase the effectivity of chicken litter hygienization.

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SUPPLEMENTARY DATA

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3.2 Publication II

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Low airborne tenacity and spread of ESBL-/AmpC-producing *Escherichia coli* from fertilized soil by wind erosion

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Summary

ESBL-/AmpC-producing *Escherichia coli* from organic fertilizers were previously detected on soil surfaces of arable land and might be emitted by wind erosion. To investigate this potential environmental transmission path, we exposed ESBL-/AmpC-positive chicken litter, incorporated in agricultural soils, to different wind velocities in a wind tunnel and took air samples for microbiological analysis. No data exist concerning the airborne tenacity of ESBL-/AmpC-producing *E. coli*. Therefore, we explored the tenacity of two ESBL/AmpC *E. coli* strains and *E. coli* K12 in aerosol chamber experiments at different environmental conditions. In the wind tunnel, ESBL/AmpC-producing *E. coli* were detected in none of the air samples (n = 66). Non-resistant *E. coli* were qualitatively detected in 40.7% of air samples taken at wind velocities exceeding 7.3 m s^{-1}. Significantly increased emission of total viable bacteria with increasing wind velocity was observed. In the aerosol chamber trials, recovery rates of airborne *E. coli* ranged from 0.003% to 2.8%, indicating a low airborne tenacity. Concluding, an emission of ESBL/AmpC *E. coli* by wind erosion in relevant concentrations appears unlikely because of the low concentration in chicken litter compared with non-resistant *E. coli* and their low airborne tenacity, proven in the aerosol chamber trials.

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Introduction

Enterobacteriaceae including *Escherichia coli* (*E. coli*) can have the capability to produce ESBL- (extended-spectrum beta-lactamase) and AmpC-enzymes. These enzymes hydrolyze the beta-lactam ring of most beta-lactam antibiotics, including extended-spectrum cephalosporins belonging to the third, fourth and fifth generation. Genes encoding for the production of ESBL- and AmpC-enzymes are found in pathogenic and commensal Enterobacteriaceae (Day *et al*., 2016). Among resistant bacteria, ESBL-/AmpC-producing Enterobacteriaceae are of outstanding importance and categorized as critical priority concerning the development of new drugs by the World Health Organization (WHO, 2017).

The presence of antimicrobial resistance in the environment represents an increasing 'One Health' problem. There is evidence of a transmission of ESBL-producing *Escherichia coli* (*E. coli*) and their mobile genetic elements encoding for antimicrobial resistance between humans, animals and the environment (Smet *et al*., 2010; Leverstein-van Hall *et al*., 2011). ESBL-producing *E. coli* are frequently detected in broiler barns, with a prevalence of up to 100% (Dierikx *et al*., 2010; Laube *et al*., 2013; Blaak *et al*., 2015; Hering *et al*., 2016; Daehre *et al*., 2017).

Besides the potential transfer of ESBL-producing *E. coli* from chickens to humans via direct contact (Dierikx

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et al., 2013; Huijbers *et al*., 2014) or contaminated meat (Leverstein-van Hall *et al*., 2011; Pietsch *et al*., 2018), ESBL-producing *E. coli* can spread in the farm environment and, thus, may act as a source for antimicrobial resistance. Apart from the spread through vectors such as flies (Blaak *et al*., 2014; von Salviati *et al*., 2015), another possibility of an environmental dissemination of ESBL-*E. coli* is the airborne route, which has been suggested from municipal sewage (Korzeniewska and Harnisz, 2013) and animal husbandries (Laube *et al*., 2014; Gao *et al*., 2015).

Applying ESBL-positive organic fertilizers to arable land presents a further important mode of transmission to the environment. For instance, ESBL-producing *E. coli* were detected in fertilized soil in studies carried out in Germany (Friese *et al*., 2013) and China (Zheng *et al*., 2017). In a French study ESBL-producing *E. coli* were even detected up to 1 year after the soil was fertilized with cow manure (Hartmann *et al*., 2012). Fields amended with ESBL-positive organic fertilizers might be affected by wind erosion, leading to bioaerosol formation (Jones and Harrison, 2004) and a possible airborne spread of these resistant bacteria. The microorganisms gain a lot of advantages from being transported together with soil particles, which ensure their survival during transport (Acosta-Martínez *et al*., 2015). The aim of this study was, therefore, to assess the relevance of airborne transmission of ESBL-producing *E. coli* by wind erosion after organic fertilization of fields. We performed three wind erosion trials with ESBL-positive chicken litter in a wind tunnel. In this context, the tenacity of resistant bacteria including ESBL-producing *E. coli* in the aerosolized state is of high interest and the current data situation is poor (Environment Agency, 2019). Therefore, we carried out a series of aerosol chamber experiments in order to investigate the airborne tenacity of two poultry-associated ESBL-/AmpC-producing *E. coli* strains and the non-resistant laboratory strain *E. coli* K12 under different conditions. This enabled a more in-depth interpretation of the results of the wind tunnel trial.

Results

Results from the wind tunnel trials

Concentrations of ESBL-/AmpC-producing and non-resistant E. coli *in the litter and soil-litter mixtures.* The results of the quantitative and qualitative analysis of ESBL-producing and non-resistant *E. coli* in the chicken litter and soil-litter mixtures for all trials are displayed in Table 1.

The molecular characteristics of the *E. coli* isolate taken in the three wind tunnel trials are summarized in Table S1 (supplementary material).

Wind velocity, relative humidity (RH) and temperature measured in the wind tunnel trials and detection of ESBL-/AmpC-producing and non-resistant E. coli *in the air samples.* The wind velocity measured for the different levels of wind tunnel engine power and in different distances to the soil-litter mixture is depicted in Table 2. The wind velocity increased with increasing wind tunnel engine power and increasing distance to the soil-litter mixture because friction decreases with increasing distance to soil. Marginal deviations were observed in the wind velocity measurements between the three trials caused by changes in atmospheric conditions outside the tunnel, like air temperature and atmospheric pressure. For that reason, the arithmetic mean of the wind velocity of all three trials is depicted. A distinct variation of the environmental conditions (relative humidity (RH) and temperature) was observed between the three wind tunnel trials. The highest mean temperature was measured in the first trial at 25.7°C. The temperature was the lowest for the second trial with 16.1°C. The RH was highest for the first trial with 56% and lowest for the third wind tunnel trial with 29.7% (Table 2).

Sixty-six air samples were analysed throughout the three trials at different wind velocities (11 AGI-30 and 11 Coriolis μ air samples per trial). Neither ESBL/AmpC-producing nor non-resistant *E. coli* were detected in the AGI-30 samples. In the Coriolis μ samples, only non-resistant *E. coli* were detected, albeit in levels below the detection levels of the quantitative assay (Table 2). Statistical analysis using logistic regression revealed that an increased wind velocity significantly correlated with a qualitative detection of *E. coli* in the air samples ($p = 0.022$).

Total viable bacterial count, PM_{10} and $PM_{2.5}$ emitted per m^2 of soil-litter mixture, soil aggregate size and collection efficiency of the AGI-30 and Coriolis μ air sampler. The geometric mean of total viable bacterial count emitted per m^2 of soil-litter mix for each wind tunnel trial is depicted in Fig. 1. Figure 1A shows the data for the AGI-30, Fig. 1B shows the data for the Coriolis μ air sampler. A similar emission was observed when comparing the three trials concerning the total viable count emitted at each wind velocity. However, with increasing wind velocity, a significant increase concerning the emission of total viable bacteria was observed in some trials for both air samplers. As all three trials showed a similar emission of total viable bacteria per m^2, a pooled analysis was performed for both air sampling devices. The data are shown in the supplementary material in Fig. S1 for the AGI-30 (a) and the Coriolis μ air sampler (b). Here it becomes evident that there was a significantly increased emission between 5.6 and 7.3 m s^{-1} and also between 7.3 and 9.8 m s^{-1} but not between 9.8 and 10.6 m s^{-1}. Particle emission in

Table 1. Quantitative and qualitative detection of ESBL-/AmpC-producing and non-resistant *E. coli* in the chicken litter directly before and after mixing with soil for the three wind tunnel trials.

ESBL-/AmpC-producing *E. coli*			
	Trial 1	Trial 2	Trial 3
Litter (directly before mixing)	Below detection limit (+)	Barn A: 3.2×10^5 cfu g^{-1} Barn B: 4.2×10^5 cfu g^{-1}	1.2×10^4 cfu g^{-1}
Soil-litter mixture	Below detection limit (+/–)	2.9×10^3 cfu g^{-1}	Below detection limit (+)
Non-resistant *E. coli*			
	Trial 1	Trial 2	Trial 3
Litter (directly before mixing)	Not determined (+)	Barn A: 2.0×10^7 cfu g^{-1} Barn B: 9.5×10^6 cfu g^{-1}	8.0×10^5 cfu g^{-1}
Soil-litter mixture	Not determined (+)	2.4×10^4 cfu g^{-1}	8.2×10^2 cfu g^{-1}

The result of the qualitative analysis is displayed in brackets when samples were not quantifiable.

Table 2. Qualitative detection of non-resistant *E. coli* in the Coriolis μ air samples at different wind velocities and environmental conditions measured for the three wind tunnel trials.

Wind tunnel engine power (%)	x̄ wind velocity 5 cm (m s^{-1})	x̄ wind velocity 30 cm (m s^{-1})	x̄ wind velocity 60 cm (m s^{-1})	x̄ wind velocity suspension chamber (m s^{-1})	Trial 1 T: 25.7°C RH: 56%	Trial 2 T: 16.1°C RH: 54.4%	Trial 3 T: 23.1°C RH: 29.7%
0	0	0	0	0	–	–	–
40	5.6	7.8	8.1	3.3	–	–	–
60	7.3	10.6	10.9	5.1	– – –	+ + –	– – –
80	9.8	14.5	15.4	6.6	+ + +	+ + +	– – –
100	10.6	15.4	16.1	6.9	+ – +	– + –	– – –

(+) *E. coli* qualitatively detectable (–) *E. coli* qualitatively undetectable; T: Temperature; RH: Relative humidity.

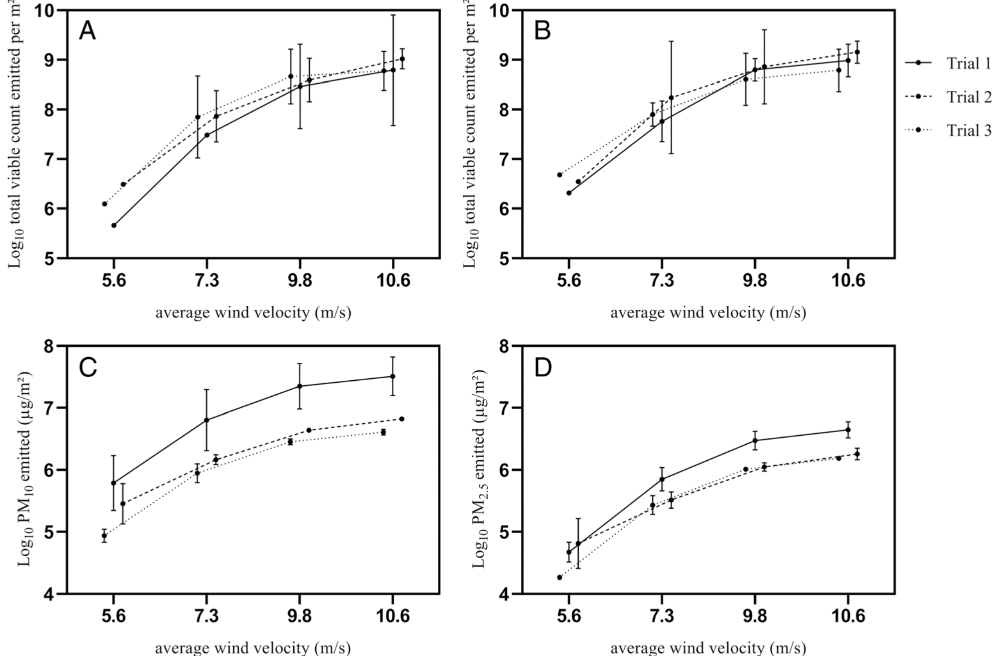

Fig. 1. LOG_{10} total viable count emitted per m^2 of soil for each level of wind velocity determined with the all-glass-impinger 30 (A) and the Coriolis μ (B) for the three wind tunnel trials. PM_{10} (C) and $PM_{2.5}$ (D) emitted per m^2 of soil in μg for the three wind tunnel trials. The error bars indicate the upper and lower 95% confidence intervals.

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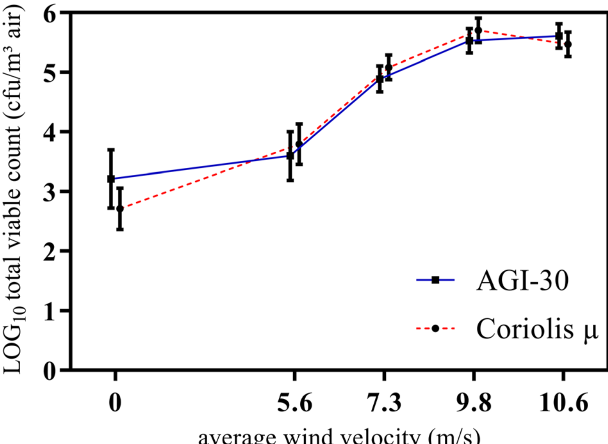

Fig. 2. Influence of the air sampling device used on total viable count detected per m^3 of air in the wind tunnel trials at different wind velocities. The error bars indicate the upper and lower 95% confidence intervals.

μg m^{-2} of soil-litter mixture is additionally depicted in Fig. 1 for PM_{10} (c) and $PM_{2.5}$ (d). At wind velocities of 7.3, 9.8 and 10.6 m s^{-1}, significantly increased PM emissions were observed for the first trial. Soil-litter mixture aggregates were the smallest in the first trial with only 33.8% of the particle mass having a diameter of >500 μm. For the second and third wind tunnel trial, aggregates were coarser with 45.2% and 49.4% of the particle mass having a diameter of >500 μm respectively.

Statistical modelling was used to investigate the influence of the air sampling device on the total viable bacterial count detected per m^3 of air in the wind tunnel trials. The three wind tunnel trials were not stratified for the analysis. The data, including the upper and lower 95% confidence intervals, are visualized in Fig. 2. Overall and between the different wind velocities, no significant differences were observed between the collection efficiency of the air sampling devices and confidence intervals show low maximum deviations.

