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

und

dem Leibniz-Institut für Agrartechnik und Bioökonomie

Manure management measures to reduce the risk of spreading ESBL-/AmpC-producing *Escherichia coli* from chicken manure into the food chain

Inaugural-Dissertation
zur Erlangung des Grades
einer Doktorin der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Corinna Thomas, geb. Roth
Tierärztin aus Karlsruhe

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Für Stephan, Annika und Luis

Für Mama

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List of Abbreviations

AIEC	adherent-invasive *Escherichia coli*
AmpC	AmpC beta–lactamase
APEC	avian pathogenic *Escherichia coli*
ARG	antibiotic resistance gene
BLE	Federal Office for Agriculture and Food
BMEL	German Federal Ministry of Food and Agriculture
C	carbon
CaO	calcium oxide
C/N ratio	carbon/nitrogen ratio
cfu	colony forming units
CH_4	methane
CMY	cephamycinase beta-lactamase
CO_2	carbon dioxide
CTX-M	cefotaximase-Munich beta-lactamase
DAEC	diffusely adherent *Escherichia coli*
DNA	deoxyribonucleic acid
D-value	decimal reduction time
E. coli	*Escherichia coli*
EAEC	enteroaggregative *Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ESBL	extended-spectrum beta-lactamase
ETEC	enterotoxigenic *Escherichia coli*
EU	European Union
ExPEC	extra-intestinal pathogenic *Escherichia coli*
H_2	hydrogen
HRT	hydraulic retention time
H_2S	hydrogen sulfide
h_{total}	total hours
HUS	hemolytic uremic syndrome
InPEC	intestinal pathogenic *Escherichia coli*
K	potassium
K_2O	potassium oxide

K. pneumoniae	*Klebsiella pneumoniae*
log	logarithm
Mbp	mega base pair
MC	moisture content
MgO	magnesium oxide
N	nitrogen
N_2O	nitrous oxide
NH_3	ammonia
NH_4^+	ammonium
NH_4-N	ammoniacal nitrogen
NMEC	neonatal meningitis-causing *Escherichia coli*
OLR	organic loading rate
OXA	oxacillinase beta-lactamase
P	phosphorus
P_2O_5	phosphorus pentoxide
SEPEC	sepsis-associated *Escherichia coli*
SHV	sulfhydryl reagent variable beta-lactamase
TEM	Temoneira beta-lactamase
UK	United Kingdom
UPEC	uropathogenic *Escherichia coli*
USEPA	United States Environmental Protection Agency
VDI	Verein Deutscher Ingenieure
VFA	volatile fatty acid
WGS	whole genome sequencing
WHO	World Health Organization

1 Introduction

The discovery and development of antibiotics to treat bacterial diseases was a milestone in the history of medicine. Within recent decades, however, antibiotic resistance has become a serious and urgent public health issue since an increasing number of antibiotics are ineffective against some life-threatening infections (Casadevall, 2017). In particular, extended-spectrum beta-lactamase (ESBL)- and AmpC beta-lactamase-producing enterobacteria are seen as massive threats to public health and are rated among the three most critical antibiotic-resistant pathogens in the “priority pathogens” list published by the World Health Organization (WHO) (Tacconelli et al., 2018). The emergence of ESBL-/AmpC-producing enterobacteria, predominantly *Escherichia (E.) coli* and *Klebsiella (K.) pneumoniae*, has become a severe problem in both human and veterinary health (EFSA and ECDC, 2021; Ewers et al., 2012). ESBL-/AmpC-producing *E. coli* can lead to serious infections such as urinary tract infections or bacteremia and can also be found as commensals in humans and animals (Karanika et al., 2016; Ewers et al., 2012; Pitout and Laupland, 2008; Luzzaro et al., 2006). The latter leads to a wide spread of these bacteria in European livestock populations (Velasova et al., 2019; Hille et al., 2017; Von Salviati et al., 2014; Hartmann et al., 2012), with a particularly high prevalence in broiler production. Here, ESBL-/AmpC-producing *E. coli* can be found in high numbers in animals, feces and farm surroundings (Blaak et al., 2015b; Laube et al., 2013). This prevalence leads to the growing concern that these bacteria can enter the human food chain not only by contaminated meat but also by contaminated water, vegetables or fruit, mainly due to the use of chicken manure as fertilizer on agricultural fields (Zhao et al., 2017; Chen and Jiang, 2014; Heuer et al., 2011). Since ESBL-/AmpC-producing *E. coli* often carry resistance genes on mobile genetic elements such as plasmids (Hawkey and Jones, 2009), both the horizontal transmission of strains and clones via contact or contamination from animals and foods to humans, and the horizontal gene transfer from one *E. coli* strain to another or even to other bacterial species, are possible (Mo et al., 2017; Smet et al., 2011). Hence, commensal bacteria carrying these antibiotic resistance genes (ARGs) can serve as reservoirs for pathogens (Smet et al., 2010).

Chicken manure is a valuable fertilizer that is applied to land in massive amounts each year (Statistisches Bundesamt, 2021). This procedure raises concerns about the spread of ESBL-producing *E. coli* to the environment (Chen and Jiang, 2014). *E. coli* can survive for extended periods of time in manure and soil (Bolton et al., 2011; Johansson et al., 2005); therefore, appropriate manure treatment methods are needed to reduce ESBL-/AmpC-producing *E. coli* in chicken manure before it is applied to land. Composting and anaerobic digestion are two manure treatment methods known for their potential to reduce pathogens (Chen and Jiang,

2014; Goberna et al., 2011; Pandey and Soupir, 2011; Erickson et al., 2010; Martens and Böhm, 2009). Temperature is considered the main inactivation factor for both treatments (Jiang et al., 2020; Chen and Jiang, 2014). However, further factors can influence the survival of bacteria during composting and anaerobic digestion: i) chemical factors, such as ammonia (NH_3) and volatile fatty acid (VFA) concentration and pH, ii) microbial factors, such as microbial competition and the tolerance of specific bacterial species and strains to heat and chemical conditions, and iii) operational factors, e.g., the choice of manure type with substrate-specific differences in chemical composition and nutrient availability (Erickson et al., 2014; Singh et al., 2011; Lang and Smith, 2008; Salsali et al., 2006; Sahlström, 2003). During composting, environmental conditions as well as the chemical factors carbon/nitrogen (C/N) ratio, moisture content (MC), and oxygen level can also play roles in the successful reduction of bacteria (Erickson et al., 2014; Singh et al., 2011; Erickson et al., 2010). These influential factors, partially influencing each other as well, lead to difficulty in predicting the extent of bacterial reduction during anaerobic digestion or composting. Studies focusing on the survival of *E. coli* during anaerobic digestion and composting, therefore, show a wide range of results, from unsatisfying reduction to successful elimination of *E. coli* within hours (Schauss et al., 2015; Pandey and Soupir, 2011; Wilkinson et al., 2011; Erickson et al., 2010). Studies also indicate that the use of different manure types and different *E. coli* strains in particular can lead to great variance in results (Xing et al., 2019; Jang et al., 2017; Smith et al., 2005). Therefore, better knowledge of the reduction kinetics of ESBL-/AmpC-producing *E. coli* during the treatment of chicken manure and the influencing factors is necessary since studies focusing on chicken manure and ESBL-/AmpC-producing *E. coli* are scarce.

The overall aims of this work were therefore to investigate the influence of composting and anaerobic digestion of chicken manure on the reduction kinetics of ESBL-/AmpC-producing *E. coli* and to evaluate the factors responsible for the reduction to define critical process parameters. Composting and anaerobic digestion were chosen because both processes are common treatment methods in European agriculture based on the microbial degradation of organic material with opposing mechanisms: anaerobic and aerobic degradation. With that aim, laboratory-scale composting of chicken manure with different C/N ratios and MCs was performed, as well as anaerobic digestion tests at mesophilic and thermophilic temperatures. In both studies, ESBL-/AmpC-producing *E. coli* were added in high numbers to the substrates, and decimal reduction times (D-values) as well as the influences of further factors such as pH, VFA or NH_3 concentration were analyzed. In a third study, chicken manure composting was performed under pilot-scale conditions, comparing the influences of three different management methods (static piles, static covered piles, periodically turned piles) on the survival of a nonresistant *E. coli* strain.