Results from the aerosol chamber trials

Concentration of E. coli *in the bacterial suspensions, concentration and recovery rates of aerosolized* E. coli

The mean concentration in the bacterial suspensions for aerosolization was 9.8×10^8 cfu ml^{-1} for *E. coli* K12 (range: 2.6×10^8 to 3.8×10^9 cfu ml^{-1}), 9.0×10^8 cfu ml^{-1} for *E. coli* R56 (range: 2.7×10^8 to 3.4×10^9 cfu ml^{-1}) and 2.0×10^9 cfu ml^{-1} for *E. coli* G-148-1 (range: 4.1×10^8 to 3.9×10^9 cfu ml^{-1}).

The airborne concentrations of the three *E. coli* strains detected in the air samples at the different AGI-30 impinger heights are depicted in Fig. 3.

In the trials without organic soiling, the geometric mean concentration was lowest at 30% RH for all strains. The highest mean concentration in the trial without organic soiling was detected at 70% RH for *E. coli* K12 and *E. coli* R56 and at 50% RH for *E. coli* G-148-1. However, corresponding recovery rates were highest at 70% RH for all three strains.

In the trials with organic soiling, the highest airborne concentrations were detected at 30% RH for all three *E. coli* strains. The lowest concentrations were detected at 70% RH for all strains. The airborne concentrations and the recovery rates for all *E. coli* strains under different conditions are depicted in Table 3.

Differences in the recovery rates of the three E. coli *strains*

For all experimental replicates under different conditions, the recovery rate of *E. coli* K12 from the aerosol was 0.16%. For the ESBL-producing *E. coli* R56, the recovery rate was 0.76% and for the AmpC-producing *E. coli* strain G-148-1, it was 0.79%.

The results of the repeated measures negative binomial model revealed that the recovery rate or the tenacity in the airborne status of the AmpC-producing *E. coli strain* G-148-1 was 4.1-fold higher compared with the non-resistant strain *E. coli* K12 (p-value = 0.016), taking all experimental replications under different conditions into consideration. For the ESBL-producing strain *E. coli* R56, the recovery rate was 3.5-fold higher compared with *E. coli* K12 ($p = 0.041$). The recovery rate of *E. coli* G-148-1 was 1.2-fold higher compared with *E. coli* R56 ($p = 0.64$).

Influence of RH and organic soiling on the recovery rate of airborne E. coli

The influence of organic soiling on the recovery rate of *E. coli* was analysed in the repeated measures negative binomial model for the different RH. Since these factors had a similar effect on all strains, we did not stratify the three strains for the analysis. The calculated cfu of *E. coli* per m^3, including the upper and lower 95% confidence intervals, are visualized in Fig. 4. At an RH of 30%, the recovery rate of *E. coli* from the aerosol is 41.3-fold higher when adding 10 g L^{-1} yeast extract and bovine serum albumin (BSA) ($p < 0.001$). At 50% RH, the recovery rate was only marginally (1.1-fold) higher with organic soiling ($p = 0.91$). At 70% RH, the addition of organic substances had an adverse effect. The recovery rate of aerosolized *E. coli* significantly decreased by 13.3-fold compared with the experiments where no organic substances were added ($p = 0.003$).

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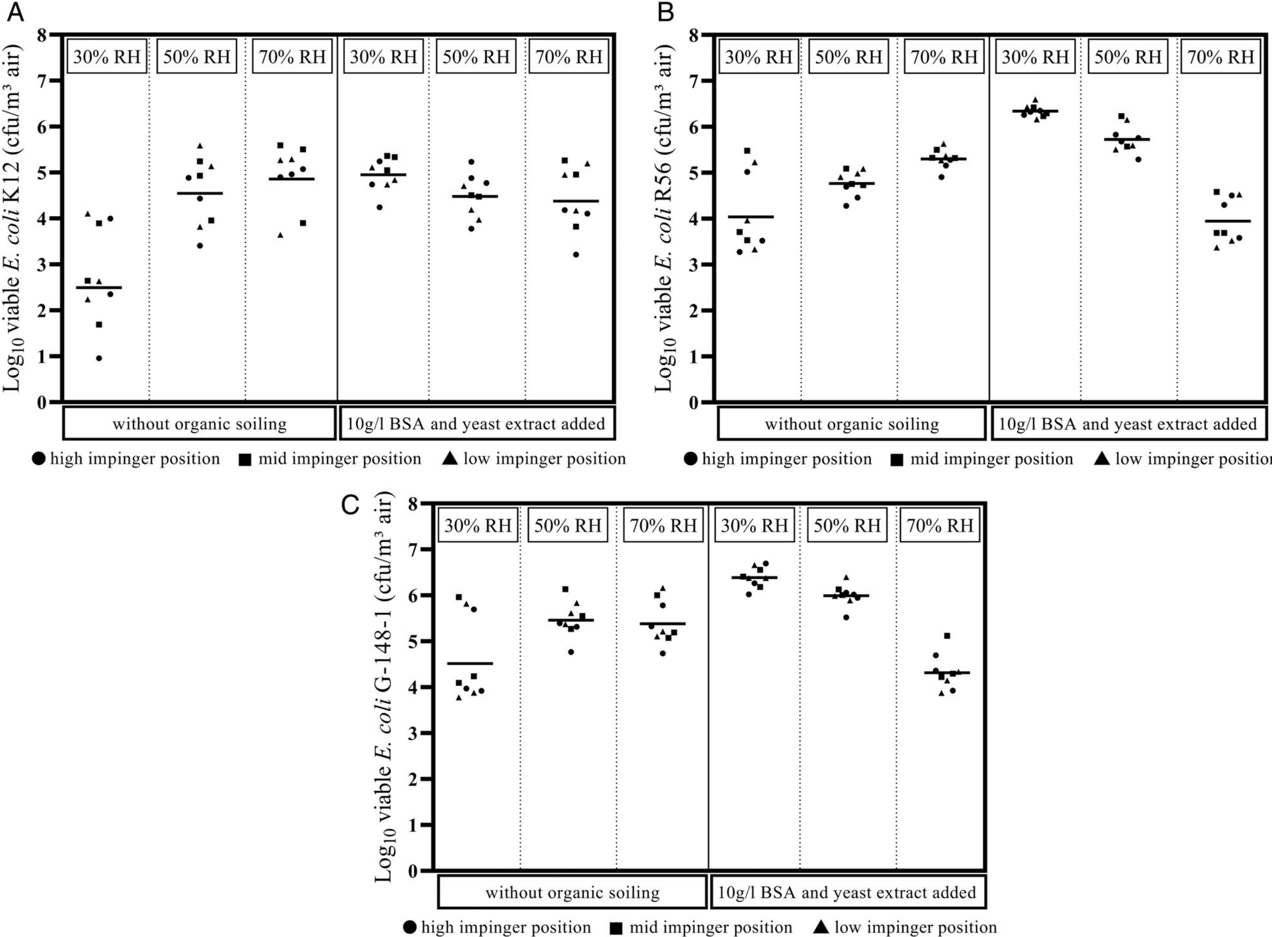

Fig. 3. LOG_{10} airborne concentration of *E. coli* K12 (A), *E. coli* R56 (B) and *E. coli* G-148-1 (C) in cfu m^{-3} air for 30%, 50% and 70% RH, with and without the addition of 10 g/L BSA and yeast extract. The horizontal line indicates the geometric mean. The different symbols indicate the sampling height of the AGI-30 impingers.

Table 3. *E. coli* concentration detected per m^3 of air for different strains and conditions, including the respective recovery rates.

E. coli strain	RH (%)	Organic soiling	cfu m^{-3}	Recovery rate (%)
E. coli K12	30	None	5.2×10^2	0.003
	50	None	2.8×10^4	0.28
	70	None	7.7×10^4	0.3
	30	10 g L^{-1} yeast extract+BSA	8.45×10^4	0.26
	50	10 g L^{-1} yeast extract+BSA	2.8×10^4	0.05
	70	10 g L^{-1} yeast extract+BSA	2.0×10^4	0.06
E. coli R56	30	None	1.1×10^4	0.11
	50	None	5.9×10^4	0.14
	70	None	2.0×10^5	0.7
	30	10 g L^{-1} yeast extract+BSA	2.2×10^6	2.66
	50	10 g L^{-1} yeast extract+BSA	5.4×10^5	0.93
	70	10 g L^{-1} yeast extract+BSA	7.6×10^3	0.01
E. coli G-148-1	30	None	3.8×10^4	0.12
	50	None	2.9×10^5	0.26
	70	None	2.5×10^5	0.42
	30	10 g L^{-1} yeast extract+BSA	2.4×10^6	2.8
	50	10 g L^{-1} yeast extract+BSA	9.8×10^5	1.17
	70	10 g L^{-1} yeast extract+BSA	1.9×10^4	0.03

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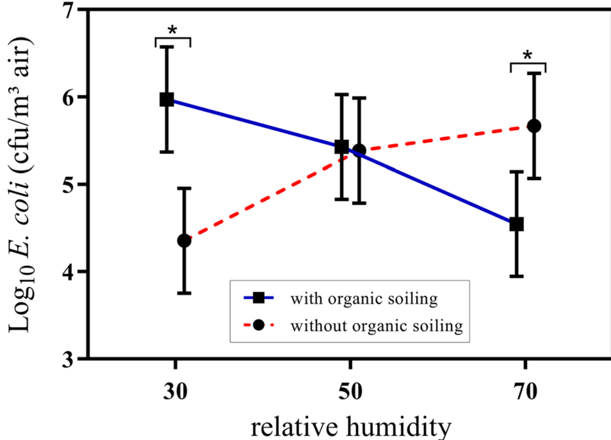

Fig. 4. Influence of organic soiling on the recovery rate of *E. coli* for the different RH, considering all three *E. coli* strains tested. The error bars indicate the upper and lower 95% confidence intervals.

Discussion

This study aimed to assess the relevance of an airborne spread of ESBL/AmpC-producing *E. coli* from soil fertilized with chicken litter to the environment by wind erosion.

The high total viable bacterial counts detected in the air samples indicate notable bio-aerosol formation, especially at high wind velocities. Wind erosion occurs when wind velocities exceed 6 m s^{-1} over dry soil (Zobeck and Pelt, 2015). This is consistent with the significantly increased average total viable bacterial count (7.1×10^7 cfu m^{-2}) detected at a wind velocity of 7.3 m s^{-1} in 5 cm distance to the soil-litter mix compared with the bacterial count detected at 5.6 m s^{-1} (2.0×10^6 cfu m^{-2}). Additionally, only for wind velocities exceeding 7 m s^{-1}, non-resistant *E. coli* were detected in the Coriolis μ air samples. However, we did not detect ESBL/AmpC-producing *E. coli* in all air samples taken in the wind tunnel trials. Within each wind velocity, no significant difference was observed in the total viable count emitted for the three wind tunnel trials, which indicates a good predictability of total viable bacteria emitted from fertilized loamy sand soil for certain wind velocities. Although the emitted total viable count was similar for all trials, a significantly increased $PM_{2.5}$ and PM_{10} emission was observed for the first wind tunnel trial. This increased fine dust emission was likely linked to the finer soil structure with smaller aggregates in the first wind tunnel trial, compared with the subsequent trials. The similar amount of total viable bacteria emitted in all three trials might be explained by assuming that larger particles, which were present in the second and third trials, might carry more total viable bacteria (Clauß, 2015). A further explanation for the similar bacterial emission for the three trials may be that the largest fraction of bacteria collected in the air samples was derived from the chicken litter and not from the soil, as recently suggested by Thiel *et al*. (2020), especially considering that the same amount of litter was used for all three trials. Based on our results on bacterial and particle emission, it can be assumed that fine dust and total viable count emission correlate. However, predicting wind-driven bacterial emissions from fertilized soil on the basis of particle emission is difficult, because it is influenced by many factors like soil type, soil structure, bacterial concentrations and composition of fertilizer and environmental conditions.

Data on airborne ESBL/AmpC-producing *E. coli* in the environment are scarce and few studies investigating this issue were conducted to date. Laube *et al*. (2014) detected ESBL-/AmpC-producing *E. coli* in 7.5% (3/40) of air samples taken from the exhaust air of chicken barns, but a quantification was not possible for any of the samples. While Blaak *et al*. (2015) detected ESBL-producing *E. coli* in 7.7% of air samples taken inside broiler barns, all air samples taken in the vicinity of the barns were negative for ESBL-producing *E. coli*, which might indicate a low environmental tenacity of poultry-associated ESBL-*E. coli* in the airborne state. In a study by Korzeniewska and Harnisz (2013), 23.8% of air samples collected at a wastewater treatment plant tested positive for phenotypic ESBL-producing *E. coli*. In that study, a significantly positive correlation between the wind velocity and the number of ESBL-positive air samples was shown.

In our study, aerosol chamber trials were performed to gain additional knowledge on the tenacity of ESBL-/AmpC-producing *E. coli* compared with non-resistant *E. coli* in the airborne state, which has never been investigated systematically to date. However, experimental studies on the airborne tenacity of non-resistant *E. coli* date back several decades (Poon,1966; Benbough, 1967; Cox, 1968; Wathes *et al*., 1986).

The airborne survival of bacteria under experimental conditions is influenced by various factors, including the bacterial strain, the composition of the culture and suspension fluid, the growth conditions, processing conditions of the bacterial cultures and the atmosphere into which the bacteria are released (Wathes *et al*., 1986). There are further important environmental factors, including the RH, oxygen concentration, temperature, ozone concentration, UV-radiation and air pollutants, which influence the tenacity of microorganisms in the aerosolized state (Zhao *et al*., 2014). The existence of overlapping lethal mechanisms for aerosolized bacteria renders the exact diagnosis of which mechanisms and conditions cause bacterial death difficult (Benbough, 1967).

Several studies have investigated the influence of temperature on the survival of *E. coli* in the aerosolized state.

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Hoeksma *et al*. (2014) investigated the survival of the *E. coli* strain DSM-1936 between 10°C and 30°C and was able to show that after the initial decay, the bacteria survived the longest at 30°C. In contrast, Wathes *et al*. (1986) reported approximately four times higher death rates at 30°C compared with 15°C for a nalidixic acid–resistant strain of *E. coli* (serotype 0149). A possible explanation might be that the influence of temperature on the survival of airborne *E. coli* is strain-specific. Zhao *et al*. (2014) stated that, in general, the decay of microorganisms is faster at higher ambient temperatures. In our aerosol chamber experiments, a uniform temperature of 24°C was used for all experiments. The temperature in the wind tunnel trials varied between 16.1°C in the first trial and 25.7°C in the third trial. We did not observe a correlation between the temperature and the number of air samples positive for non-resistant *E. coli* or the total viable count in the wind tunnel trials.