2 Literature

2.1 Extended-spectrum beta-lactamase-/AmpC-producing bacteria

2.1.1 Beta-lactam antibiotics and extended-spectrum/AmpC beta-lactamases

Ever since the discovery and introduction of antibiotics as a treatment option for bacterial infections in humans and animals - starting with the discovery of penicillin as the first beta-lactam antibiotic in 1928 (Kong et al., 2010) – a correlated emergence of antimicrobial resistance in bacteria can be observed (Fisher and Mobashery, 2020; Allen et al., 2010; Davies and Davies, 2010; Medeiros, 1997). The bactericidal effect of beta-lactam antibiotics is based on their ability to inhibit the synthesis of the bacterial cell wall (Fisher and Mobashery, 2020; Kong et al., 2010). The central chemical structure of all beta-lactam antibiotics is the beta-lactam ring (Figure 1). These antibiotics can be divided into penicillins, and cephalosporins – themselves divided into 1st to 5th generation cephalosporins – and carbapenems and monobactams (Fisher and Mobashery, 2020; Lima et al., 2020a).

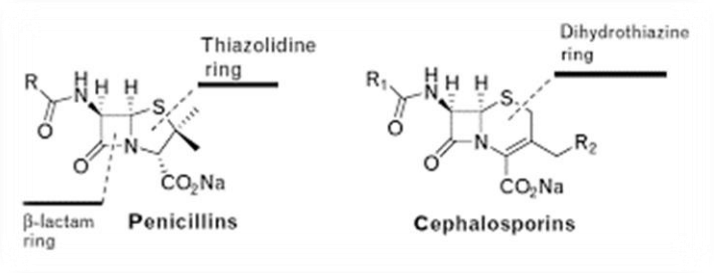

Figure 1. Core structure of A: penicillins and B: cephalosporins (Perez-Inestrosa et al., 2005)

However, the beta-lactam ring is also the point of attack for resistant bacterial strains. These bacteria produce enzymes called beta-lactamases, which are able to hydrolyze the beta-lactam ring, making these drugs ineffective (Kong et al., 2010). In general, beta-lactamases are classified either according to molecular characteristics or to functional characteristics. The Ambler classification was first established in 1980 and modified over the years. It divides beta-lactamases into four groups due to their molecular structure: Class A, Class C, and Class D belong to the serine beta-lactamases, whereas Class B belongs to the metallo beta-lactamases possessing a zinc atom (Sawa et al., 2020; Ambler, 1980). Bush et al. (1995) and Bush and Jacoby (2010) established a new classification due to the functional characteristics of beta-lactamases, such as hydrolytic activity and susceptibility to inhibition (Table 1).

Table 1. Classification of beta-lactamases according to their functional characteristics (Bush, 2013; Bush and Jacoby, 2010; Bush et al., 1995)

Group	Enzyme type
1 (Ambler Class C)	Cephalosporinases, not inhibited by clavulanic acid, e.g., AmpC beta-lactamases
2 (Ambler Classes A and D)	Extended-spectrum beta-lactamases, inhibited by clavulanic acid
3 (Ambler Class B)	Resistance against carbapenems and all other beta-lactams except monobactams. Not inhibited by clavulanic acid, but by ethylenediaminetetraacetate (EDTA)

There is no single definition of extended-spectrum beta-lactamases (ESBLs) found in literature since the ongoing discovery of new variants has led to the adjustment of the definition. However, ESBLs are beta-lactamases that hydrolyze an extended spectrum of beta-lactam antibiotics, including 3rd- (e.g., cefotaxime, ceftazidime) and 4th-generation cephalosporins (e.g., cefepime, cefozopran) and monobactams (Pitout and Laupland, 2008). They can usually be inhibited in vitro by the beta-lactamase inhibitors clavulanic acid, tazobactam, or sulbactam and mostly carry transmissible beta-lactamase genes on mobile genetic elements (Shaikh et al., 2015; Rubin and Pitout, 2014) that can be exchanged between bacterial strains (Smet et al., 2011) and species (Mo et al., 2017) via horizontal gene transfer. ESBLs belong to Ambler molecular class A and most often to group 2be according to their functional characteristics (Cantón et al., 2012). Horizontal gene transfer is the main reason for the successful emergence of these resistant bacteria and explains the enormous number of different variants found. ESBLs are the major cause of antibiotic resistance in Gram-negative bacteria worldwide and are most common in Enterobacteriaceae, predominantly *Escherichia (E.) coli* and *Klebsiella* spp., but are also found, for example, in *Salmonella* spp., *Enterobacter* spp., *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Cantón et al., 2008). In 1983, the first ESBL was detected in Germany and soon after was also described in France (Sirot et al., 1987; Knothe et al., 1983). Since then, ESBLs have been discovered all over the world in hospitalized patients, in the community, and in healthy humans and animals (Ewers et al., 2012; Cantón et al., 2008). ESBLs can be divided into different groups according to their amino acid sequences. The first ESBLs found belonged to the TEM (Temoneira – the patient's name) and SHV (sulfhydryl reagent variable) types (Cantón et al., 2008). In 1989, a new ESBL group was discovered in Germany, France, Italy, and Argentina. This group was named CTX-M because of its stronger resistance to cefotaxime rather than ceftazidime (Cantón et al., 2008). The CTX-

M group emerged as the predominant group of ESBLs worldwide, both in humans and animals, both associated with nosocomial infections and found in the healthy community (D'Andrea et al., 2013; Cantón et al., 2012). In addition, AmpC beta-lactamases, as class C beta-lactamases, were found, predominantly in animals. AmpC beta-lactamases can additionally hydrolyze cephamycins whereas they are in general not affected by beta-lactam inhibitors. The most frequently detected plasmid-mediated AmpC beta-lactamase is CMY-2 (Ewers et al., 2012; Philippon et al., 2002).

2.1.2 Escherichia coli as one of the main producers of extended-spectrum/AmpC beta-lactamases

E. coli was first discovered in 1885 by Theodor Escherich. The species *E. coli* belongs to the family Enterobacteriaceae (Table 2) and is a common resident of the gastrointestinal tract of humans and animals. It is a Gram-negative, facultative anaerobic, motile by peritrichous flagellae or nonmotile, nonspore forming, rod-shaped bacterium with a diameter of 1.1-1.5 µm and a length of 2.0 and 6.0 µm (Wieler et al., 2011; Tenaillon et al., 2010).

Table 2. Taxonomy of *E. coli* (Scheutz and Strockbine, 2015)

Level	
Domain	Bacteria
Phylum	Proteobacteria
Class	γ-Proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli*

E. coli is a very complex and diverse organism with a multitude of different strains. Many of these strains are commensals of the intestinal flora of humans and animals and do not cause any harm to their hosts (Kaper et al., 2004). However, there are also various pathogenic strains which cause serious infections. These pathogenic *E. coli* are divided into intestinal (InPEC) and extraintestinal (ExPEC) pathogenic *E. coli*. ExPEC can cause, for example, meningitis, wound infections, septicaemia, mastitis and urinary tract infections. The ExPEC group includes uropathogenic *E. coli* (UPEC), avian pathogenic *E. coli* (APEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-associated *E. coli* (SEPEC) (Santos et al., 2020; Pitout, 2012). InPEC are divided into enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent

E. coli (DAEC), enteroinvasive *E. coli* (EIEC), and adherent-invasive *E. coli* (AIEC) (Santos et al., 2020; Robins-Browne et al., 2016; Kaper et al., 2004). EHEC, in particular, is known to a broader public since this group of *E. coli* has been responsible for major foodborne and waterborne outbreaks worldwide and can cause serious infections and deaths due to bloody diarrhea and hemolytic uremic syndrome (HUS) (Heiman et al., 2015; Saxena et al., 2015; Pennington, 2014). In 2011, the EHEC strain O104:H4 was responsible for a large foodborne outbreak in Germany (Burger, 2012).

The diversity of *E. coli* strains is complex, with a shared core genome of only 20 to 30%. Up to 80% of the DNA therefore belongs to highly flexible genes and mobile genetic elements such as plasmids, transposons or bacteriophages. These elements usually harbor virulence factors, pathogenicity islands or resistance genes and can easily be transferred to other *E. coli* or bacterial species by horizontal gene transfer (Robins-Browne et al., 2016; Wieler et al., 2011). The genome size of *E. coli* therefore varies from 4.6 million base pairs (mbp) to 5.5 mbp (Robins-Browne et al., 2016). With the emergence of whole genome sequencing (WGS), a rising number of genomes of *E. coli* strains can be described. In addition, the concentration in human and animal feces can be up to 10^{10} colony forming units (cfu)/g, which results in a high dynamic spread of *E. coli* clones, but also of ever-novel variants due to horizontal gene transfer. This fact complicates the search for the origin of strains, infections, or outbreaks since pathotypes may have more than one phylogenetic root and are not necessarily spread clonally (Wieler et al., 2011).