Concerning the RH, we showed a significantly higher recovery rate of different *E. coli* strains in the aerosol chamber experiments without organic soiling at an RH of 50% and 70% compared with 30% RH. This finding is in line with a series of studies that showed decreased survival rates of aerosolized *E. coli* at low RH values. Wathes *et al*. (1986) reported half-life times of 3 min for low RH (<50%) and 14 min in humid conditions for *E. coli* aerosolized from phosphate-buffered saline (PBS) at 30°C. According to Poon (1966), the death rate of aerosolized *E. coli* is directly proportional to the rate of water evaporation and the water evaporation increases with decreasing environmental RH. Hoeksma *et al*. (2014) observed a fast biological decay of *E. coli* during the first 30 s after the aerosolization and a much smaller decay in the following 30 min, which he explains by a sudden cooling down effect caused by the evaporation of water. It is hypothesized that after aerosolization in dry conditions, water in the newly formed bacterial droplet will evaporate in milliseconds, thus altering the temperature, solute content and other environmental conditions of the droplet, which might promote biological decay (Liu *et al*., 2017). Additionally, under dry conditions, the toxicity of oxygen by the formation of free oxygen radicals which damage flavin-linked bacterial enzymes is increased (Benbough, 1967) compared with humid conditions, under which oxygen-induced free radicals are present to a lesser extent (HECKLY *et al*., 1963).

At 30% RH, bacterial suspensions with organic soiling showed a significantly increased recovery rate compared with suspensions without organic soiling. The explanation might be that airborne microorganisms are protected from external influences by particles coagulated within the viable bacterial particles, thus protecting bacterial proteins and membrane phospholipids, which appear to be targets for humidity and temperature-induced bacterial inactivity (Zhao, 2011).

In the trials with organic soiling, the lowest concentrations of aerosolized *E. coli* were detected in the aerosol at 70% RH (4.5×10^4 cfu m^{-3} air). This is unexpected because a high RH and the addition of organic substances both seemed beneficial for the survival of airborne bacteria. According to Marthi *et al*. (1990), a high RH might lead to clumping of cells, potentially increasing the odds of cell survival. This clumping effect may be enhanced by organic soiling in the *E. coli* suspensions. Viable *E. coli* may therefore have quickly deposited in large aggregates on the floor of the aerosol chamber and were undetectable in the air samples. Quantification of the deposited *E. coli* fraction might have been of particular interest. A limitation of this study is that we did not generate valid information about the particle size in the aerosol chamber trials, which may have supported this hypothesis.

In the wind tunnel trials, the RH was similar for the first and second wind tunnel trials with 56% and 54.4%. In the third wind tunnel trial, the RH was considerably lower, with 29.7%. Non-resistant *E. coli* were detected in 50% (5/10) of the Coriolis μ air samples in the first trial and 60% (6/10) in the second trial but in none of the air samples taken in the third wind tunnel trial. The lower environmental RH in the third wind tunnel trial might have led to inactivation by desiccation of *E. coli* adhering to soil or litter particles. This hypothesis seems inconsistent with the results of the aerosol chamber trial, where the highest survival rates under the presence of organic soiling were found at 30% RH. This deviation might be explained by the fact that we aerosolized the *E. coli* strains in the aerosol chamber trials from bacterial suspensions. Zhao (2011) stated that the biological decay of microorganisms aerosolized from dry sources might differ from the decay in wet aerosolization. He, therefore, recommended using dry aerosolization for microorganisms released from dry sources like faeces or litter. However, Hoeksma *et al*. (2014) pointed out that *E. coli* rarely survives the procedure for preparing dry aerosols.

The recovery rate for poultry-associated ESBL-/AmpC-producing *E. coli* strains R56 and G-148-1 was significantly higher than the recovery rate of the laboratory strain *E. coli* K12 under all experimental conditions. This is in accordance with findings by Marshall *et al*. (1988), who observed a prolonged survival of aerosolized *E. coli* of wild-type strains compared with the laboratory strain *E. coli* K12. The reasons for the significant differences in the recovery rates between the *E. coli* strains are still unclear. Additional research is warranted. To estimate the influence of ESBL-/AmpC-plasmid carriage on the tenacity of *E. coli* during aerosolization, a comparison of the recovery rates of ESBL/AmpC *E. coli* strains and their plasmid-cured variants would be of interest, as Ranjan *et al*. (2018) have demonstrated that the carriage of

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certain ESBL-plasmids was beneficial regarding competition fitness *in vitro*. Little is known about the molecular mechanisms and bacterial stress response in aerosolized bacteria. In a recent study by Ng *et al*. (2018), comparative transcriptome analysis was used to gain knowledge on gene expression in *E. coli* following aerosolization. Results indicate that *E. coli* responds to environmental stimuli in the air very quickly by changing the transcriptional signature. During aerosolization, 11 stress-responsive genes and 13 stimulus-responsive genes were regulated. It might be hypothesized that wild-type strains can change their transcriptional signature faster compared with laboratory strains in reaction to environmental stimuli.

Estimation of the potential of an airborne environmental spread of ESBL-/AmpC-producing E. coli *by wind erosion*

In the wind tunnel trials, there were considerable differences in the concentrations of ESBL-producing *E. coli* in the chicken litter between the trials, reflecting realistic variations in the colonization of broiler flocks with these bacteria (Daehre *et al*., 2017). While in the first trial, ESBL-*E. coli* were only qualitatively detectable in the chicken litter, in the second wind tunnel trial, chicken litter with a high concentration (3.2×10^5 cfu g^{-1} litter in barn A and 4.2×10^5 cfu g^{-1} in barn B) of ESBL-producing *E. coli* was used for aerosolization. Blaak *et al*. (2014) reported a similar average concentration (5.3×10^5 cfu g^{-1}) of ESBL-producing *E. coli* in fresh chicken litter. The chicken litter used in the wind tunnel trials naturally contained ESBL/AmpC-producing *E. coli*, which were already adapted to the existing environmental conditions. This is beneficial because bacterial stress is minimized (Wesche *et al*., 2009) and may lead to a survival advantage over artificially added bacteria. The concentration of ESBL/AmpC-producing *E. coli* in the chicken litter and the mixing ratio of the soil-litter mixture reflected practical conditions, which ensures a good transferability of the results to field conditions.

The two air samplers used in the wind tunnel trial have different properties. The airspeed at the inlet of the AGI-30 is 4 m s^{-1}, for the Coriolis μ it is 25 m s^{-1}. Therefore, air sampling was always super-isokinetic with the Coriolis μ, while it was only super-isokinetic for the AGI-30 at the lowest wind speed, where a mean of 3.3 m s^{-1} was measured in the suspensions chamber and sub-isokinetic for higher wind speeds (Table 2). Sub-isokinetic sampling is not ideal and has higher measurement errors compared with isokinetic or super-isokinetic sampling (Friedlander, 1977). Additionally, both air samplers have different particle cut-offs. The AGI-30 has a cut-off of 0.3 μm (Yao and Mainelis, 2006). Particles with a diameter above 15 μm are also not sampled in the AGI-30 collection fluid, because they are collected at the tube wall by inertial force (Lindsley *et al*., 2017). The Coriolis μ has a cut-off size of 0.5 μm at an operation flow of 300 L min^{-1}, which means that particles of 0.5 μm are sampled at 50% efficiency and larger particles are sampled at higher efficiency (Mbareche *et al*., 2018). Because of their different cut-offs, it was unexpected that both air sampling systems showed similar collection efficiencies. However, Clauß *et al*. (2013) stated that most airborne microorganisms are bound to particles with a size of 5–10 μm. For this size fraction, both air samplers have a high collection efficiency, which might explain the similar results concerning the total viable count detected per m^3 of air. Additionally, the detection limits of both air samplers differ. In our experimental setup, the quantitative detection limit was approximately 8.5×10^2 cfu m^{-3} for the AGI-30 air samples and 1.2×10^1 cfu m^{-3} for the Coriolis μ air samples. The qualitative detection limit was 8.0×10^1 cfu m^{-3} for the AGI-30 samples and only approximately 1 cfu m^{-3} for the Coriolis μ air samples. Despite the very low qualitative detection limit of the Coriolis μ, no ESBL-/AmpC-producing *E. coli* were detected in the air samples taken in the wind tunnel trials. The reasoning for this appears to be multifactorial. ESBL-producing *E. coli* are rapidly inactivated in chicken litter. In a study recently published by Siller *et al*. (2020), ESBL-producing *E. coli* concentrations decreased from an average of 3.4×10^5 cfu g^{-1} in fresh chicken litter below the detection limit after storage periods of 36 h in the summer and 72 h in the winter. To limit this inactivation in the litter used in the wind tunnel trials, the timespan between litter collection and aerosolization was minimized. A high concentration of ESBL-producing *E. coli* in the litter or fertilized soil seems to be essential in order to detect ESBL-/AmpC-producing *E. coli* in air samples. Chinivasagam *et al*. (2009) were able to show a direct link between levels of non-resistant *E. coli* in chicken litter and airborne *E. coli* concentrations in chicken barns. At concentrations of 10^8 cfu g^{-1} *E. coli* in the litter, airborne concentrations in the barns ranged from 10^2 to 10^5 cfu m^{-3}. In our study, despite the fact that ESBL/AmpC-positive litter was used in all trials, after mixing and diluting the litter with soil, quantitative detection of ESBL-producing *E. coli* in the soil-litter mixture was only possible in the second wind tunnel trial. Presumably, the vast majority of ESBL/AmpC *E. coli* present on the soil surface in the wind tunnel trials was subsequently inactivated during aerosolization. This hypothesis is strongly supported by the results of the aerosol chamber trials, which confirmed a low tenacity of aerosolized ESBL-/AmpC-producing *E. coli* with an average reduction of 2.25 LOG_{10}.

The detection of high total viable bacterial counts and low amounts of *E. coli* in air samples taken in the wind tunnel might be explained by the fact that *E. coli* is a

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Gram-negative bacterium. Gram-negative bacteria account for a small proportion of airborne bacteria (Zucker *et al*., 2000). In poultry houses, Bakutis *et al*. (2004) reported a proportion of 2.6% of Gram-negative bacteria of the total bacterial count. Zhao *et al*. (2014) suspected that airborne Gram-negative bacteria are less frequently detected in air samples of livestock production systems, because they might be more vulnerable to environmental stress such as oxidation, radiation, and dehydration, presumably due to their thinner cell walls.

Additionally, it has to be considered that the ESBL/AmpC-*E*. *coli* producing subpopulation represents only a small proportion of the total *E*. *coli* population. This could explain why ESBL/AmpC-producing *E*. *coli* have remained below the detection limit in the air samples while non-resistant *E*. *coli* were qualitatively detected. In the second wind tunnel trial, ESBL-*E*. *coli* represented 1.6% of the entire *E*. *coli* population in the chicken litter for barn A and 4.4% for barn B. In the third wind tunnel trial, 1.5% of *E*. *coli* were ESBL-producing. Friese *et al*. (2019) recently reported an according proportion of 1.1% ESBL-producing *E*. *coli* in turkey rearing flocks.

Considering all factors discussed, we conclude that an airborne spread of ESBL/AmpC-producing *E*. *coli* in the environment by wind erosion seems unlikely. However, because non-resistant *E*. *coli* were detected in the air samples, we suspect that ESBL-/AmpC-producing *E*. *coli* may have been present below the detection limit; therefore, a potential airborne spread of extremely low quantities of ESBL/AmpC-producing *E*. *coli* cannot be excluded. In two out of three wind tunnel trials, soil-litter mixtures with a low concentration of ESBL/AmpC-producing *E*. *coli* were used. If litter with a significantly higher concentration of ESBL-*E*. *coli* would be applied to arable land, the potential of emission might be increased. However, under practical conditions, it seems unlikely that ESBL-*E*. *coli* from chicken litter are applied to arable land in relevant concentrations because the largest proportion of ESBL-producing *E*. *coli* in chicken litter is quickly inactivated when transported from barns to arable land (Thiel *et al*., 2020). Additionally, short-term storage (5 days) of chicken litter was proven to inactivate ESBL-*E*. *coli* effectively (Siller *et al*., 2020) and a recent lab-scale study by Thomas *et al*. (2019) confirmed the extinction of ESBL-*E*. *coli* in chicken litter within 2 h at temperatures exceeding 55°C. Additionally, the present study confirmed a low airborne tenacity for non-resistant and ESBL-/AmpC-producing *E*. *coli*. An airborne spread of ESBL/AmpC-producing *E*. *coli* from organic fertilizers from farm animals other than chickens seems even more improbable because ESBL/AmpC-producing *E*. *coli* are detected in broiler farms in the highest quantities and with a prevalence of up to 100% (Dierikx *et al*., 2010; Laube *et al*., 2013; Hering *et al*., 2016). Additionally, in the largest comparative study to date, by far higher concentrations of airborne bacteria were detected in poultry barns with 2.7×10^6 cfu m^{-3} air compared with 1.3×10^5 cfu m^{-3} in pig barns and 2×10^4 cfu m^{-3} in cattle buildings (Seedorf *et al*., 1998), which might be an indication for a pronounced potential of chicken litter, which typically has a high dry matter content, to form bioaerosols.

If emission of extremely low quantities of ESBL-/AmpC-producing *E. coli* to the environment via wind erosion occurs, the risk of colonization in humans and animals remains unclear. Dungan (2010) stated that information on the infectivity of aerosolized enteric pathogens is scarce. However, it was recently shown by Robé *et al*. (2019) that an oral infection dose as low as 10^1 cfu ESBL-*E*. *coli* has led to persistent colonization of broiler chicks. This suggests that even very low quantities of these bacteria emitted to the environment might lead to health implications and thus highlight the crucial role of further research in the domain of airborne environmental antimicrobial resistance.

Experimental procedures

Wind tunnel trials

Characteristics of the wind tunnel. Detailed technical descriptions of the wind tunnel located at the ZALF have been published by Funk (2000) and Funk *et al*. (2008, 2019). A modified sketch of the wind tunnel illustrating the most important technical features and the experimental setup is included in Fig. S2 in the supplementary material.

The wind tunnel has a total length of 25 m and is of the Eiffel-type (push-type), meaning the air circulation is not a closed system. The airflow is generated by two axial ventilators, which are on top of each other at the beginning of the measuring section. The wind velocity is continuously adjustable up to 18 m s^{-1}. Rectifiers reduce turbulences and the profile former creates a logarithmic wind profile before the air enters the measuring section. The measuring section of the wind tunnel has a length of 7 m and a height and width of 0.7 m, equating a cross-sectional area of 0.49 m^2. It is accessible from one side and can be closed with acrylic glass plates. The floor space on which the soil-litter mixture was deposited measures 4.9 m^2. Aerosolized particles are blown to the suspension chamber, where the air sampling devices were positioned. An exhaust air channel is located in the ceiling of the suspension chamber. The cross-sectional area of the suspension chamber is 12 times larger compared with the measuring section, resulting in a reduction of the

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average wind velocity to an eight of the original wind velocity.