The high diversity of *E. coli* strains also leads to a high adaptability of *E. coli* to different environments. Since *E. coli* are responsible for various serious infections and foodborne outbreaks, it is essential to know the factors influencing the growth and survival of *E. coli*. In general, *E. coli* is very easy and undemanding to cultivate, which is one of the reasons that *E. coli* is a popular model organism in the laboratory and probably the most intensively studied bacterium in the world (Robins-Browne et al., 2016; Tenaillon et al., 2010). The optimum growth temperature of *E. coli* is 37 °C, with minimum and maximum temperatures of 8 and 45 °C, respectively (Bell and Kyriakides, 1998). Hence, *E. coli* is not particularly heat resistant, and in general, heat resistance is often linked to other physical, chemical, and microbial factors, such as pH (growth minimum and maximum: 4.4 and 9.0, respectively), water activity, solar radiation, ammonia (NH_3), microbial competition and substrate availability. These factors can stress *E. coli*, increasing the susceptibility of these bacteria to temperature (Petersen and Hubbart, 2020; Ishii et al., 2009; Kaur et al., 1998; Bell and Kyriakides, 1998).

2.2 Occurrence and relevance of ESBL-/AmpC-producing *E. coli* in public health

2.2.1 Occurrence of ESBL-/AmpC-producing *E. coli* in humans and animals

Since the first description of ESBL-producing enterobacteria in the early 1980s (Cantón et al., 2008), numerous studies on their occurrence and prevalence in both humans and animals have been conducted worldwide. These studies revealed an increasing and threatening problem for both human and veterinary health since therapeutic options for serious infections have become limited. Until the early 2000s, ESBL-producing enterobacteria, predominantly *Klebsiella (K.) pneumoniae* and *E. coli*, carried TEM and SHV β-lactamases, and mainly ESBL-producing *K. pneumonia* was a major source of nosocomial infections (Cantón et al., 2008; Pitout et al., 2005). Then, a new ESBL-group – the CTX-M-type enzymes, mostly expressed by *E. coli* – evolved and rapidly increased since 2000, becoming the predominant ESBL type that appeared particularly in the community instead of hospital patients (Lewis et al., 2007; Livermore et al., 2007; Paterson and Bonomo, 2005; Pitout et al., 2005; Bonnet, 2004). The main CTX-M types found in humans are CTX-M-14 and CTX-M-15 (Peirano and Pitout, 2019; Ewers et al., 2012; Lewis et al., 2007). Humans infected with CTX-M-producing *E. coli* mainly suffer from urinary tract infections and, in lower cases, from bacteremia (Pitout and Laupland, 2008; Luzzaro et al., 2006); however, CTX-M-producing *E. coli* also appear as commensals in healthy humans (Karanika et al., 2016; Valverde et al., 2004).

With the increasing concern regarding the emergence of ESBL-producing *E. coli* found in humans, studies have also begun to focus on the occurrence of ESBL-producing Enterobacteriaceae in animals. Soon, it became obvious that healthy farm animals often carry ESBL-producing *E. coli* as commensals of the intestinal flora (Table 3) (Ewers et al., 2012). This topic has been addressed by many studies within the last 15 years. ESBL-/AmpC-producing *E. coli* were found in beef and dairy cattle, for example, in France (Hartmann et al., 2012), Germany (Hille et al., 2017; Schmid et al., 2013), the United Kingdom (UK) (Velasova et al., 2019), Egypt (Braun et al., 2016), and the United States (Lee et al., 2020; Davis et al., 2015). They were also found in sheep (Tello et al., 2020; Snow et al., 2011), pigs (Dohmen et al., 2017; von Salviati et al., 2014), laying hens (Blaak et al., 2015b), and turkey (Friese et al., 2013) and with especially high prevalence within the broiler production chain (Blaak et al., 2015b; Laube et al., 2013).

Table 3. Prevalence (%) of presumptive ESBL-/AmpC-producing *E. coli* from farm animals and derived meat from the specific monitoring of the EU Member States in 2018/2019, according to EFSA and ECDC (2021)

Source	**Presumptive ESBL-/AmpC-producing *E. coli***	**Presumptive ESBL-producing *E. coli***	**Presumptive AmpC-producing *E. coli***
Pigs	42.7	34.1	9.7
Bovines < 1 year	46.4	43.0	4.6
Turkeys	39.3	33.9	7.9
Broilers	48.3	31.9	18.9
Pig meat	6.8	5.6	1.5
Bovine meat	5.2	4.5	0.8
Broiler meat	39.8	25.7	16.1

Studies on companion animals are fewer in number, but ESBL-/AmpC-producing *E. coli* were also found both in diseased (mostly urinary tract infections) and healthy cats and dogs (Zhang et al., 2018; Liu et al., 2016; Bogaerts et al., 2015; Ewers et al., 2012; Sun et al., 2010) and in horses (Apostolakos et al., 2017; Dierikx et al., 2012) around the world.

In addition, ESBL-producing *E. coli* have been detected in wildlife animals, such as wild birds (Atterby et al., 2017; Alcalá et al., 2016; Guenther et al., 2010), deer (Velhner et al., 2018), fox (Costa et al., 2006), wild boars (Bonardi et al., 2018), and fish (Moremi et al., 2016). The finding of ESBL genes in wildlife – which normally has no direct contact with humans or domestic animals and faces no selective pressure through antibiotics – suggests other sources of infections: for example, through the spread of contaminated feces or wastewater into the environment. Therefore, wild animals become victims of the environmental spread of ESBL genes but can also serve as permanent reservoirs of these genes when commensal *E. coli* colonize an animal's gut and therefore act as spreaders of ESBL genes (Guenther et al., 2011).

As found in humans, also in animals, the CTX-M group is the increasingly predominant group of ESBLs found (Ewers et al., 2012). Especially worrying is the fact that animals often do not exhibit an infection but simply harbor these bacteria in the intestinal tract, being a permanent reservoir of resistance genes and a source of infection.

2.2.2 Occurrence of ESBL-/AmpC-producing *E. coli* in broiler production

With the increasing number of studies assessing the occurrence and prevalence of ESBL-/AmpC-producing *E. coli* in farm animals, it became apparent that the prevalence in the broiler production chain is particularly high and therefore poses a major concern in human and veterinary medicine (Blaak et al., 2015b; Laube et al., 2013). ESBL-/AmpC-producing *E. coli* were found all over the world in broiler chickens, for example, in Asia (Wu et al., 2018; Lim et al., 2015), Africa (Falgenhauer et al., 2019a), and South America (Casella et al., 2015). In addition, numerous studies have reported findings of ESBL-/AmpC-producing *E. coli* in European broiler farms, for example, in the UK (Randall et al., 2011), Portugal (Costa et al., 2009), France (Baron et al., 2018), and even in Norway, which is a country with a low antimicrobial usage profile (Mo et al., 2014). Furthermore, some studies, for example, from the Netherlands (Blaak et al., 2015b; Dierikx et al., 2013a), Spain (Mesa et al., 2006), and Germany (Hering et al., 2016; Friese et al., 2013; Laube et al., 2013), reported a prevalence of ESBL-producing *E. coli* of 100% on broiler farms. This high prevalence was even observed in organic broiler farms in the Netherlands, where ESBL-/AmpC-producing *E. coli* were isolated from eight of eight farms tested (Huijbers et al., 2015). However, only a few studies have focused on the quantity of ESBL-/AmpC-producing *E. coli* found in broiler feces, although high concentrations will increase the risk of shedding these bacteria into the environment. High concentrations will also magnify the potential for horizontal gene transfer and the emergence of new genetic variants. Blaak et al. (2015b), for example, found 2.3 x 10^7 cfu/kg ESBL-producing *E. coli*, and Laube et al. (2013) even described 1.25 x 10^6 cfu/g in pooled broiler feces samples.

The most frequently detected ESBL genes in European broiler production belong to the CTX-M group, mainly CTX-M-1 but also CTX-M-14, which is the predominant type in Asia, and CTX-M-15, which is together with CTX-M-14 the main type found in humans worldwide. In addition, among others, SHV-1, SHV-12, and TEM-52 were found (Blaak et al., 2015b; Ewers et al., 2012; Smet et al., 2008), and in contrast to humans and other animals, there is also a high prevalence of AmpC-producing *E. coli* carrying the CMY-2 gene in broiler production (Apostolakos et al., 2019; Daehre et al., 2018a; Ewers et al., 2012).