Experimental design. To obtain ESBL-/AmpC-positive chicken litter for the wind tunnel trials, an initial screening was carried out to identify ESBL/AmpC-positive broiler barns. In total, 35 barns of two different broiler farms were tested by taking boot swab samples, pooled faeces and litter samples. All samples were qualitatively and quantitatively analysed for ESBL-/AmpC-producing *E. coli* (please see below for laboratory protocols). Litter from the barns with the highest ESBL/AmpC-producing *E. coli* concentration was chosen for further investigations in the wind tunnel trials. The concentration is shown in Table 1. In the first wind tunnel trial, the result of the qualitative analysis of the soil-litter mixture concerning AmpC-producing *E. coli* is uncertain, because only a few small colonies, which phenotypically resembled *E. coli*, were detected on the MC+ plates. These small colonies were transferred to a new set of plates and incubated overnight. The next day, no visible growth of *E. coli* was observed. In the second wind tunnel trial, litter from two barns was mixed because the concentration of ESBL-*E. coli* in the screening was similarly high.

For each trial, approximately 10 kg of chicken litter from the selected barns was collected in sterile plastic bags and stored in polystyrene boxes with ice packs until use. Trial 1 took place 1 day after litter collection; the chicken litter was stored overnight at 4°C. Trials 2 and 3 took place on the same day as litter collection. Two samples were stored for microbiological analyses: One directly after litter collection and one upon arrival in the wind tunnel.

The wind tunnel trials were carried out at the Leibniz Centre for Agricultural Landscape Research (ZALF). A concrete mixer was used to mix 3 kg of chicken litter with 250 dm^3 of loamy sandy soil taken from a trial plot of land. This ratio was chosen based on the German legislation, Paragraph 6 (4) of the Düngeverordnung (German Fertilizer Ordinance) which limits total nitrogen to 170 kg per hectare and data from the Leibniz-Institute of Vegetable and Ornamental Crops (2017), which estimates the average total nitrogen of fresh chicken litter from Brandenburg at 17.5 kg per metric ton. The grain size of the soil was measured by wet sieving (2000–63 μm) and the sedimentation method (<63 μm; DIN ISO 11277, 2002). This resulted in the following soil particle composition: Sand (2000–63 μm): 69%; Silt (63–2 μm): 25%; Clay (<2 μm) 6%. Derived from the particle composition, the soil can be classified as a 'loamy sand' and is therefore susceptible to wind erosion.

In the second wind tunnel trial, chicken litter from two barns was mixed at a ratio of 1:1, because these barns showed similarly high concentrations of ESBL-producing *E. coli* in the screenings. To ensure that the soil was negative for ESBL/AmpC-producing and non-resistant *E. coli*, soil samples for microbiological analysis were taken before mixing. After the mixing procedure, the soil-litter mixture was sampled again for microbiological analysis.

The wind tunnel was then filled with a 5 cm layer of soil-litter mixture and exposed to different wind velocities (5.6, 7.3, 9.8 and 10.6 m s^{-1} in 5 cm distance to the soil surface on average) for a time span of 10 min each. The wind velocity was monitored at a height of 5, 30 and 60 cm in the wind tunnel section using a hot wire anemometer (Lambrecht Thermal Anemometer 642, Lambrecht, Goettingen, Germany). The RH, temperature and PM_{10}, as well as the $PM_{2.5}$ concentration [μg m^{-3}], were measured using an Environmental Dust Monitor (EDM 164, GRIMM-Aerosol Technik, Ainring, Germany).

There was no exchange of the soil-litter mixture between wind velocities and the soil surface remained unaffected for each replicate of the wind tunnel trials. Therefore, total viable bacterial counts and particle concentrations were calculated by gradually adding up the total viable count and particles for each wind velocity, since a proportion of particles were already aerosolized at lower wind velocities and are therefore missing at higher wind velocity measurements, as described by Funk *et al*. (2019). Particle concentrations and total viable counts measured per m^3 of air were multiplied with the air volume (in m^3), which passed the wind tunnel for each wind velocity and divided by 4.9 (floor space in m^2) to calculate the emission of particles and total viable bacteria per m^2 of soil-litter mixture and therefore considering the dilution factor at increasing wind velocity. The experiments were carried out in triplicate for each trial. After one cycle, the soil was turned and mixed to create a fresh surface. Air samples were taken at the terminus of the wind tunnel using all-glass impingers 30 (AGI-30; Neubert Glas GbR, Geschwenda, Germany, VDI Norm 4252-3) and a Coriolis μ cyclone air sampler (Bertin Instruments, Montigny-le-Bretonneux, France). The impingers were filled with 30 ml of PBS (Oxoid, Wesel, Germany) and connected to a vacuum pump with a plastic tube. The airflow was monitored using a rotameter. It was approximately 12.5 L min^{-1}. Coriolis μ cones were filled with 15 ml PBS. The airflow for the Coriolis μ was set to 300 L min^{-1}. Sampling times for the AGI-30 impinger and Coriolis μ air samples were 10 min for each level of wind velocity. Impingers and Coriolis cones were stored at cool temperatures before and after air sampling. The air sampling devices were positioned 1.5 m above the ground, to imitate the height at which average humans respire. In total, 11 air samples were taken with the AGI-30 impingers and Coriolis μ per wind tunnel trial: Directly after the wind tunnel was filled with soil-litter

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mixture, a check plot sample was taken using impingement and cyclone sampling when the wind tunnel was turned off. At 5.6 m s^{-1}, only one air sample was taken for each trial. At 7.3, 9.8 and 10.6 m s^{-1}, three air samples were taken with each air sampling device per trial.

Laboratory analyses. All boot swabs, pooled faeces, litter, soil and air samples from the screenings of the barns and the wind tunnel trials were analysed qualitatively and quantitatively for ESBL/AmpC-producing and non-resistant *E. coli* as described by Siller *et al*. (2020). The air samples were additionally analysed for the total viable bacterial count.

Twenty grams of pooled faeces, litter and soil samples were mixed at a ratio of 1:10 with Luria/Miller-broth (LB; Roth, Karlsruhe, Germany) in stomacher bags. Boot swabs were put in stomacher bags and 200 ml LB was added. The samples were homogenized using a Stomacher 400 Circulator (Seward Limited, West Sussex, UK) at 200 rpm for 2 min. The quantitative analysis was performed by directly streaking 100 μl of the homogenized samples in triplicates on specific agar plates after serial dilution. For the quantification of *E. coli*, MacConkey agar No. 3 (Oxoid) was used (MC-). To quantify ESBL/AmpC-producing *E. coli*, 1 mg L^{-1} Cefotaxime (AppliChem, Darmstadt, Germany) was added to the MacConkey agar plates (MC+), as recommended by the EFSA (EFSA, 2011). Under optimal conditions, the quantitative detection limit of this method is 3.3×10^1 cfu g^{-1} and the qualitative detection limit is 1 cfu/20 g.

For the air samples, 100 μl were directly streaked on MC+ and MC- plates in triplicates. Additionally, 100 μl of the air samples were plated out on blood base agar (Blood agar Base No. 2, Oxoid) after serial dilution to determine the total viable bacterial count.

For qualitative analysis, the homogenized samples were incubated in LB medium for 24 h at 37°C. Three millilitre of air sample fluids were incubated under the same conditions after a 1:10 dilution with LB medium in Erlenmeyer flasks. Subsequently, 10 μl of each sample was streaked on MC+ and MC- agar with an inoculation loop and incubated again for 24 h at 37°C. The quantitative detection limit for the AGI-30 air samples was 8.5×10^2 cfu m^{-3}, the qualitative detection limit was 8.0×10^1 cfu m^{-3}. For the Coriolis μ air samples, the quantitative detection limit was 1.2×10^1 cfu m^{-3}, the qualitative detection limit was approximately 1 cfu m^{-3}.

Species confirmation of colonies suspected to be *E. coli* was achieved using MALDI-TOF Mass Spectrometry (MALDI Microflex LT and Biotyper database, Bruker Daltonics, Bremen, Germany).

Real-time PCR and sequencing. To detect the most important ESBL and CIT-type AmpC resistance genes (bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{CMY}), isolates from the pre-trial screenings and the wind tunnel trials were tested using real-time PCR, as previously described by Roschanski *et al*. (2014). To identify the present ESBL-/AmpC-variants, a selection of isolates was sequenced using Sanger-sequencing for each trial. The fewest isolates were analysed for the first wind tunnel trial (n = 6) because no AmpC-producing *E. coli* colonies were isolated from the soil-litter mixture. Most isolates (n = 16) were analysed for the second wind tunnel trial because isolates from two barns were characterised in this trial. For the third trial, 10 isolates were analysed. For Sanger sequencing, DNA isolation and PCR were performed as published previously (Projahn *et al*., 2017). Purified PCR products were sent to LGC Genomics (Berlin, Germany), who provided the sequences. The obtained sequences were analysed using DNASTAR Lasergene (Madison, Wisconsin) and compared with the reference sequences of GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Phylotyping. Phylotyping of the isolates acquired from the litter used in the wind tunnel trials was performed as published by Clermont *et al*. (2013) with a modified PCR according to Projahn *et al*. (2017). Isolates that could not be assigned to one phylogroup due to unspecific band patterns were assigned a combined phylogroup.

Aerosol chamber trials

Characteristics of the aerosol chamber. The aerosol chamber trials were carried out in the aerosol chamber of the Institute for Animal Hygiene and Environmental Health (Freie Universtität Berlin).

Detailed technical descriptions of the aerosol chamber, including a Figure, have been published previously (Rosen *et al*., 2018). In brief, the aerosol chamber generates bioaerosols under different standardized climatic conditions (temperature, airflow rate and RH). The volume of the aerosol chamber is 7 m^3. A perfusion pump transports the bacterial suspensions with a rate of 9 ml h^{-1} to an ultrasonic nebulizer. There is an axial ventilator in the ceiling of the chamber to disperse the aerosol and a separate opening in the ceiling for fresh air. The airflow rate was 100 m^3 h^{-1} and the temperature was 24°C for all experiments. The RH was set at 30%, 50% or 70% depending on the experimental setup.

The aerosol was sampled using three AGI-30 impingers at different heights (0.3, 0.8 and 1.3 m), each filled with 30 ml PBS. The airflow was approximately 12.5 L min^{-1} and the sampling time was 30 min.

Experimental design. In this experimental series, one non-resistant and two poultry-associated ESBL-/AmpC-producing strains of *E. coli* were aerosolized under

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various conditions. The ESBL and AmpC-producing strains were aerosolized together in a mixed bacterial suspension. Air samples were taken during aerosolization. We investigated the effect of different RH (30%, 50% and 70%) and the influence of organic soiling (10 g L^{-1} yeast extract and BSA, in combination) on the bacterial tenacity in the airborne state. Each *E. coli* strain was aerosolized at the three different RHs and with and without the addition of organic soiling respectively. Each experimental combination was carried out in triplicates.

The recovery rates of airborne *E. coli* were calculated by dividing the concentration of *E. coli* per m^3 measured in the aerosol chamber in the experiments by the expected concentration per m^3. This expected concentration was calculated for each experiment individually by multiplying the concentration of the *E. coli* suspensions (per ml) with the forward speed of the perfusion pump (9 ml h^{-1}) divided by the air exchange rate of the aerosol chamber (in m^3 h^{-1}).

Preparation of the bacterial suspension and organic soiling solution. We used the well-described, non-resistant commensal laboratory strain *E. coli* K12 (DSM 423; https://www.dsmz.de/collection/catalogue/details/culture/dsm-423) and two resistant, poultry-associated strains that were isolated from healthy chickens in a previous project in 2011 (Hering *et al*., 2016). The *E. coli* strain G-148-1 belongs to the multilocus sequence type (MLST) 10 and the phylogroup A. It harbours the CIT-type AmpC resistance gene $bla_{\text{CMY-2}}$ and the *mcr-1* gene, which mediates colistin resistance. The second strain, first named R56 by Falgenhauer *et al*. (2016), belongs to the MLST 410 and phylogroup B1. It harbours the ESBL resistance gene $bla_{\text{CTX-M-15}}$ and is additionally resistant to enrofloxacin.

Three colonies of the specific *E. coli* strain were added to 10 ml LB and incubated overnight at 37°C and 200 rpm in a shaking incubator (Multitron, Infors HT, Germany). The following day, 5 ml of this suspension was added to 100 ml of LB and incubated for 8 h. Then, 100 μl of the suspension was plated to blood base agar and incubated overnight (8 h) to achieve the exponential growth phase. The bacteria were removed from the agar plates with a plate spreader by adding 3 ml PBS and homogenized on a vortex mixer for 3 min with glass beads. To achieve the targeted concentration of 10^9 cfu (colony forming units)/ml, a fraction of the bacterial suspension was diluted with PBS at a ratio of 1:10. A measured value of 0.5 McFarland standards in the diluted fraction verified the targeted concentration of 10^9 cfu ml^{-1} in the bacterial suspension. The optical density was measured at 600 nm to validate the McFarland measurement (reference range: 0.073–0.11).

The organic soiling solution containing 10 g L^{-1} yeast extract (Merck, Darmstadt, Germany) and 10 g L^{-1} BSA (Sigma, St Louis, USA) was prepared by adding yeast extract to distilled water at a ratio of 1:50 and autoclaving. BSA was dissolved in distilled water at the same ratio and sterilized by membrane filtration. Afterward, the solutions were mixed at a ratio of 1:1.

For the addition of organic soiling, bacterial suspensions were centrifuged at 3000 rpm for 10 min, the supernatant was discarded and the pellet was resuspended with the organic soiling solution.

Microbiological analysis of the bacterial suspensions and air samples. All bacterial suspensions and air samples were analysed quantitatively. Serial dilutions were prepared for all samples.

In the experiments with *E. coli* K12, 100 μl was streaked in triplicates on MC- agar and incubated for 24 h at 37°C. In the experiments with ESBL-/AmpC-producing *E. coli*, all samples were streaked out in triplicates on two types of MacConkey agar No. 3 plates. Both plate types were supplemented with 2 mg L^{-1} cefotaxime. One set of plates additionally contained 4 mg L^{-1} of enrofloxacin, allowing a phenotypical selection for the R56 strain. The other set of plates additionally contained 7 mg L^{-1} colistin, leading to a phenotypical selection for the G-148-1 strain.

Statistical analysis

All statistical analysis was performed using R version 3.62 (R Foundation Vienna). Since bacterial counts (cfu) were lognormal distributed, we used the geometric mean for averaging. For statistical analysis, we used a mixed count regression. Due to overdispersion, we choose a negative binomial distribution. For the wind tunnel experiments, we used two hierarchical random effects. One random effect was used for the three soil-litter mixtures, which were measured at multiple wind velocities and within this effect, we used a random effect for the cfu plating triplicates as a repeated measures adjustment. The wind velocity and impinger type were modelled as a fixed effect interaction to assess the difference in measured cfu between AGI-30 and Coriolis μ at different wind velocities. Additionally, a model with the type of air sampler as the only fixed effect was run to compare air sampler performance overall. The probability of a qualitative detection of *E. coli* in the air samples depending on the wind velocity was analysed using logistic regression.

For the aerosol chamber experiments, a random effect for each of the experiments was utilized to account for repeated measures due to three AGI-30 impinger measurements per experiment and the combined aerosolization of two strains (*E. coli* R56 and G-148-1). We

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checked for interactions using interaction plots and found an interaction between humidity and organic soiling. Additionally, the strain was included as a categorical variable in the model. *Post hoc* comparisons between all strains were adjusted for multiple comparisons using the Tukey method. For a better comparison between the three strains, the concentrations in the bacterial suspensions were normalized for this analysis. All mixed models were performed using the R package lme4 (version 1.1-21). Estimated marginal means and multiple comparison post hoc tests were performed using the emmeans R package (version 1.4.5). Results are reported with 95% confidence intervals. A significance threshold of 0.05 was used. Figures were created using Graphpad Prism 8 (GraphPad Software, San Diego, CA).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Molecular characterization of the isolates from the chicken litter taken in the barns, which was subsequently used in the wind tunnel trials.

Fig. S1. LOG_{10} of the geometric mean total viable count emitted per m^2 of soil for each level of wind velocity determined with the all-glass-impinger 30 (a) and the Coriolis μ (b) for the three wind tunnel trials. The error bars indicate the upper and lower 95% confidence intervals.

Fig. S2. Sketch of the wind tunnel; modified according to Funk (2000) and Funk *et al*. (2019).

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4. Unpublished Data

4.1 Screenings for ESBL/AmpC-producing *E. coli* prior to the field, wind tunnel and litter storage trials

To identify chicken barns with a high concentration of ESBL/AmpC-producing *E. coli* in the chicken litter for usage in the field, litter storage and wind tunnel trials, two large broiler farms (farm A and farm B) were regularly screened for the prevalence of these bacteria. In the first screenings, boot swabs and pooled feces were taken in the barns. In subsequent screenings, also litter samples were collected, because the concentration of ESBL/AmpC *E. coli* in the litter samples was most relevant for the subsequent trials. An identical sampling path for the collection of boot swabs was taken in all chicken barns (Figure 2). On the sampling path, pooled feces and litter samples were taken by picking up 10 individual feces or litter samples in a sterile container with a spatula. All barns were sampled at the end of the fattening period, one to two weeks before the animals were housed out and consequently had high stocking densities. For that reason we only entered the barns halfway, to reduce stress in the chickens, which gathered in the back part of the barn when walking through it.

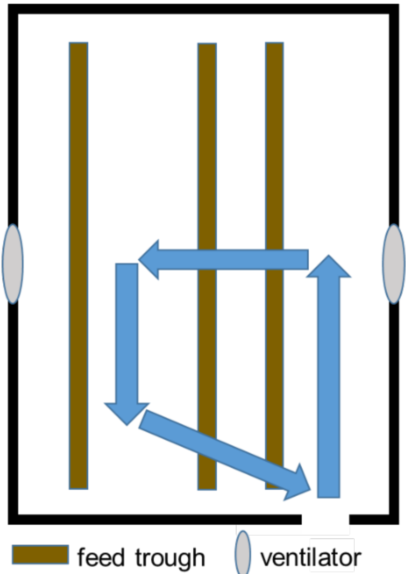

Figure 2: Sampling path for the collection of boot swab, pooled feces and litter samples in the chicken barns

All samples were microbiologically analyzed. As a first step, a qualitative analysis for ESBL/AmpC-producing *E. coli* was performed for all samples after overnight enrichment. Qualitatively positive samples were assessed semi-quantitatively and categorized according to the number of phenotypic ESBL/AmpC *E. coli* detected on the agar plates in four categories: over 100 colonies (+++), 10-100 colonies (++), 1-10 colonies (+) and no visible colonies (-). Samples that showed over 100 colonies in the semi-quantitative analysis were subsequently analyzed quantitatively and additionally the present ESBL/AmpC-gene family was determined via real-time multiplex PCR as previously described by Roschanski et al., (2014).

4.1.1 Detection of ESBL/AmpC-producing *E. coli* in samples from Farm A

All 15 barns of farm A were screened for ESBL/AmpC-producing *E. coli* three times, approximately every six months. ESBL/AmpC-producing *E. coli* were qualitatively detected in 100% (15/15) barns for each of the three screenings. Quantitatively, the highest concentrations

of ESBL/AmpC *E. coli* detected on farm A was 3.7 x 10^7 cfu in a boot swab, 1.8 x 10^6 cfu/g in a pooled feces sample and 8.6 x 10^6 cfu/g in a litter sample. An overview of the qualitative and quantitative microbiological analysis of the samples from farm A is depicted in Table 3.

Table 3 Qualitative and quantitative detection of ESBL/AmpC-producing *E. coli* for broiler farm A at different points in time

Sampling date	Sampling pattern	No. of barns sampled	No. of barns qualitatively positive for ESBL/AmpC *E. coli*	Highest ESBL- *E. coli* concentration detected in the samples
05/15/2017	1 boot swab 1 pooled feces	15	15	1.3 x 10^6 cfu/boot swab 2.1 x 10^5 cfu/g pooled feces
11/01/2017	1 boot swab 1 pooled feces 2 litter samples	15	15	3.7 x 10^7cfu/boot swab 1.8 x 10^6/ cfu g pooled feces 8.6 x 10^6 cfu /g litter
05/08/2018	1 boot swab 1 pooled feces 2 litter samples	15	15	1.4 x 10^7 cfu /boot swab 9.7 x 10^4 cfu /g pooled feces 3.6 x 10^5 cfu /g litter

In the first and second screening isolates from all barns and in the third screening isolates from three barns were analyzed for the prevalent ESBL/AmpC gene family via real-time multiplex PCR. Hence, this analysis was carried out for isolates taken in 33 barns. Resistance genes belonging to the *bla*$_{SHV}$ gene family were most commonly detected, with 84.8% positive barns (28/33), *bla*$_{TEM}$ was detected in 30.3% of the barns (10/33), *bla*$_{CMY-2}$ was detected in 15.2% of the barns (5/33) and *bla*$_{CTX-M}$ was detected in 3% of the barns (1/33).

4.1.2 Detection of ESBL/AmpC-producing *E. coli* in samples from Farm B

For farm B, on the first screening, only five barns were analyzed for ESBL/AmpC-producing *E. coli*. On the second and third screening, all 40 barns of the farm were screened for the presence of ESBL/AmpC-producing *E coli*. ESBL/AmpC *E. coli* were qualitatively detected in 60% of the barns (3/5) for the first screening, in 20% of the barns (8/40) for the second screening and in 32.5% of the barns (13/40) for the third screening. Overall ESBL/AmpC-producing *E. coli* were qualitatively detected in 28.2% (24/85) of the investigated flocks for farm B. The highest concentration of ESBL/AmpC-producing *E. coli* detected on farm B was 3.6 x 10^6 in a boot swab and 6.3 x 10^5 cfu/g in a litter sample. An overview of the qualitative and quantitative microbiological analysis of the samples from farm B is depicted in Table 4.

Table 4 Qualitative and quantitative detection of ESBL/AmpC-producing *E. coli* for broiler farm B at different points in time

Sampling date	Sampling pattern	No. of barns sampled	No. of barns qualitatively positive for ESBL/AmpC *E. coli*	Highest ESBL *E. coli* concentration detected in the samples
08/08/2017	1 boot swab 2 pooled feces	5	3	qualitative detection only
02/07/2018	1 boot swab 1 litter sample	40	8	1.1×10^6 cfu /boot swab 3.9×10^4 cfu /g litter
05/22/2018	1 boot swab 1 litter sample	40	13	3.6×10^6 cfu /boot swab 6.3×10^5 cfu /g litter

For farm B only isolates from three barns per screening were investigated for the present ESBL/AmpC-genes via real-time multiplex PCR. Resistance genes belonging to the *bla*TEM gene family were most commonly detected in 66.7% (6/9) of the barns, *bla*SHV was detected in 44.4% (4/9) of the barns, *bla*CMY was detected in 33.3% (3/9) of the barns and *bla*CTX was detected in 11.1% (1/9) of barns.

4.2 Field trial with pig slurry

4.2.1 Background

The field trial with pig slurry was performed, to expand the spectrum of organic fertilizers concerning the investigation of a potential spread of ESBL/AmpC *E. coli* to the environment, because in the field trials with chicken litter, ESBL *E. coli* were not detected in litter samples taken after the transport from the barns to the field (Thiel et al., 2020). As mentioned previously, ESBL-producing *E. coli* have been detected in soil amended with pig slurry in various studies and the physicochemical properties of pig slurry appear to be more favorable for the survival of Enterobacteriaceae when compared to chicken litter. Pig slurry has a higher moisture content than poultry litter, which makes bacterial desiccation unlikely, the bacteria are less likely to be exposed to free oxygen radicals in slurry compared to litter and additionally, in slurry, there will be no self-heating process when removed from the barns (Strauch, 1991).

4.2.2 Experimental design

The field trial with pig slurry was carried out in March 2019 on a trial plot in Paulinenaue (Brandenburg, Germany). The trial plot had a size of 120 x 120 m and was fertilized with 30 m³ of untreated pig slurry. The slurry was tested for the concentration of ESBL/AmpC - producing *E. coli* in advance. Slurry application and incorporation were performed on the same day. The slurry was applied near-ground with a slurry tanker equipped with a drag hose and

incorporated with a disc harrow. Slurry samples were taken before application. Soil samples were taken before slurry application, after slurry application and after slurry incorporation. Boot swabs were taken on the field surface after slurry application and incorporation. Air samples were taken downwind with the AGI- 30 and the Coriolis µ air sampler during manure application and incorporation for 15 minutes, respectively. The airflow of the vacuum pump connected to the AGI-30 impinger was verified using a rotameter. It was approximately 9.5 l/min. The airflow of the Coriolis µ was set to 300 l/min. A picture of the air sampling setup is shown in Figure 3. The weather conditions were measured on the trial day.

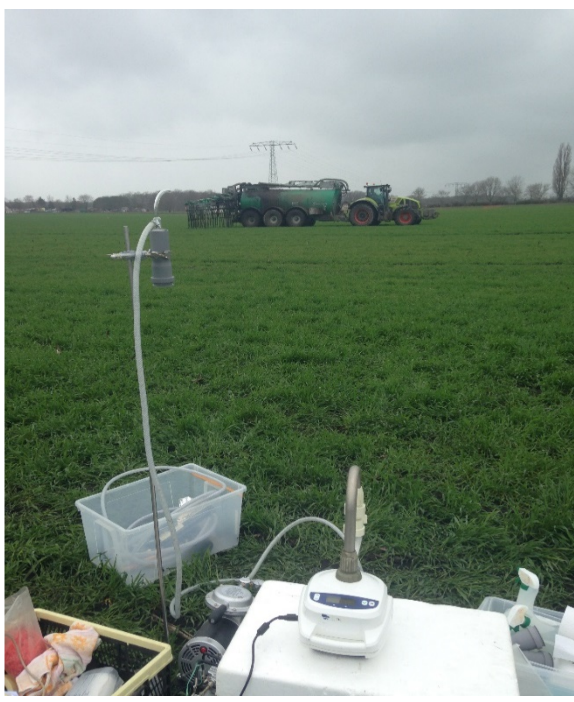

Figure 3: Air sampling setup for the field trial with pig slurry

Subsequent to the trial day, soil samples were taken on the trial plot in time intervals of approximately 14 days. Additionally, dust was collected in four dust traps positioned on the trial plot for 12 weeks.

All samples were quantitatively and qualitatively investigated for ESBL/AmpC-producing and non-resistant *E. coli*. For the air samples the total viable count was additionally determined. Real-time multiplex PCR was performed to identify the present ESBL/AmpC-resistance genes in isolates phenotypically suspected to be ESBL/AmpC *E. coli*.

4.2.3 Results

The relevant weather data for the trial day is summarized in Table 5.

Table 5 weather data for the pig slurry trial day

temperature	**RH**	**wind velocity**	**precipitation**
9.1 °C	82.6%	2.9 m/s	0.2 l/m²

No ESBL/AmpC-producing or non-resistant *E.* coli were detected in the soil samples taken before manure application. The concentration of ESBL-producing *E. coli* in the slurry samples was 5.2×10^2 cfu/g of slurry. Non-resistant *E. coli* were detected in the slurry at a concentration of 1.5×10^4 cfu/g.

After slurry application, the soil sample and boot swab taken on the field were quantitatively positive for ESBL-producing *E. coli* in concentrations of 3.3×10^1 cfu/g soil and 9.3×10^3 cfu/ boot swab, respectively. Non- resistant *E. coli* were detected in concentrations of 2.5×10^3 cfu/g of soil and 2.4×10^5 cfu/ boot swab.

After slurry incorporation, ESBL-producing *E. coli* were only qualitatively detectable in the soil sample and boot swab. The concentration of non- resistant *E. coli* detected after slurry incorporation was 6.7 x 10^1 cfu/g of soil and 4.3 x 10^4 cfu/ boot swab.

In the air samples, no Enterobacteriaceae were detected. No viable bacteria were detected in the AGI-30 air samples after direct plating on blood base agar. In the Coriolis µ air sample taken during manure application, only one colony of *Bacillus licheniformis* was detected. No viable bacteria were detected for the Coriolis µ air samples taken during manure incorporation.

Sixteen isolates of phenotypic ESBL-producing *E. coli* taken in the field trial were analyzed concerning their resistance genes. All Isolates (16/16) harbored a *bla*$_{CTX-M}$ gene. In 56.3 % (9/16) an additional *bla*$_{TEM}$ was detected. In one *K. pneumoniae* Isolate isolated from a boot swab, *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ were detected

In the soil samples taken on the trial plot in the weeks after the field trial, ESBL-producing and non- resistant *E. coli* were detected qualitatively only. Qualitative detection of ESBL-producing *E. coli* in soil samples was possible for approximately seven weeks after the trial. Non- resistant *E. coli* were detected in the soil samples for approximately nine weeks. In the soil samples taken 12 weeks after the field trial, neither ESBL-producing, nor non-resistant *E. coli* were detected. This data including the exact sampling dates and the resistance genes detected in the ESBL *E. coli* isolates from the soil samples is depicted in Table 6.