Several studies have focused on the transmission routes of ESBL-/AmpC-producing *E. coli* along the broiler production chain. These studies showed that ESBL-/AmpC-producing *E. coli* were found at all levels of the broiler production pyramid from breeding farms to hatcheries to fattening farms to slaughterhouses (Apostolakos et al., 2019; Mo et al., 2014; Dierikx et al., 2013b; Reich et al., 2013). In addition, ESBL-producing *E. coli* were found in the surrounding

environment of broiler farms, such as in dust samples, slurry, rinse water, runoff water, surface water, soil, ambient air and barn air, and even in flies (Blaak et al., 2015b; Blaak et al., 2014; Laube et al., 2014; Friese et al., 2013). A high prevalence of ESBL-producing *E. coli* was also found in retail chicken meat (Kaesbohrer et al., 2019; Randall et al., 2017; Kola et al., 2012).

The genetic background of these ESBL-/AmpC-producing *E. coli* is complex, implying different transmission routes via vertical and horizontal transmission of clones and genes (Apostolakos et al., 2019; Projahn et al., 2018; Nilsson et al., 2014). The finding of these bacteria was not necessarily linked to treatment with antibiotics, indicating that no selective pressure by antibiotics was needed (Daehre et al., 2018b; Huijbers et al., 2016; Mo et al., 2014). This emphasizes the possibility of vertical transmission from grandparent flocks or parent flocks to fattening broilers or horizontal transmission by direct contact or by a contaminated housing environment (Daehre et al., 2018b; Dierikx et al., 2013b). The latter was also discussed by Robé et al. (2019), who demonstrated that even a very low dose of only 10^1 cfu ESBL-/AmpC-producing *E. coli*, for example, in an insufficiently cleaned and disinfected poultry house, can lead to rapid colonization of broilers during the fattening period. The different transmission routes were analyzed in a review by Dame-Korevaar et al. (2019) and four major transmission routes were described: i) vertical transmission from generation to generation, ii) transmission at hatcheries, iii) horizontal transmission in fattening farms, and iv) horizontal transmission between farms or from the environment.

2.2.3 Fecal emissions of ESBL-/AmpC-producing *E. coli* and survival in the environment

Since studies show an increasing intestinal colonization of healthy humans and animals with ESBL-/AmpC-producing *E. coli* (Karanika et al., 2016; Ewers et al., 2012), fecal emissions of ESBL-/AmpC-producing *E. coli* into the environment are of great concern (Chen and Jiang, 2014; Heuer et al., 2011). This can occur, for example, by spreading manure as fertilizer on fields (Zheng et al., 2017; Gao et al., 2015b) or through the waterborne route by contaminated rinse water or wastewater that contaminates surface, ground, and drinking waters as well as growing plants (Gekenidis et al., 2020b; Njage and Buys, 2015). In addition, an airborne transmission route is discussed (Gao et al., 2015a; Laube et al., 2014), although a recent study by Siller et al. (2021) demonstrated a low airborne tenacity of ESBL-/AmpC-producing *E. coli* and only limited spread by wind erosion. The fourth possible transmission route is the spread of ESBL-/AmpC-producing *E. coli* and ESBL/AmpC genes by vectors such as flies and wild animals that can spread these bacteria and genes over long distances (Blaak et al., 2014; Guenther et al., 2011).

In fact, ESBL-/AmpC-producing *E. coli* were found in the direct environment of animal farms. In a German study by Laube et al. (2014), an intensive spread of ESBL-/AmpC-producing *E. coli* to the surrounding areas of broiler farms was reported. ESBL-/AmpC-producing *E. coli* were detected in slurry samples, in boot swabs from various surfaces of the surrounding areas, and in air samples. Von Salviati et al. (2015) described similar results in the surrounding environment of German pig farms, including ESBL-/AmpC-positive samples from the digestate of biogas plants. Additionally, small proportions of fecal samples of flies and mice were positive for ESBL-/AmpC-producing *E. coli*. Blaak et al. (2015b) found ESBL-producing *E. coli* in the environment of broiler and laying hen farms in the Netherlands, for example, in rinse water and runoff water, in other farm animals and dust, in surface water next to farms, in soil, on flies, and in barn air. Hartmann et al. (2012) detected ESBL-producing *E. coli* isolates in the surroundings of cattle farms, in soil and composted manure in France. In an American study, ESBL-/AmpC-producing *E. coli* were even isolated from animals and soil and feed samples of extensive beef cattle farms with seldom use of antibiotics (Lee et al., 2020). In all five studies, a clonal relatedness of the *E. coli* isolates found in animal feces and the environment was demonstrated, indicating transmission routes from barns to the environment.

Furthermore, ESBL-/AmpC-producing *E. coli* have been found worldwide in the wastewater of hospitals, communities and slaughterhouses; in wastewater treatment plants, including wastewater treatment plant effluents (Savin et al., 2020; Gomi et al., 2017; Jørgensen et al., 2017); in surface water (Falgenhauer et al., 2019b; Blaak et al., 2015a) and rivers (Zarfel et al., 2017; Gao et al., 2014); and in irrigation (Gekenidis et al., 2018) and drinking water (Madec et al., 2016). ESBL-/AmpC-producing *E. coli* were also detected in soils (Song et al., 2021; Blaak et al., 2015b; Hartmann et al., 2012) and on plants, vegetables and fruit (Freitag et al., 2018; Njage and Buys, 2015; van Hoek et al., 2015). In many of these studies, the CTX-M genes were the most prominent ESBL-group found, and a clonal relationship was demonstrated between isolates from humans, animals, and the environment.

These findings show the already expansive dissemination of ESBL-/AmpC-producing *E. coli* in the environment. Therefore, a growing concern of public health is that these bacteria can enter the human food chain by contaminating water and foods (Gekenidis et al., 2020b; Freitag et al., 2018; Hu et al., 2013). Furthermore, the spread of ESBL-/AmpC-producing *E. coli* in the environment leads to infected wildlife being both victims of and reservoirs for ESBL-/AmpC-producing *E. coli* (Guenther et al., 2011). However, to date, it remains very difficult to predict the survival of ESBL-/AmpC-producing *E. coli* or *E. coli* in general in the environment (Franz et al., 2014). Although there are several studies addressing the survival of *E. coli* in soil, the

time of survival can substantially vary. In a review by Fremaux et al. (2008), for example, the survival of *E. coli* O157:H7 in soil ranged from 25 to more than 231 days in different studies. Islam et al. (2005) detected *E. coli* O157:H7 for 154 to 196 days in soils amended with contaminated composts and until days 74 and 168 on onions and carrots, respectively, as well as on lettuce and parsley until days 77 and 177, respectively (Islam et al., 2004). Some authors even hypothesize that *E. coli* can become soil commensals and therefore able to survive and grow in soil under certain circumstances, yielding reservoirs for pathogenic and antibiotic-resistant *E. coli* and permanent sources of infection (Montealegre et al., 2018; NandaKafle et al., 2018; Kim et al., 2009; Ishii et al., 2006). Interestingly, poultry manure is not only the manure type burdened in particular with ESBL-/AmpC-producing *E. coli*, but it also seems that *E. coli* survive longer in poultry manure-amended soil compared to other manure types (Sharma et al., 2016; Islam et al., 2005). However, most of these studies focused on the survival of *E. coli* in general or on *E. coli* O157:H7. Little is known about the survival of ESBL-/AmpC-producing *E. coli* in the environment, though. In a study by Gekenidis et al. (2020b), ESBL-producing *E. coli* were undetectable on lettuce after seven days, while they survived for up to nine weeks in soil when introduced via manure. Hartmann et al. (2012), on the other hand, reported the finding of an ESBL-producing *E. coli* strain in soil one year after it was amended with cattle manure. The main reason for such variance in results is the strong dependency of *E. coli* survival on environmental factors. Therefore, differences in i) ambient and soil temperature, ii) soil moisture, iii) soil composition/texture, including pH and soil chemistry, iv) solar radiation, v) rainfall events, vi) substrate availability, vii) microbial composition and competition, and viii) characteristics of the individual *E. coli* strain make it extremely difficult to forecast how long ESBL-/AmpC-producing *E. coli* and *E. coli* in general will survive in the environment (Sharma et al., 2019; Xing et al., 2019; Jang et al., 2017; Franz et al., 2014; Van Elsas et al., 2011).

2.2.4 Transmission of ESBL-/AmpC-producing *E. coli* from animals to humans

Finding the same groups of ESBL genes, mainly the CTX-M group, in humans and animals leads to the concern that ESBL-producing bacteria and genes can be transferred to humans and to bacterial species or strains that cause serious infections. This horizontal transfer can occur through direct contact with companion or food animals or when these bacteria enter the human food chain by contaminated meat, vegetables and fruit, or drinking water.

The successful dissemination of ESBL/AmpC genes worldwide in animals, humans, and foods is due to the spread of bacterial clones but also due to horizontal gene transfer of mobile genetic elements such as plasmids (Sharp et al., 2014; Valentin et al., 2014). Since commensal

E. coli carrying ESBL/AmpC genes can colonize the animal's intestine without causing an infection, healthy animals can serve as gene reservoirs (Smet et al., 2010; Carattoli, 2008). This can lead to the transfer of these genes to other potentially pathogenic *E. coli* strains (Gekenidis et al., 2020a; Smet et al., 2011) or even to other bacterial groups (Mo et al., 2017), for example, to pathogens causing serious infections. Therapeutic treatment then becomes dramatically limited when these pathogens show resistance to antibiotics.

Several studies have aimed to find the relationship between ESBL genes of human and animal origin. However, due to horizontal gene transfer, the genetic variability is very complex and makes it difficult to understand transmission routes of a certain strain or clonal complex and to find the original source of ESBL-/AmpC-producing *E. coli* (Sharp et al., 2014; Valentin et al., 2014). Nonetheless, the same ESBL/AmpC genes, the same plasmids, or even closely related or identical isolates were found, for example, between broilers and broiler farmers (van Hoek et al., 2016; Huijbers et al., 2014; Dierikx et al., 2013a), broilers and hospitalized children (Falgenhauer et al., 2019a), pigs and pig farmers (Fischer et al., 2017), humans and companion animals (Hong et al., 2019), wild yellow-legged gulls and humans (Bonnedahl et al., 2009), chicken meat and humans (Kluytmans et al., 2013; Overdevest et al., 2011), broilers, chicken meat and patients (Voets et al., 2013; Leverstein-van Hall et al., 2011), and humans, companion animals and horses (Schmiedel et al., 2014).

Therefore, the transmission of ESBL-/AmpC-producing bacteria or genes from food-producing animals into the human food chain must be disrupted. It is essential to pay more attention to possible treatment methods for potentially ESBL-/AmpC-contaminated feces and manure of animals, especially chicken manure, before it is applied to agricultural land in order to stop the spread of ESBL/AmpC-producing *E. coli* to food and water.

2.3 Manure treatment methods

Animal manure consists mainly of feces, urine, and bedding material. Depending on the animal species and farming management, solid manure and liquid manure called slurry must be distinguished (Christensen and Sommer, 2013). Animal manure is often considered waste that must be disposed of and as a pollutant to the environment. It contributes to environmental pollution by odors, hydrogen sulfide (H_2S), nitrate, NH_3, greenhouse gases, such as methane (CH_4) and nitrous oxide (N_2O), heavy metals and pathogens, especially when poorly managed. However, animal manure is also rich in plant nutrients, such as nitrogen (N), phosphorus (P), and potassium (K) (Christensen and Sommer, 2013). Therefore, manure is a valuable fertilizer that is worth recycling. The fertilizer value of animal manure is dependent on animal species,

feeding and management practices, bedding material, and storage time (Jensen and Sommer, 2013). Farmers can utilize different methods to handle the manure of their animals. Often, manure is applied to land directly or stored for some time before it is spread to the fields. Manure can also be sold as valuable fertilizer to other customers. In addition, farmers can decide to compost their manure to gain an even more valuable and safer product that they can sell or use for their own purposes. They may also transport their manure to biogas plants or establish their own biogas plants on their farms to produce biogas for use or sale. Therefore, manure is normally less a waste but more a useful and saleable product. However, a proper manure management is essential to gain a valuable and safe product and to lower the risk of environmental pollution. Animal manures can be burdened with a variety of pathogenic bacteria, including *Salmonella* spp., *E. coli*, *Listeria* spp., and *Campylobacter* spp., as well as with viruses, e.g., rotavirus and fungi, and with parasites, such as *Cryptosporidium* and *Giardia* (Chen and Jiang, 2014; Martens and Böhm, 2009). Thus, the spread of animal diseases and zoonotic diseases via animal manure used as fertilizer must be minimized by manure treatment options. Manure management therefore also includes the treatment of manure before application to fulfill the task of minimizing the levels of pathogens in the manure and the spread of these pathogens to the environment.

2.3.1 Storage

Typically, farmers collect the manure of their animals and either use it fresh as fertilizer on their fields, store it for later use, or sell it (Statistisches Bundesamt, 2021). Depending on the animal species and the farm management, manure can be stored as solid manure in piles or in moveable silos, or it can be stored as slurry in tanks. Manure piles can additionally be covered with straw or a tarp, or they can be positioned under a roof to keep rainfall out and to decrease odors (Sommer and Christensen, 2013).

The storage of manure is a prerequisite to realizing closed nutrient cycles and high nutrient efficiency. Storage ensures that manure is applied to fields with the optimal timing when plant nutrients can be optimally utilized, e.g., application of the manure prior to the growing season. Storage is also necessary in times when the amount of manure exceeds the need on the fields and in times when regulations restrict the use of fertilizer with respect to the amount and the time of application.

Many countries have regulations concerning the storage and application of manure. These regulations define how manure has to be stored, e.g., on sealed grounds to avoid leakage, and they define the type, the maximum amount, and the time of application (Sommer et al.,

2013). In the European Union (EU), for example, the 1991 nitrates directive (91/676/EEC) was adopted to prevent the pollution of ground and surface waters caused by nitrates from agricultural origin. In Germany, the nitrates directive was implemented in 1996 with the Düngeverordnung (BMELV, 2017). In addition, this national regulation also defines the minimum storage capacity for solid manure (2 months) and slurry (6 months) that farmers must ensure on their farms.

In the literature, static piles are often considered in the composting methods, although no active management of composting is used here. The piles are only stored without controlling the composting process. However, static piles also heat up because of microbial activity and the degradation of organic material (Berry et al., 2013; Erickson et al., 2010). This degradation is performed both aerobically and anaerobically because, with a lack of management of the pile, parts of the pile will turn anaerobic during storage, causing more odors, less heat and insufficient decomposition of the material, which will not lead to a stable compost. However, the storage of manure for some amount of time is the most practiced method performed on animal farms (Vinnerås, 2013).

2.3.2 Composting

Composting is the biological, aerobic decomposition of organic matter, such as animal manures, food waste or garden waste, under controlled conditions (Epstein, 2019; Rynk, 1992). This definition includes every form of composting from simple backyard composting to on-farm composting to composting in large industrial facilities.

Composting reduces the weight, volume and moisture content (MC) of the original mass. The stable, humus-like final product called compost is a valuable material that can be used and sold, for example, as a soil conditioner and fertilizer. Another benefit of composting is the easier handling of finished compost (e.g., storage, transport, land application) since the mass is extensively reduced compared to the original mass. Furthermore, finished compost is free of odors, no further self-heating can occur, and pathogens and weed seeds are reduced during the composting process (Epstein, 2019; Rynk, 1992). In particular, the reduction of pathogens during the composting process is of great value from a public health perspective. The high temperatures during composting are considered the main inactivation factor (Chen and Jiang, 2014). The potential to reduce *E. coli* during composting and the factors involved will be further discussed in Chapter 2.4.

Composting is a common and traditional treatment method for solid wastes and was performed in a broader sense by the Romans, Greeks, and Israelites (Epstein, 2019). Interest in the science and technology of composting grew throughout the twentieth century. Composting should now become more efficient and faster and should achieve better results and a stable, high-quality product. Therefore, questions have arisen with respect to which factors influence the composting process, and as a consequence, composting has become a better understood and controlled process. New and optimized technologies have made it possible to operate large-scale and industrial composting facilities, which currently play important roles in recycling waste material such as municipal or animal wastes back to land (De Bertoldi-Schnappinger, 2007; Rynk, 1992).

The composting process starts almost as soon as the organic matter is mixed and piled up (Rynk, 1992). When the initial particle size of the substrate is too large, earthworms, nematodes and soil insects start to break down the material into smaller particles or the organic materials are mechanically broken into smaller pieces. Then, microorganisms such as bacteria, fungi, actinomycetes, and protozoa already present in the organic materials or mixed in from the soil feed on the organic substrates (Cooperband, 2002). To function best, microorganisms need warm temperatures, easily degradable material and sufficient oxygen for decomposition. The composting process is an exothermic process. Therefore, carbon (C) is used as an energy source and transformed into carbon dioxide (CO_2) and water, generating a considerable amount of heat. CO_2, water vapor, and heat are released into the air (Insam and de Bertoldi, 2007; Rynk, 1992). The temperature of the pile is directly related to the microbial activity and is therefore a good process indicator. With the increase in microbial activity, the temperature rises rapidly. Within the first 24 to 72 h, the temperature rises to its maximum of 54 to 65 °C or even 70 °C and remains at a high level for several weeks. This period is called the active (thermophilic) phase of composting, with thermophilic microorganisms decomposing the organic material. Temperatures above 70 °C are possible but not desirable since many microorganisms, mesophilic in particular, are killed during the temperature peak (Epstein, 2019; Chen et al., 2011; Insam and de Bertoldi, 2007; Cooperband, 2002). With a decrease in oxygen, the temperature also falls as the microbial activity slows down; therefore, aeration or turning of the pile leads to higher temperatures over a longer period and is crucial for successful composting (Rynk, 1992).