Table 6 Qualitative detection of ESBL-producing and non- resistant *E. coli* and resistance genes detected in the soil samples collected after slurry incorporation

Sampling date	days after incorporation	ESBL *E. coli*	*E. coli*	resistance genes (RT- PCR)
04/05/2019	9	+	+	*bla*$_{CTX-M}$, *bla*$_{TEM}$
04/12/2019	16	+	+	*bla*$_{CTX-M}$, *bla*$_{TEM}$
04/30/2019	34	+	+	*bla*$_{CTX-M}$, *bla*$_{TEM}$
05/17/2019	51	+	+	*bla*$_{CTX-M}$
05/28/2019	62	-	+	-
06/17/2019	82	-	-	-

(+) qualitatively detectable (-) qualitatively undetectable

No ESBL-producing and non- resistant *E. coli* were detected in the dust samples collected from the dust traps 12 weeks after the field trial. The mean weight of dust collected in the dust traps was 18.1g (range 17.6 to 18.3 g).

5. Discussion

ESBL/AmpC-producing *E. coli* are frequently detected in livestock, especially in the feces of broiler chickens (Daehre et al., 2017; Blaak et al., 2015a; Laube et al., 2013) and fattening pigs (Hering et al., 2014; von Salviati et al., 2014). These ESBL/AmpC-positive feces are applied to arable land as organic fertilizers. ESBL/AmpC-producing *E. coli* might be aerosolized from organic fertilizers during tillage operations. Additionally, these resistant bacteria could survive on the soil surface and might be subsequently aerosolized by wind erosion, which poses a potential environmental emission source and a health hazard. The airborne environmental emission of ESBL/AmpC *E. coli* by tillage and wind erosion has never been investigated before and was therefore investigated in this study.

In the field trials, tillage operations with ESBL-positive solid and liquid organic fertilizers were carried out under practically relevant, realistic conditions. No ESBL-producing or non-resistant *E. coli* were detected in the air samples taken in the field trials. Therefore, further experimental series investigating the tenacity of ESBL/AmpC-producing *E. coli* in the airborne state were carried out. Wind erosion events affecting ESBL/AmpC -positive fertilized soil were simulated in wind tunnel trials. For these trials, the experimental setup, including the amount of ESBL/AmpC-positive chicken litter mixed with soil was chosen to reflect practical relevant conditions. Also in the wind tunnel trials, no ESBL/AmpC *E. coli* were detected in the air samples, however, we detected non-resistant *E. coli* in air samples taken at wind speeds exceeding 7.3 m/s. The low tenacity of ESBL/AmpC producing *E. coli* in the airborne state was confirmed in the aerosol chamber trials with recovery rates ranging from 0.003 to 2.8 % depending on the environmental conditions.

Furthermore, we were able to show that short- term anaerobic storage of chicken litter represents an effective tool for the reduction of ESBL-producing *E. coli* in this organic fertilizer, which is recommended prior to land application.

5.1 Detection of ESBL/AmpC *E. coli* in the investigated broiler barns

Even though it was not the main focus of the project, a considerable amount of 130 broiler flocks were investigated for the presence of ESBL/AmpC-producing *E. coli* on two broiler farms. A remarkable difference in the colonization of the broiler flocks was observed between the farms, with 100% (45/45) qualitatively ESBL/AmpC-positive broiler flocks for farm A and 28.2% (24/85) positive flocks for farm B. The explanation for the difference in the colonization rate with ESBL/AmpC *E. coli* for farm A and farm B remains speculative. However, the difference observed in the colonization rate between the two farms might be attributed to better hygiene management on farm B. Dierikx et al. (2013b) detected ESBL-producing *E. coli* in

poultry barns after intensive cleaning and disinfection and suspected that insufficient decontamination of the broiler barns might lead to an ESBL colonization of the consecutive flock.

The quantity of ESBL/AmpC-producing *E. coli* detected in the samples taken in the broiler flocks showed a high variation. For both farms, in some flocks ESBL/AmpC-producing *E. coli* were detected qualitatively only, while in other flocks high concentrations of up to 1.8 x 10^6 cfu/g in pooled feces samples and 8.6 x 10^6 cfu/g litter were detected for farm A and up to 6.3 x 10^5 cfu/g litter for farm B. Blaak et al. (2015a) reported similar maximum ESBL *E. coli* concentrations in broiler feces of 8.3 x 10^6 cfu/g broiler feces. Laube et al. (2013) reported higher ESBL *E. coli* concentrations of up to 8.5 x 10^7 cfu/g pooled feces.

Similarly to the studies conducted by Laube et al. (2013) and Daehre et al. (2017) resistance genes belonging to all four investigated ESBL/AmpC gene families bla_{SHV}, bla_{TEM}, bla_{CTX} and bla_{CMY} were detected on both farms in the screened barns, indicating a high genetic diversity of ESBL/AmpC-producing *E. coli* colonizing the broiler flocks.

5.2 Molecular characteristics of *E. coli* Isolates from the wind erosion and litter storage trials

Molecular characterization of the isolates taken in the wind tunnel trials revealed, that the *E. coli* isolates harbored resistance genes that are typically detected in livestock. Of the isolates from the first wind tunnel trial, 100% harbored the bla_{CMY-2} gene, which is the AmpC-type beta-lactamase that is most common in livestock (EFSA, 2011). In the second and third wind tunnel trials, 100% of the *E. coli* isolates were assigned as bla_{SHV-12}, which is one of the most commonly identified ESBL-genes and the most predominant gene of the bla_{SHV} gene family (EFSA, 2011). The Isolates from all three trials were assigned either to the Phylogroups A, A/C or B1, which might indicate, that they belong to nonpathogenic *E. coli* strains, as avian pathogenic *E. coli* (APEC) strains are mainly assigned to the Phylogroup B2 in Europe (Cordoni et al., 2016).

Of the *E. coli* Isolates taken in the litter storage trials, 44 were analyzed by WGS, leading to a detailed molecular characterization of these isolates. In the winter trial, once again bla_{SHV-12} was identified in all analyzed isolates. In the summer trial bla_{CTX-M1}, the most commonly identified variant of the bla_{CTX-M} gene family (EFSA, 2011) was identified in all analyzed isolates. It is remarkable, that only a single predominant resistance gene was detected in all analyzed isolates for each of the wind tunnel and litter storage trials, respectively. Van Hoek et al. (2018) explains this phenomenon by a rapid horizontal transfer of ESBL-plasmids in the *E.coli* population, which they were able to demonstrate in their study for an IncI1/ST3 plasmid carrying bla_{ctx-M1} in a broiler flock.

In the litter storage trial in winter, we isolated ESBL-producing *E. coli* of the multilocus sequence type (MLST) 117, expressing the H-4 antigen. Strains with these characteristics are known to be pathogens and cause avian colibacillosis (Mora et al., 2012) or even have a zoonotic potential and cause urinary tract infections in humans (Vincent et al., 2010). However, in these studies, the pathogenic *E. coli* strains belonged to the Phylogroup D. The predominant strain isolated in our winter trial belonged to the Phylogroup F, rendering pathogenicity of the isolated *E. coli* strain unlikely because *E.coli* of Phylogroup F have not been described as avian pathogenic to date (Vangchhia et al., 2016). However, we did not analyze virulence genes in the *E. coli* isolates taken in our study, which might have been of interest retrospectively.

5.3 Importance of fertilizer management to disrupt the transmission of ESBL *E. coli* to the environment

In a recently published review, it was pointed out, that proper manure management is a key element in the reduction of public health hazards and environmental impacts originating from manure- borne pathogenic and antibiotic-resistant bacteria (Alegbeleye and Sant'Ana, 2020).

For the short-term litter storage trials carried out in this study, it was concluded, that short-term storage of chicken litter in piles is a useful and cost-efficient tool to reduce the concentration of ESBL-producing *E. coli* in the litter. The average initial concentrations of 3.4 x 10^5 cfu/g ESBL *E. coli* in fresh chicken litter decreased below the quantitative detection limit after storage periods of 36 h in the summer and 72 h in winter and below the qualitative detection limit after 72 h in summer for litter samples taken in a depth of 55 cm from the heap. However, incomplete inactivation was observed for litter samples taken at the surface of the pile in summer and winter and for the deep samples in winter.

Knowledge of the inactivation of ESBL/AmpC-producing *E. coli* in organic fertilizers is scarce. Two laboratory-scale studies concerning the inactivation of ESBL/AmpC-producing *E. coli* in chicken litter were recently published by Thomas et al. (2019, 2020). In the first study ESBL/AmpC-producing *E. coli* were added in a concentration of over 10^7 cfu/ml to a mixture of chicken litter and an inoculum from a biogas plant and digested anaerobically. At a constant temperature of 55°C, both *E. coli* strains were quantitatively undetectable by direct count after 2 h of incubation. The ESBL and AmpC producing *E. coli* strains used in this study were the same strains (*E. coli* R56 and *E. coli* G-148-1), which we used in the aerosol chamber experiments in our study. In the second study, they showed, that the fastest decrease of ESBL-producing *E. coli* occurs at a C/N ratio of 10:1 and that dry mixtures with a moisture content of 20% resulted in a faster reduction in the concentration of ESBL *E. coli* compared to the moist mixtures, despite showing lower maximum temperatures.

The studies by Siller et al. (2020) and Thomas et al. (2019, 2020) accordingly indicate, that a reliable inactivation of ESBL *E. coli* in chicken litter by storage or composting can be expected, mainly because chicken litter is typically dry and heats up fast (Wilkinson et al., 2011). In our study, we measured a maximum temperature of 58.5 °C in a depth of 55 cm in the summer and 50.4 °C in the winter. Erickson et al. (2010) reported similar temperatures for static piles of chicken litter with 54.4 °C in the summer and 51.8 °C in the winter, measured in a depth of 30 cm. Additionally, the moisture content measured in the chicken litter in our study was low, with none of the samples having a moisture content above 30%. The pH- value is a further environmental factor that might influence bacterial concentrations in chicken litter (Erickson et al., 2014) and was monitored in the litter samples taken in our study. At the beginning of the experiment, the pH was in the alkaline range, which is typical for chicken litter (Huang et al., 2017). In the following five days the pH value dropped to 5.6 in winter and 6.5 in summer in the depth of the litter heap, presumably due to the formation of organic acids like propionic acid, butyric acid and acetic acid under anaerobic conditions (Ugwuanyi et al., 2005). However, the minimum pH-values measured in the litter were likely not sufficient to inactivate ESBL *E. coli.*, because *E. coli* was shown to have a high probability of surviving pH values as low as 1.5 to 4, which are present during the stomach passage (Takumi et al., 2000).

In conclusion, regarding chicken litter an effective reduction of ESBL/AmpC *E. coli* by storage and composting can be expected, but no studies concerning the tenacity of ESBL/AmpC-producing *E. coli* in organic fertilizers originating from livestock species other than chickens were carried out to date. However, several studies investigated the tenacity of non-resistant *E. coli* in organic fertilizers from other animal species. Wroński et al. (2012) calculated survival times of 50 days at 20°C and 83 days at 4°C for *E. coli O157:H7* in cattle slurry. For pig slurry, Côté et al. (2006) calculated storage times of 15 to 26 days to reduce *E. coli* levels by 90% and 54 to 114 days to reduce *E. coli* below the detection limit. Both studies confirm greatly prolonged survival times of *E. coli* in liquid organic fertilizers. This is in accordance with the observations in our field trial with pig slurry, in which both, non-resistant *E. coli* and also the ESBL-producing subpopulation survived the transport to the test field in quantifiable concentrations. This highlights the importance of proper fertilizer management especially for liquid organic fertilizers. Thermophilic inactivation was shown to be similarly efficient for the inactivation of Gram-negative bacteria in liquid fertilizers. Martens et al. (1998) exposed cattle slurry to temperatures of approximately 55 °C in a slurry treatment plant and observed a complete inactivation of all *Salmonellae* within a time span of 3 h. Thus, thermophilic treatment of liquid organic fertilizers prior to land application should be recommended to increase biological safety regarding ESBL/AmpC *E. coli*.

5.4 Detection of ESBL/AmpC-producing and non-resistant *E. coli* in the air samples taken in the experimental series

Three out of four experimental series carried out in the course of the project, namely the field trials, the wind tunnel trials and the aerosol chamber trials investigated the tenacity of airborne ESBL/AmpC-producing *E. coli.* In the field trials, neither ESBL/AmpC-producing nor non-resistant *E. coli* were detected in the air samples, presumably due to the low number of *E. coli* in the organic fertilizers and the limited bioaerosol formation in the trial with pig slurry.

In the wind erosion trials, ESBL/AmpC-producing *E.coli* were below the detection limit in all air samples taken as well. However, non-resistant *E. coli* were qualitatively detected in 33% of the air samples (11/33) taken with the Coriolis µ air sampler at different wind velocities, but in none (0/33) of the air samples taken with the AGI-30, even though a similar collection efficiency, which showed no significant differences concerning the total viable bacterial count, was demonstrated for both air samplers. Therefore it can be assumed that *E. coli* were below the detection limit in the air samples taken with the AGI-30, mainly due to the lower airflow of this air sampler with 12.5 l/min compared to 300 l/min collected with the Coriolis µ. The results of our study are in accordance with the findings of Langer et al. (2012), who compared the collection efficiency of the AGI-30 and Coriolis µ air samplers for the collection of *Legionella pneumophila*. They also reported similar recovery rates for both air samplers but an approximately 100-fold lower detection limit for the Coriolis µ air sampler. Thus, we recommend the use of cyclone air samplers with high airflows rate over the use of AGI-30 impingers for sampling bioaerosols for which a low quantity of the investigated microorganisms is assumed.

Non- resistant *E. coli* were detected qualitatively in the Coriolis µ air samples, while ESBL/AmpC-producing *E. coli* remained below the detection limit. This might be explained by the fact, that the ESBL/AmpC *E. coli* producing subpopulation represents only a small proportion of the total *E. coli* population. In the wind tunnel trials, the average proportion of ESBL/AmpC-producing *E. coli* compared to the total *E. coli* population in the chicken litter was 2.5%. This proportion is slightly higher compared to the average proportion of 1.3% ESBL *E. coli* we detected in the chicken litter used for the litter storage trials or the proportion of 1.1% recently reported for turkey rearing flocks by Friese et al. (2019).