When the active phase slows, temperatures gradually drop to 37 °C and finally reach ambient temperature after some time. Mesophilic microorganisms recolonize the pile and continue to compost the material at a slower pace. Therefore, oxygen addition is no longer necessary, and the compost pile can be simply stored. This phase is called the curing phase of composting.

The curing phase is important to produce stable compost and can last from several weeks to months or even years, depending on the original substrate, composting methods, and environmental factors (Figure 2) (Diaz and Savage, 2007a; Rynk, 1992).

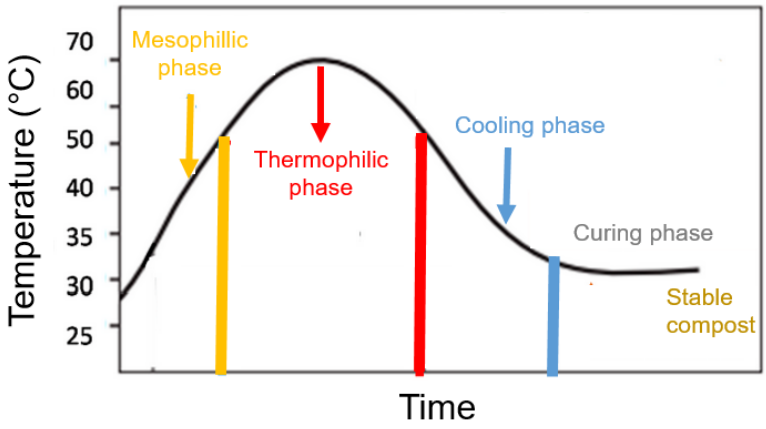

Figure 2. The phases of composting, adapted from Papale et al. (2021)

Compost is called finished when the raw materials are no longer actively decomposing, or only decomposing at a very low rate, when the compost therefore becomes biologically and chemically stable. Even when stored in large piles, compost will no longer heat up or become anaerobic, causing unpleasant odors. There are different ways to measure stability, and it is difficult to offer clear instructions describing when compost is stable. In general, it is a good sign when the temperature in the center of the pile is near ambient temperature and the oxygen level within the pile remains above 10 to 15%. Additionally, a low C/N ratio can be a sign of stable compost (Epstein, 2019; Cooperband, 2002; Rynk, 1992).

To achieve good composting results, it is crucial to create the right environment for microorganisms. Many factors influence microbial activity and the composting process (Table 4): the most important factors are oxygen and the MC (Epstein, 2019). Microorganisms need oxygen for the decomposition of organic substrates, and they release water and CO_2. Therefore, oxygen decreases during the process, and the shrinking particle size also reduces the air space within the mass. A minimum oxygen concentration of 5% is necessary to allow aerobic composting; otherwise, the pile will turn anaerobic, producing volatile fatty acids (VFAs) and intensive odors. This also leads to reduced microbial activity and temperature. Therefore, it is important to apply oxygen during the active phase either by mechanical turning or by forced aeration (Epstein, 2019; Diaz and Savage, 2007a; Rynk, 1992). Since water is essential for microbial activity, MC also plays an important role in the composting process. The MC of the mass ideally lies between 40 and 65% (Rynk, 1992). However, because of different

water-holding capacities, generalization is not possible (Diaz and Savage, 2007a). If the MC of the organic mass is too low, microorganisms may dehydrate, and therefore, microbial activity and temperature will decline. Excessive MC will lead to difficulties in gas exchange, lower air space between particles, and subsequently to anaerobic conditions. With the lack of available oxygen, the microbial activity will again decline, as will temperature. Therefore, especially in modern composting facilities, the possibility of adding water during the process should be enabled (Epstein, 2019; Diaz and Savage, 2007a).

Nutrients are also important factors for the composting process. Not only P and K, but especially C and N influence microbial activity. Microorganisms need C as an energy source and for their growth. N in turn is essential for protein synthesis and reproduction. It is important to provide both in a balanced ratio, called the C/N ratio. Raw materials with a C/N ratio of 20:1 to 40:1 are considered acceptable for good composting results. If the C/N ratio is too low, N is lost to the atmosphere as NH_3 or N_2O, leading to both excessive odors and greenhouse gas emissions. (Rynk, 1992).

Another important factor is temperature; however, it is the result of microbial activity and therefore an indicator factor for a working process. Nonetheless, temperature also helps microorganisms colonize the mass and increases the activity when the temperature is in the right range of 43-66 °C (Rynk, 1992). When temperatures become too high during the composting process, on the other hand, microorganisms can be destroyed. Here, aeration, turning or adding water can help to reduce the temperature (Epstein, 2019; Diaz and Savage, 2007a; Rynk, 1992). Another aspect is the temperature stratification within the compost pile. It is nearly impossible to attain the same temperature throughout the composting mass. In the center of each pile, the temperature is the highest, whereas the surface, on the other hand, is significantly cooler. Hence, turning of the composting mass is an important tool to mix particles from the cooler outside into the hotter center of each pile (Epstein, 2019; Insam and de Bertoldi, 2007).

The pH value is also a factor important for composting, but is also very difficult to describe. Since composting materials can differ greatly, both the microorganisms and the pH value and its course during the composting process can vary significantly. Composting is considered effective at pH levels between 5.5 and 9, being most effective near neutral (pH=7) (Diaz and Savage, 2007a). It is very uncommon that high or low pH values inhibit the composting process, though. However, if a compost material has a high N content, causing a high pH above 8.5, the N will turn to NH_3. This effect will add to the alkalinity, may inhibit the microorganisms and will also lead to the loss of valuable N in the compost (Diaz and Savage,

2007a; Rynk, 1992). During composting, in most cases, the pH falls during the initial phases of composting and then increases to a level between 6.5 and 7.5. However, as mentioned above, exceptions can be frequently observed (Epstein, 2019).

The particle size and structure of the material also affect microbial activity and therefore the composting process. On the one hand, smaller particles are easier for microorganisms to feed on; on the other hand, when particles are too small, this will lead to a very small air space between particles and therefore to a lack of oxygen, which is necessary for the microorganisms to function. Particle size can be adjusted by grinding, chopping or mixing in amendments (Epstein, 2019; Rynk, 1992).

Table 4. Conditions acceptable for composting, adapted from Rynk (1992)

Composting condition	**Acceptable**
C/N ratio	20:1-40:1
MC	40-65%
Oxygen concentration	> 5%
Particle size	Variable (< 3 cm)
Temperature	43-66 °C
pH	5.5-9.0

Composting can take place in many different forms, depending on the substrate, purpose, large-scale or small-scale, and industrial or backyard composting. However, there are two main groups that must be distinguished: windrow composting and in-vessel composting. In windrow systems, compost material is piled up to 1.5 to 2.5 m height and a width of 2.5 to 4 m with greater or less elongation. The piles should be conically formed in cross-section. To ensure the aeration of the windrows during composting, they can either be turned manually, for example, by a bucket loader or with a windrow turner, or they can be left static, with air forced in through pipes. The turned windrow system is the traditional system and ensures that every part of the composting material will reach the more active and hotter interior zone of each pile. Windrow composting can either take place unsheltered from the environment or under a roof or cover (Diaz et al., 2007b). In-vessel composting can be divided into vertical or horizontal bioreactors. Composting material is filled in bioreactors, mainly consisting of metal, of different sizes and shapes, many of them with a rotating drum. MC and oxygen are normally monitored, and water and air can be supplied if necessary (Diaz et al., 2007b). However, with animal manure, windrow composting is still the most common method. In general, farmers who use their manure themselves try to keep composting as simple as possible, normally using

either static or turned windrows. If farmers decide to produce a saleable product, they will have more interest in better management of composting and use more advanced technologies and monitoring systems (Cooperband, 2002).

2.3.3 Anaerobic digestion

Anaerobic digestion is a biological process wherein organic matter is decomposed by microorganisms in an environment without oxygen. During this fermentation process, biogas, mainly CH_4 and CO_2, is produced, and the remaining mass is called digestate. With the global energy need rapidly growing and the focus on renewable energies, biogas production from agricultural, industrial or municipal wastes and residues or energy crops (e.g., maize, grass, sugar beet) has been of growing interest for years (Bauer et al., 2010; Amon et al., 2007a; Amon et al., 2007b). Biogas can be used to generate electricity and heat and can also be a source of fuel. In addition, anaerobic digestion is also a way to handle sewage or animal manures and to gain two valuable products at the same time: saleable biogas and digestate as an improved fertilizer (Achinas et al., 2017; Weiland, 2010). The potential of anaerobic digestion to produce CH_4 was already discovered in the 19th century, and some single biogas plants were already operated in the mid-twentieth century, for example, in Germany. However, interest in this form of energy source was growing enormously with the energy crisis in the 1970s and the growing importance of renewable energies in the 1980s (Bond and Templeton, 2011). Since then, boosted by governmental financial support, Germany has been leading in terms of the number of biogas plants and the amount of biogas production for many years. Recently, other European countries, as well as China, have been catching up (Gustafsson et al., 2022; Pelkmans, 2021). Biogas production has increased over the last ten years by more than 90% and is expected to grow further (Abanades et al., 2021; Scarlat et al., 2018).

Many different types of organic feedstocks can be used as substrates for biogas production. The most commonly used feedstocks in Europe are animal manures and slurries, as well as sewage sludge from aerobic wastewater treatment plants, municipal waste, and food wastes. These feedstocks are often combined with so-called cosubstrates such as energy crops, harvest residues, and other organic wastes from agricultural industries, foods, or households to achieve a higher methane yield (Achinas et al., 2017; Weiland, 2010).

Anaerobic digestion is a complex process involving many different groups of microorganisms that are responsible for different steps during degradation. In general, four phases can be divided (Figure 3). The first phase is called hydrolysis. During this phase, hydrolytic microorganisms feed on complex polymers such as carbohydrates, proteins, and fats and

decompose these into easily degradable oligomers and monomers such as sugars, amino acids, and fatty acids. Then, in the second phase, called acidogenesis, sugars, amino acids, and fatty acids are converted into acetate, hydrogen (H_2), and VFAs, such as propionate and butyrate, by acidogenic bacteria. The VFAs are further converted into acetate, CO_2, and H_2 by acetogenic bacteria (acetogenesis). Acetate, CO_2, and H_2 are then the main substrates to produce biogas, which is the last phase, called methanogenesis, and is performed by methanogenic microorganisms (Theuerl et al., 2019; Achinas et al., 2017; Weiland, 2010). Impressively, the microbiome of each single fermenter is unique, with a large number of bacterial species that are still unknown and their ecological function and interactions unclear (Theuerl et al., 2019; Weiland, 2010).

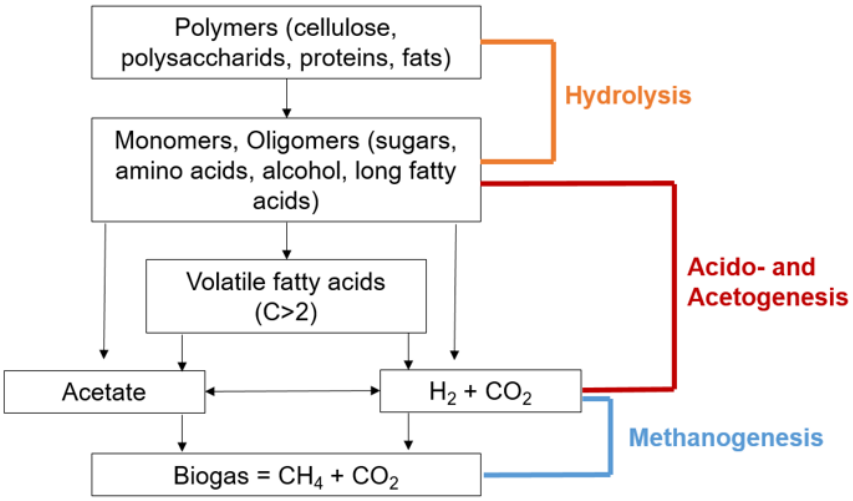

Figure 3. The stages of the biogas process, adapted from Theuerl et al. (2019) and Weiland (2010)
C = carbon, CH_4 = methane, CO_2 = carbon dioxide, H_2 = hydrogen

With all the different bacteria involved, anaerobic digestion is not only complex but is also a highly sensitive process. Every imbalance can lead to the inhibition of the microorganisms and therefore to a decrease in the biogas yield. Many different factors play a role in the biogas process: i) wrong or fluctuating temperature within the digesters, ii) variance in the substrate availability, iii) accumulation of inhibiting metabolites (e.g., H_2, ammonium/ammonia (NH_4^+/NH_3), VFAs), and iv) inhibition by other substances (e.g., heavy metals, antibiotics, disinfectants). In general, it is almost impossible to identify a single factor that is responsible for a disturbance (Theuerl et al., 2019).

Process temperature is one of the most important factors influencing microbial activity. The higher the temperature is, the faster the anaerobic digestion process. However, this is limited

due to the temperature range in which different microorganisms are active, and every change in temperature will lead to a shift in the microbial community and can lead to disturbances in biogas production (Theuerl et al., 2019). In the agricultural sector, anaerobic digestion is performed under mesophilic (35-42 °C) or thermophilic (45-60 °C) conditions (Theuerl et al., 2019; Weiland, 2010). In Germany, many digesters are operated at 40-45 °C, which is between the perfect range of either mesophilic or thermophilic microorganisms in order to ensure a temperature suitable for both mesophilic and thermophilic microorganisms (Theuerl et al., 2019). Temperatures should remain at a constant level or be changed only slowly to allow the adaptation of the microorganisms to the new conditions. Thermophilic anaerobic digestion is a less stable process and more susceptible to failure since the increased microbial activity leads to faster degradation of the biomass, which also leads to a higher risk for inhibition by released NH_4^+/NH_3, VFAs, or H_2S (Theuerl et al., 2019; Weiland, 2010).

The feedstocks used for biogas production are also crucial for a stable digestion process. The feedstocks supply the macronutrients (e.g., C) and micronutrients (e.g., P, sulfur, nickel, selenium) that are necessary for the growth and survival of the microorganisms in the digesters. The C/N ratio, for example, should range between 15:1 and 30:1 to avoid process disturbance by the accumulation of NH_4^+/NH_3 (Weiland, 2010). Additionally, the mixing of the tank content is necessary to allow contact between nutrients and microorganisms. Both the composition and the yield of biogas/CH_4 as well as the quality of the digestate depend on the feedstock used. When feedstocks with high N content are used, for example, chicken manure or grass silage, the risk of NH_4^+/NH_3 inhibition/toxicity is high. The equilibrium of NH_4^+ with NH_3 shifts to NH_3 with increasing temperature and pH (Theuerl et al., 2019). In general, it is not easy to define maximum NH_4^+/NH_3 values acceptable for a functioning anaerobic digestion process; however, ranges from 3 to 5 g/L and from 80 to 400 mg/L are considered acceptable for NH_4^+ and NH_3, respectively (Theuerl et al., 2019). If a disturbance of the process by NH_3 occurs, this leads to accumulation of VFAs in the process, which in turn leads to a decrease in pH. This at least helps to buffer the NH_3 effect. On the other hand, a decrease in pH below 6.0 due to high VFA accumulation leads again to inhibition, similar to a pH above 8.5. The pH range for optimal CH_4 formation lies between 7.0 and 8.0 (Weiland, 2010). Both VFAs and H_2 are key metabolites of the anaerobic digestion process and can also inhibit the process in high concentrations. VFAs are even more inhibiting in the undissociated form and therefore at low pH values. When, for example, the first phases of anaerobic digestion happen too fast, perhaps due to increased temperature or too easily degradable substrates, accumulation of VFAs, H_2, or NH_4^+/NH_3, or all together, can be observed, which will affect each other and will lead to disturbance of the process (Weiland, 2010).

There are different systems used for anaerobic digestion in the agricultural sector. The main process type used is wet fermentation, wherein the feedstocks have a total solid concentration of below 10%. Solid substrates, such as energy crops or chicken manure, are mixed with liquid manure, liquid digestate, or recycled process water to manifest pumpable slurries or are directly added to the liquid digester content. Only a very small number of biogas plants use the dry fermentation process with a dry matter between 15 and 35%. Wet fermentation always takes place as a continuous process with substrate being continuously added to the digesters and digestate being released. Dry fermentations are operated either continuously or as batch processes, wherein the substrate is added in portions to the digester, which contains the liquid digestate of a previous batch digestion, called inoculum. This is used mainly for the monofermentation of energy crops (Weiland, 2010).