Assuming, that non-resistant *E. coli* were detected in the air samples taken in the wind tunnel trials, while ESBL/AmpC-producing *E. coli* remained below the detection limit due to their higher quantity in the chicken litter leaves open the question, whether livestock-associated ESBL/AmpC-producing *E. coli* have survival advantages or disadvantages compared to the non-resistant *E. coli* population in the aerosolized state. In the aerosol chamber trials, we were

able to demonstrate significantly increased recovery rates of 4.1- fold for the poultry associated AmpC-producing *E. coli strain* G-148-1 (*p*-value= 0.016) and 3.5- fold for the poultry-associated strain *E. coli* R56 compared to the non-resistant strain *E. coli* K12 (*p*= 0.041). However, this finding is may also be attributed to the survival advantage of "wild-type" *E. coli* strains compared to "laboratory" strains and not due to the ESBL/AmpC plasmids, as Marshall et al. (1988) have demonstrated prolonged survival times of aerosolized *E. coli* of wild-type strains compared to the laboratory strain *E. coli* K12. Interestingly, Laube (2015) reported a high proportion of 17% ESBL/AmpC producers among total *E. coli* detected in air samples taken with AGI-30 impingers in chicken barns, which might be an indication for a survival advantage of *E. coli* harboring ESBL/AmpC plasmids. For finally estimating the influence of carriage of ESBL/AmpC plasmids on the survival of *E. coli* during aerosolization, a direct comparison of the recovery rates of *E. coli* strains carrying ESBL/AmpC plasmids and plasmid-cured variants of the same strain would be of outstanding interest, as Ranjan et al., (2018) have demonstrated, that the carriage of certain ESBL plasmids was beneficial regarding competition fitness in vitro. Additional research concerning this issue is warranted.

In all experimental series carried out in the project, the air samples were analyzed by direct cultivation only, which might have led to an underestimation of the bacterial counts detected in the air samples. Heidelberg et al., (1997) pointed out, that aerosolized bacteria might lose their ability to form colonies and thus become viable but non-culturable (VBNC). They stated that less than 10% of Gram-negative bacteria aerosolized with a collision nebulizer in their study were capable of forming visible colonies. By using culture-independent methods we might have been able to detect ARG of ESBL/AmpC *E. coli* or the respective plasmids in the air samples taken in the field and wind tunnel trials, which were all assigned as ESBL/AmpC negative in our study. Airborne ARG or plasmids may pose a hazard to public health. Maamar et al., (2020) highlited the role of combining cultivation with metagenomics when analyzing airsamples. In their study they were able to identify 52 potentially mobile ARGs in 166 dust metagenomes. Gaviria-Figueroa et al. (2019) were able to identify 44 ARGs in air samples taken at a wastewater treatment plant. This indicates that further research concerning the emission of mobile genetic elements from agriculture-associated bioaerosols is warranted.

Pienaar et al. (2016) pointed out, that pathogenic *E. coli* in the VBNC state may be resuscitated and thus become infectious again, which leads to the assumption, that ESBL/AmpC *E. coli* might also have the ability to resuscitate from the VBNC state. For the aerosol chamber trials, it remains uncertain if the low recovery rates of 0.003% to 2.8% depending on the *E. coli* strain and the environmental conditions were caused by a complete inactivation (cell death) of *E. coli* upon aerosolization or if an unknown proportion of the aerosolized *E.coli* entered the VBNC state and were thus undetectable by direct plating. However, Kaushik and Balasubramanian

(2013) analyzed air samples concerning the quantity of Gram-negative bacteria including *E.coli* detected by bacterial cultivation and propidium monoazide (PMA) -assisted qPCR and concluded that the overall counts obtained by PMA- PCR and bacterial cultivation were similar. This study, in the context of the results of our aerosol chamber trials, leads to the assumption that only a very small fraction of *E. coli* remains viable in the aerosolized state, another very small proportion of the *E. coli* may enter the VBNC state, but the vast majority of *E. coli* are being inactivated through cell death upon aerosolization.

The exact inactivation mechanisms of *E. coli* during aerosolization are still unknown. However, Thomas et al. (2011) have demonstrated that the cell membrane represents a major site of damage during aerosolization of *E. coli* with 32.6% of the bacteria showing impaired membrane integrity after five seconds of aerosolization with a collision nebulizer. This very rapid onset of inactivation during aerosolization is typically observed for *E. coli.* Benbough (1967) stated that the initial death rate of aerosolized *E. coli* is high and subsequently diminishes. Very little is known about the molecular mechanisms and bacterial stress response in aerosolized bacteria as well. In a recent study, Ng et al. (2018) have used comparative transcriptome analysis in aerosolized *E. coli* and showed a fast response to environmental stimuli in the air by the regulation of 11 stress-responsive genes and 13 stimulus-responsive genes.

5.5 Factors influencing the tenacity of ESBL/AmpC -producing and non-resistant *E. coli* in the aerosolized state

Apart from the aerosol chamber trials performed in this study, no study investigated the influence of environmental factors on the airborne tenacity of ESBL/AmpC *E. coli* under experimental conditions to date. However, experimental studies concerning the tenacity of airborne non-resistant *E. coli* have been conducted for decades (Poon,1966; Benbough, 1967; Cox, 1968; Wathes et al., 1986). The findings of these studies can be useful to interpret results concerning the tenacity of ESBL/AmpC-producing *E. coli*. However, as mentioned previously we observed significant differences concerning the recovery rates of the different *E. coli* strains analyzed in our study. Therefore, strain specify in *E. coli* concerning airborne survival rates under different environmental conditions should be considered when extrapolating the results of studies, which used other *E. coli* strains.

Under experimental conditions, the airborne tenacity of bacteria is influenced by various factors including the strain, the composition of the culture and suspension fluid, the growth conditions, the processing conditions of the bacterial cultures and the atmosphere into which the bacteria are released (Wathes et al. 1986). In the environment, further factors including the RH, oxygen concentration, temperature, ozone concentration, UV-radiation and air pollutants influence the tenacity of aerosolized bacteria (Zhao et al., 2014). It is difficult to assess, which environmental

conditions cause bacterial decay in the aerosolized state, because of the existence of overlapping lethal mechanisms (Benbough, 1967). In the environment, temperature and UV-radiation have been identified as the most important meteorological factors related to the viability of airborne bacteria in a recent study (Bragoszewska and Pastuszka, 2018).

The influence of the temperature on the survival of airborne *E. coli* was investigated in several studies. Generally, bacterial inactivation is faster at higher ambient temperatures (Zhao et al., 2014). Wathes et al. (1986) compared the inactivation of a nalidixic acid-resistant strain of *E. coli* (serotype 0149) in the airborne state depending on the ambient temperature and reported an approximately four times higher death rate at 30 °C compared to 15 °C. We did not investigate the influence of temperature on the survival of the three *E. coli* strains in the aerosol chamber experiments, as the targeted temperature was 24°C uniformly.

The influence of the RH on the recovery rates of the three *E. coli* strains from the aerosol was investigated in the aerosol chamber trials. In the trials, in which the *E. coli* strains were aerosolized from phosphate-buffered saline (PBS) without organic additives, we observed that the recovery rate for all three *E. coli* strains was significantly higher at an RH of 50% and 70% compared to 30%. This finding is supported by the study of Wathes et al. (1986), who aerosolized *E. coli* from PBS at different RH at 30 °C and reported half-life times of 3 min for low RH (<50%) and 14 min in humid conditions. A study by Ng et al. (2017) confirmed, that low relative humidity (30–40%) is unfavorable for the survival of aerosolized *E. coli* compared to a high RH (>90%), and showed an increased stress response of aerosolized *E. coli* at low RH. However, in a study by Hoeksma et al. (2014) *E.coli* (strain DSM-1936) was aerosolized from buffered peptone water and a larger decay at higher relative humidity, especially at 80% was observed. This divergence might be explained by the fact that different *E. coli* strains were used in the studies. But presumably the composition, or more precisely the organic content of the media from which wet aerosols are released seem to play a crucial role for the recovery of bacteria at different RHs.

This assumption is supported by the observation, that the recovery rates at 30% and 70% RH significantly differed for all three *E. coli* strains aerosolized in our study when adding 10g/l yeast extract and BSA to the bacterial suspensions. At an RH of 30% the recovery rate significantly increased when compared to the trials, in which the *E. coli* strains were aerosolized from PBS. The explanation might be that the airborne *E. coli* were protected from desiccation by organic particles coagulated within the viable bacterial particles (Zhao, 2011).

Although, a high RH and the addition of organic substances both seem to be beneficial factors, concerning bacterial survival in the aerosol, at an RH of 70% the recovery rate of all three *E. coli* strains decreased significantly when adding 10g/l yeast extract and BSA in our study. This is in accordance with the study by Hoeksma et al. (2014), in which the lowest concentrations

of *E. coli* were recovered from the aerosol at an RH of 80% when aerosolized from an organic-rich substance. A high RH in combination with a high concentration of organic substances in the aerosolization fluid might lead to a clumping of cells (Marthi et al. 1990). In our study, viable *E. coli* may have deposited in large aggregates on the floor of the aerosol chamber and have therefore been undetectable in the air samples, which might have led to an underestimation in the concentration of *E coli* detected under humid conditions.

In our study, we did not investigate the influence of further environmental factors like oxygen concentration in air or UV-radiation on the survival of ESBL/AmpC *E. coli* in the airborne state. Additional research in this field is would be of interest.

5.6 Relevance of an airborne environmental ESBL/AmpC *E. coli* spread from arable land during and following tillage operations

A large quantity of solid and liquid organic fertilizers are applied to arable land in Germany annually: Approximately 135 million m³ of liquid slurry, 16 million metric tons of manure and 1.1 million metric tons of poultry litter (Statistisches Bundesamt, 2016), which might lead to an airborne spread of ESBL/AmpC-producing *E.coli* from ESBL/AmpC-positive fertilizers to the environment during tillage. In our study, we performed two field trials with chicken litter and one field trial with pig slurry under practical conditions and did not detect ESBL-producing or non-resistant *E. coli* in all air samples taken during the tillage operations.

Assumingly, the main causes for the undetectability of *E.coli* in the air samples differ between tillage operations with liquid and solid organic fertilizers: In the field trials with chicken litter, ESBL-producing *E. coli* were below the detection limit after fertilizer transport from the barn to the field (Thiel et al., 2020) and the number of non-resistant *E. coli* dropped to a mean concentration as low as 1.4 x 10^3 cfu/g of chicken litter. Consequently, the very low *E. coli* concentrations detected in the chicken litter explain why these bacteria were undetectable in the air samples, despite the pronounced bioaerosol emission of up to 1.6 x 10^8 cfu/m³ total viable bacteria we detected in a distance of 20 m during the application of chicken manure (Münch et al., 2020).

In the trial with pig slurry, ESBL-producing and non-resistant *E. coli* were detected in higher concentrations of 5.2 x 10^2 and 1.5 x 10^4 cfu/g of slurry after the transport to the field, respectively. However, despite higher concentrations of ESBL-producing and non-resistant *E. coli* in the slurry in this trial, these bacteria were also undetectable in air samples taken during tillage. The main cause in this trial was presumably the almost non-existent bioaerosol emission we observed during the application and incorporation of the slurry, with just one colony of *Bacillus licheniformis* detected in all air samples taken. The main causes for the low bioaerosol emission were most likely the weather conditions and probably even more

important, the lower bioaerosol emission potential of liquid fertilizers compared to solid fertilizers (Boutin et al., 1988) and the mode of fertilizer application. On the trial day, a high RH of 82.2 % accompanied by slight precipitation and a relatively low wind velocity of 2.9 m/s was measured. These weather conditions seem unfavorable for a pronounced bioaerosol formation (Šantl-Temkiv et al., 2020). The slurry was incorporated near ground with a slurry tanker equipped with a drag hose, this reduces emissions compared to slurry application with a baffle plate (Bayerische Landesanstalt für Landwirtschaft, 2020), which is prohibited in Germany since February 01, 2020, according to the German fertilizing ordinance (§ 6 (3)). Tanner et al. (2005) applied liquid class B biosolids (~8% solids) containing 9.6×10^4 to 2.3×10^7 cfu/ml *E. coli* with a spray tanker to arable land and took air samples via impingement with five SKC Biosamplers in a distance of only 2 m downwind from the emission source. Accordingly, in this study no *E. coli* were detected in all samples taken. They concluded, that aerosolization of coliform bacteria after liquid biosolid application to land does not occur at detectable levels.

In order to reduce fine dust and bioaerosol emission from solid fertilizers, treatment by either composting or storage prior to land application seems beneficial (Münch et al., 2020). In the first field trial, for which chicken litter was treated in four different ways (fresh, stored, composted and dried) the lowest total viable bacterial counts during manure application were detected for the stored litter with 1×10^5 cfu/m³ followed by 3×10^6 cfu/m³ for the composted litter and 4.4×10^7 cfu/m³ for the fresh chicken litter in 20 m distance from the manure spreader. The highest total viable count was detected during the application of dried chicken litter with 1.6×10^8 cfu/m³, therefore drying chicken litter prior to land application should be considered disadvantageous concerning bioaerosol formation during litter application. For all types of litter pretreatment, the total viable count detected in the air samples considerably decreased with increasing distance to the manure spreader. In a distance of 20 m from the emission source, the total viable counts detected in the air samples were three to almost four LOG_{10} higher compared to the total viable count detected at a distance of 100 m.

In conclusion, an airborne spread of ESBL/AmpC-producing *E.coli* during tillage operations appears to be very unlikely for both, solid and liquid organic fertilizers. However, we recommend to pretreat solid organic fertilizers by composting or storage to reduce bioaerosol emission and to conduct fertilizer application on days with weather conditions for which a reduced bioaerosol emission can be expected. Also, for people passing fields, on which tillage operations are performed keeping an appropriate distance of > 100m to the emission source is recommended to greatly reduce exposition to bioaerosols.

In the time following fertilizer application, if farrow fields are affected by wind erosion, this might lead to an airborne emission of dust-borne ESBL/AmpC -producing *E. coli* from ESBL/AmpC - positive soil. Soil samples taken in the field trials with chicken litter were ESBL/AmpC -negative

because the litter used for fertilization was ESBL/AmpC -negative. However, in soil samples taken after the trial with pig slurry, we detected ESBL-producing *E. coli* up to seven weeks and non- resistant *E. coli* up to nine weeks after slurry incorporation, indicating that ESBL *E. coli* can survive on the soil surface for several weeks. Hartmann et al. (2012) even reported a survival time of one year in amended soil for a $bla_{CTX\text{-}M1}$ ESBL *E. coli* strain originating from cattle manure.

The survival of *E. coli* in fertilized soil is influenced by various factors, of which temperature seems to be the most important (Jang et al., 2017). *E. coli* can potentially regrow in soil at temperatures over 30°C, but the survival rate is highest in cold temperatures under 15°C (Ishii et al., 2010), which were predominating in the weeks following the trial carried out in march. Additionally, rainfall can promote the regrowth of *E. coli* in soil, (Stocker et al., 2015). The survival of *E. coli* in soil is also influenced by the strain (Topp et al., 2003) and the soil type. For clay soil, Boes et al. (2005) did not detect non-resistant *E. coli* after a time span of 21 days following fertilization with pig slurry. Semenov et al. (2008) reported a significantly more stable survival of *E. coli* O157:H7 in sandy soil-manure mixtures compared to loam soil-manure mixtures. This is in accordance with the findings of our study, in which the trial plot fertilized with pig slurry consisted of sandy soil with a top layer of turf, which might explain the prolonged survival time of seven weeks for ESBL-producing and nine weeks for non-resistant *E. coli.*

In the pig slurry trial, dust samples collected from dust traps during 12 weeks after fertilization tested negative for ESBL-producing and non-resistant *E. coli*. Interestingly it was shown, that *E. coli* seems to have an increased tenacity when adhering to dust. Schulz et al. (2016) reported survival times of dust-bound *E. coli* of up to 20 years and Zhao et al. (2014) stated that bacteria in the airborne state have an increased tenacity when adhering to dust. However, *E. coli* were below the detection limit in the dust samples presumably due to two main reasons: Firstly, probably only a small proportion of the dust captured in the dust traps originated from the trial plot because under field conditions the atmosphere has a strong diluting capability; making microbiological events in the air difficult to capture (Šantl-Temkiv et al., 2020). Secondly, a large proportion of *E. coli* emitted from the trial plot was likely inactivated during the aerosolization process, despite being bound to dust. In the experimental series carried out in the wind tunnel and aerosol chamber, we observed a low tenacity of *E. coli* in the aerosolized state, even in the presence of organic substances (Siller et al., 2021).

Fast incorporation of organic fertilizers to soil appears to be an important agricultural management factor, which assumingly decreases the airborne emission of fertilizer-associated bacteria. Incorporating organic fertilizers to the top layers of soil leads to a dilution of the fertilizer by a factor of 100-150 (Rasschaert et al., 2020). This estimation which corresponds to a dilution of slightly more than 2 LOG_{10} was confirmed for ESBL/AmpC -producing *E. coli*

when mixing sandy soil with ESBL/AmpC -positive chicken litter in the wind tunnel experiments, where a reduction of 2.2 LOG_{10} was observed. Accordingly, in the field trial with pig slurry, ESBL-producing *E. coli* were detected in soil samples taken from the soil surface after slurry application in low concentrations (6.7 x 10^2 cfu/g) and after incorporation only a qualitative detection was possible.

Concluding, an airborne spread of relevant quantities of ESBL/AmpC-producing *E. coli* from fertilized soil by wind erosion under field conditions appears to be very unlikely.

6. Conclusion

The hypothesis of the present study was that there is an airborne environmental dissemination of relevant quantities of ESBL/AmpC-producing *E. coli* from organic fertilizers due to tillage operations and by wind erosion affecting soil fertilized with ESBL/AmpC-positive organic fertilizers.

The first prerequisite for an environmental dissemination of ESBL/AmpC *E. coli* from organic fertilizers is that viable ESBL/AmpC *E. coli* are present in the organic fertilizers in relevant concentrations after being transported to arable land. In this study, we could show, that this is not the case for chicken litter because ESBL/AmpC *E. coli* are quickly inactivated after the litter is removed from the barns. In contrast, we detected ESBL-producing *E. coli* in quantifiable concentrations in pig slurry after the transport to the field. However, the second prerequisite for an airborne environmental spread of bacteria is a sufficient bioaerosol formation. While we observed a marked bioaerosol formation during the application and incorporation of chicken litter to arable land, a negligible bioaerosol formation of culturable ESBL/AmpC *E. coli* was observed when applying and incorporating pig slurry. The third prerequisite for an airborne environmental spread of ESBL/AmpC *E. coli* is a high tenacity in the aerosolized state. However, in this study, it was demonstrated in the practically oriented wind erosion trials and in the systematic aerosol chamber trials, that ESBL/AmpC-*E. coli* have a very low tenacity in the aerosolized state.

The results of the experimental series carried out in this study strongly indicate, that the hypothesis of an airborne environmental spread of relevant quantities of culturable ESBL/AmpC *E. coli* from organic fertilizers due to tillage operations and by wind erosion has to be rejected.

7. Summary

ESBL/AmpC-producing *E. coli* are resistant to a variety of beta-lactam antibiotics, which drastically limits therapeutic options for bacterial infections in veterinary and human medicine. The airborne emission of ESBL/AmpC-producing *E. coli* from livestock has previously been published in several studies. However, these direct emissions from livestock facilities are limited to small local areas in the vicinity of the stables. Airborne emissions of ESBL/AmpC-producing *E. coli* may also result from the agricultural use of ESBL/AmpC-positive fertilizers and wind erosion, which could affect the upper soil layers of soil amended with ESBL/AmpC-positive fertilizers. These potential emission routes could lead to an airborne exposure of the general population to ESBL/AmpC *E. coli* and therefore pose a health risk.

This hypothesis was examined in detail in this study by four practical and experimental test series with different approaches. In the short-term chicken litter storage trials, which were carried out under practical conditions, the inactivation kinetics of ESBL-producing *E. coli* was investigated under different environmental conditions (summer and winter). In the field trials, also carried out under practical conditions, it was investigated whether the application of ESBL/AmpC-positive organic fertilizers leads to an airborne spread of culturable ESBL/AmpC -producing *E. coli* during agricultural operations (application and incorporation). In the wind erosion trials, the airborne dissemination of ESBL/AmpC -producing *E. coli* from sandy soil fertilized with ESBL/AmpC-positive chicken litter was investigated under practically relevant conditions. Finally, aerosol chamber trials were carried out to explore the tenacity of ESBL/AmpC-producing *E. coli* in the airborne state under different environmental conditions.

In field trials with ESBL-positive chicken litter, even for organic fertilizers with high concentrations of ESBL-producing *E. coli* (2.1×10^5 cfu/g pooled feces), the number of ESBL *E. coli* dropped below the detection limit after the chicken litter was transported to the field edge overnight. This observation was systematically confirmed in the manure storage experiments, in which the ESBL *E. coli* concentration dropped below the quantitative detection limit in stored chicken manure after 72 h in winter and after 36 h in summer.

In the wind erosion trials carried out under practically relevant conditions, no ESBL-producing *E. coli* were detected in the air samples despite high concentrations of ESBL-producing *E. coli* in the chicken litter used (up to 4.2×10^5 cfu/g litter).

The results of the aerosol chamber experiments confirmed a low tenacity of ESBL/AmpC-producing *E. coli* in the airborne state. Depending on the environmental conditions low recovery rates ranging from 0.003 to 2.8 % in the aerosol were observed.

Considering the results of all experimental series in context, it can be concluded that an airborne dissemination of culturable ESBL/AmpC-producing *E. coli* from livestock by agricultural land utilization and wind erosion appears very unlikely.

8. Zusammenfassung

Luftgetragene Emission ESBL/AmpC-produzierender *E. coli* aus Wirtschaftsdüngern durch landwirtschaftliche Bodenbearbeitung und Winderosion

ESBL/AmpC-produzierende *E. coli* weißen Resistenzen gegen eine Vielzahl von Beta-Lactam Antibiotika auf, was die Therapieoptionen bakterieller Infektionen in der Veterinär- und Humanmedizin drastisch einschränken kann. Eine luftgetragene Emission ESBL/AmpC-produzierender *E. coli* aus Nutztierhaltungen wurde bereits in verschiedenen Studien gezeigt. Diese direkten Emissionen aus Tierhaltungen sind jedoch auf kleine lokale Bereiche in der Stallumgebung beschränkt. Eine luftgetragene Emission ESBL/AmpC-produzierender *E. coli* ist jedoch auch durch die landwirtschaftliche Nutzung ESBL/AmpC-haltiger Wirtschaftsdünger denkbar, sowie durch Winderosion, welche die oberen Bodenschichten von mit ESBL/AmpC-positivem Wirtschafsdünger gedüngten Boden betreffen könnte. Diese Emissionswege könnten zu einer großflächigen luftgetragenen Exposition der Bevölkerung mit ESBL/AmpC *E. coli* und somit zu einem Gesundheitsrisiko führen .

Diese Hypothese wurde im Rahmen der vorliegenden Arbeit mithilfe von praxisnahen und experimentellen Versuchsreihen mit verschiedenen, komplementären Untersuchungsansätzen und Fragestellungen untersucht. In den unter Praxisbedingungen durchgeführten Kurzzeit – Mistlagerungsversuchen mit Masthähnchenmist wurde die Absterbekinetik ESBL-bildender *E. coli* abhängig von den Umweltbedingungen (im Sommer und im Winter) erfaßt. In den ebenfalls unter Praxisbedingungen durchgeführten Feldversuchen wurde untersucht, ob es bei der Ausbringung ESBL/AmpC-positven Wirtschaftsdüngers zu einer luftgetragenen Verbreitung während landwirtschaftlicher Bearbeitungsmaßnahmen (Ausbringung und Einarbeitung) kommt. In den anschließenden Winderosionsversuchen wurde der luftgetragene Austrag ESBL/AmpC-bildender *E. coli* aus mit Masthähnchenmist gedüngtem Sandboden unter praxisnahen Bedingungen untersucht. Schließlich wurde eine Testreihe in einer Aerosolkammer zur Tenazität ESBL/AmpC-bildender *E. coli* im luftgetragenen Zustand durchgeführt.

In Feldversuchen mit ESBL-positivem Masthähnchenmist, zeigte sich, dass selbst bei hohen Konzentrationen ESBL-bildender *E. coli* (2.1 x 10^5 KbE/g Sammelkot), deren Zahl nach dem Transport des Masthähnchenmists zum Feldrand über Nacht unter die Nachweisgrenze sank. Diese Beobachtung wurde nochmals systematisch unter Praxisbedingungen in

Mistlagerungsversuchen bestätigt, in welchen in gelagertem Masthähnchenmist im Winter nach 72 h und im Sommer bereits nach 36 h ESBL *E. coli* quantitativ nicht mehr nachzuweisen waren.

In den unter praxisnahen Bedingungen im Windkanal durchgeführten Winderosionsversuchen konnten trotz teils hoher Konzentrationen ESBL/AmpC-produzierender *E. coli* im Masthähnchenmist (bis zu 4.2 x 10^5 KbE/g Einstreu) und einer schnellstmöglichen Aerosolisierung keine ESBL-bildenden *E. coli* in den Luftproben nachgewiesen werden.

Durch die Ergebnisse der Aerosolkammerversuche wurde eine geringe Tenaziät ESBL/AmpC-produzierender *E. coli* im luftgetragenen Zustand bestätigt. Abhängig von den Umweltbedingungen zeigten sich sehr geringe Wiederfindungsraten zwischen 0,003% und 2,8% im Aerosol.

Die Betrachtung der Ergebnisse der durchgeführten Versuchsreihen im Gesamtkontext läßt die Schlussfolgerung zu, dass ein luftgetragener Austrag kultivierbarer ESBL/AmpC-produzierender *E. coli* aus Tierhaltungen durch landwirtschaftliche Flächennutzung und Winderosion sehr unwahrscheinlich erscheint.

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IV. List of Publications

Publications

Siller, P.; Daehre, K.; Thiel,N.; Nübel U.; Roesler, U. (2020**) “Impact of short-term storage on the quantity of extended-spectrum beta-lactamase–producing Escherichia coli in broiler litter under practical conditions”** Poultry Science 99, 2125-2135 https://doi.org/10.1016/j.psj.2019.11.043)

Siller, P; Daehre, K; Rosen, K; Münch,S; Bartel, A; Funk, R; Nübel, U; Amon, T; Roesler, U (2021): **“Low airborne tenacity and spread of ESBL-/AmpC-producing *Escherichia coli* from fertilized soil by wind erosion”**. Environmental microbiology, in press https://doi.org/10.1111/1462-2920.15437

Frentrup, M., Thiel, Junker, V., N., Behrens, W., Münch, S., Siller, P., Kabelitz, T., Faust, M., Indra, A., Baumgartner, S., Schepanski, K., Amon, T., Roesler, U., Funk, R., Nübel, U. (2021) **“Agricultural fertilization with poultry manure results in persistent environmental contamination with the pathogen Clostridioides difficile**” Environmental microbiology, in press https://doi.org/10.1111/1462-2920.15601

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Presentations

Siller, P.; Daehre, K.; Roesler, U. (2017**): Dissemination of ESBL-producing *E. coli* from broiler manure to fields and surrounding; DRS Doktorandensymposium 2017** 22.09.2017

Siller, P.; Daehre, K.; Roesler, U. (2018**): Is wind erosion capable of spreading resistant *E. coli* from broiler stables in the environment? DRS Doktorandensymposium 2018** 21.09.2018.

Siller, P.; Daehre, K.; Roesler, U. (2019**): How likely is an environmental spread of ESBL–producing *E. coli* out of manure?; XIX International Congress of ISAH 2019 Wrocław, Poland** – 08.09.-12.09.2019. In: Proceedings of the XIXth International Congress of the International Society for Animal Hygiene : animal hygiene as a fundament of one health and

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Siller, P.; Dähre, K.; Rösler, U. (2019): **Are ESBL-producing *E. coli* spread in the environment by manure application, soil cultivation and wind erosion?; National Symposium on Zoonoses Research 2019** Berlin – 16.10.-18.10.2019.In: Zoonoses 2019 - International Symposium on Zoonoses Research : Book of Abstracts – International Symposium on Zoonoses Research (Hrsg.) Berlin, S.53

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Posters

Siller, P.; Dähre, K.; Rösler, U. (2017): **Ausbringungsbedingte Emission ESBL-bildender *E.Coli* aus Broilermist auf Felder und deren Umgebung;. 3. Tagung der DVG-Fachgruppe Umwelt- und Tierhygiene** 30.11.-01.12.2017.
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Siller, P.; Daehre, K.; Rösler, U. (2019):**Emission of ESBL – producing E. coli to the environment through soil cultivation and wind erosion;.Junior Scientist Zoonoses Meeting** 20.06.2019

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VI. Selbständigkeitserklärung

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

Berlin, den 11.02.2022 Paul Siller

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