There are various ways and concepts to design a wet fermentation biogas plant. The most typical reactor used is the vertical, continuously stirred tank fermenter. Stirring of the digester mass is necessary to allow contact between the microorganisms and the newly added substrate, to maintain constant temperature conditions, and to help the biogas reach the surface. Stirring is performed by pneumatic, mechanical, or hydraulic stirrers. Horizontal digesters with a horizontal paddle mixer are often used as the first-stage fermenter when a two-stage reactor biogas plant is used. Since operation at a higher dry matter is possible, two-stage reactor systems are preferred when energy crops, municipal or industrial organic wastes, or solid manure are used as feedstocks. Here, the second fermenter digests the digestate of the first fermenter (Weiland, 2010).

Two important factors in biogas management are the hydraulic retention time (HRT) and the organic loading rate (OLR). The HRT is the average amount of time that substrates remain in the digesters and differs greatly between feedstocks and biogas plants. Feedstocks with a high amount of easily degradable compounds decompose faster and therefore need a shorter HRT. In addition, anaerobic digestion at thermophilic temperatures also has a shorter HRT (Dobre et al., 2014; VDI, 2006). In Germany, for example, HRT can vary from 1.6 days to 140 days (Gemmeke et al., 2009). The OLR is defined as the daily amount of raw materials fed per unit volume of the digester capacity. An increase in OLR results in an increase in biogas yield. However, when the OLR is too high, process inhibition due to the accumulation of process metabolites, and therefore a decrease in biogas yield, can occur (Gautam et al., 2022).

As with composting, anaerobic digestion is a possible treatment option to reduce pathogens in the substrate (Vinnerås, 2013). Thermophilic temperature, high NH_3 content and retention time are considered important factors influencing the survival of pathogenic bacteria (Jiang et al.,

2020). The potential to reduce *E. coli* during anaerobic digestion will be further discussed in Chapter 2.4.

2.3.4 Chicken manure treatment challenges

Chicken manure, as referred to in the following work, is manure produced on fattening chicken farms. Here, minimal bedding material, such as wood pellets or straw pellets, is used at the beginning of one fattening period and is removed at the end of each fattening period (normally lasting four to six weeks). The low amount of bedding material and the characteristics of the chicken excrements with a high N content and a very low MC lead to the special characteristics of chicken manure compared to other animal manure types (Table 5). Consequently, the C/N ratio and the MC of chicken manure are low, of course varying from chicken farm to chicken farm and due to the bedding material and the amount used, as well as the food fed and the climate conditions inside and outside the barns. C/N ratios of 10:1 and MCs of only 30 to 20% can often be found (Siller et al., 2020; Wiesler et al., 2016; Wilkinson et al., 2011).

Table 5. Nutrient contents of chicken manure compared to other solid animal manure types (Topagrar, 2022)

Solid manure type	MC (%)	Total N[a)] (kg/t)	NH_4[b)] (kg/t)	P_2O_5[c)] (kg/t)	K_2O[d)] (kg/t)	MgO[e)] (kg/t)	CaO[f)] (kg/t)
Cow manure	77	5.6	-	2.9	9.6	1.7	-
Pig manure	77	7.4	-	6.5	7.4	2.7	-
Horse manure	68	4.9	-	3.2	9.8	1.9	-
Chicken manure	40	29.9	10.0	22.0	20.2	8.2	41.6

[a)] N = nitrogen, [b)] NH_4 = ammonium, [c)] P_2O_5 = phosphorus pentoxide, [d)] K_2O = potassium oxide, [e)] MgO = magnesium oxide, [f)] CaO = calcium oxide

In Germany alone, over 600,000 tonnes of chicken manure was applied to land in 2020 (Statistisches Bundesamt, 2021). Since chicken manure is a very valuable fertilizer due to its high N content, it is often used directly on fields or sold to others. If there is no direct need for it, it is mainly stored for some amount of time before it is applied to land (Statistisches Bundesamt, 2021). However, chicken manure can also be treated in compost piles or biogas plants. These treatment methods also support the reduction of pathogenic bacteria (Vinnerås, 2013), which is of great importance from a public health perspective. However, because of its characteristics, chicken manure is a challenging substrate both for composting and for anaerobic digestion. A C/N ratio between 20:1 and 40:1 and an MC between 40 and 65% are considered acceptable for good composting results (Rynk, 1992). For anaerobic digestion, the

C/N ratio should range between 15:1 and 30:1 to avoid process disturbance by the accumulation of NH_4^+/NH_3 (Weiland, 2010). Therefore, to common knowledge, chicken manure should be mixed with a carbon-rich amendment and water before being composted, and it is also easier to use chicken manure with cosubstrates in biogas digesters instead of performing monofermentation. In particular, thermophilic anaerobic digestion is challenging since the risk of NH_3 inhibition due to the shift from NH_4^+ to NH_3 rises significantly with increasing temperature (Bi et al., 2020; Theuerl et al., 2019). However, in recent years, interest in chicken manure as a substrate for biogas plants has been growing, and its potential has been recognized. Therefore, an increasing number of studies have focused on techniques to overcome the limitations of this nitrogen-rich substrate: for example, using stripping and membrane extraction to lower the NH_3 concentration in digesters (Bi et al., 2020; Fuchs et al., 2018; Nie et al., 2015).

2.4 Manure treatment methods and the survival of *E. coli*

Both composting and anaerobic digestion have the potential to reduce pathogens and other microorganisms during the process (Lima et al., 2020b). However, success is highly dependent on many factors: for example, the temperatures achieved during the process, the type of substrate used, the management of the process, the bacterial type present, and in terms of composting also many environmental influences (Chukwu et al., 2022; Chen and Jiang, 2014; Martens and Böhm, 2009). In addition, time also serves a relevant role in bacterial reduction. To express the extent of pathogen reduction over time in a substrate, the decimal reduction time (D-value) is commonly used. Hereby, the D-value describes the time needed for a 1 log reduction in bacteria.

During the composting process, temperatures often rise to levels of 60 °C and above. Since *E. coli* is not particularly heat resistant, composting leads to a reduction in *E. coli* when performed properly with temperature being the main inactivation factor (Chen and Jiang, 2014). In an on-farm experiment, for example, *E. coli* was reduced from 9 $\log_{10}$ to undetectable levels after seven days of bovine manure composting (Millner et al., 2014). Erickson et al. (2010) showed that also in static piles of chicken manure mixed with peanut hulls, *E. coli* were no longer detectable after two days of composting with temperatures above 50 °C. On the other hand, when temperatures remain lower, for example, due to environmental conditions, e.g., ambient temperature or rainfall, management problems or at surface locations, *E. coli* can survive for prolonged times. This was demonstrated, for example, by Berry et al. (2013), who found naturally occurring *E. coli* in top samples of turned bovine manure compost piles after 28 days, whereas in unturned piles, *E. coli* remained detectable in the top samples for up to

42 days. In addition, Siller et al. (2020) conducted a short-term chicken manure storage trial with ESBL-positive manure and could no longer quantitatively detect ESBL-producing *E. coli* in the pile centers after 36 hours and 72 hours in summer and winter, respectively. Qualitatively, ESBL-producing *E. coli* were detected until 72 hours in summer and until the end of the trial (96 h) in winter. Surface samples even remained qualitatively positive for ESBL-producing *E. coli* both in summer and winter throughout the trials.

As mentioned above (Section 2.3.2), the temperature of a compost pile is a process factor and is dependent on many other factors, including the C/N ratio, the MC, the substrate availability, and the oxygen level, which all influence the microbial activity and therefore the process temperature. In addition, the C/N ratio and MC as well as further chemical factors, such as pH level, VFA and NH_3/NH_4^+ concentration, microbial factors, such as microbial competition and the bacterial strain, and operational factors, such as sufficient turning of the composting mass and covering, might also directly influence the reduction in *E. coli* (Chen and Jiang, 2014; Erickson et al., 2014; Berry et al., 2013; Singh et al., 2011; Wilkinson et al., 2011) (Table 6). Erickson et al. (2014), for example, found faster reductions in *Salmonella* spp. when the C/N ratio was 20:1 compared to 30:1 or 40:1. Singh et al. (2011) observed longer survival times of *E. coli* in cow manure compost with a C/N ratio of 16:1 than in a mixture with a C/N ratio of 25:1. In addition, Wilkinson et al. (2011) demonstrated greater reductions in *E. coli* in poultry manure at 50 and 65% MC than at 30% MC during thermophilic conditions, whereas 30% MC led to greater reductions when held at 35 and 45 °C. The authors concluded that the sensitivity of bacteria to heat increased with increasing MC. Low MC, on the other hand, can lead to the desiccation of *E. coli* irrespective of temperature, and low MC values can also reduce the heat tolerance of *E. coli* (Hutchison et al., 2005; Himathongkham and Riemann, 1999). Furthermore, *E. coli* strains can differ in their tolerance to heat, MC, and further factors such as pH and NH_3 and VFA levels due to their high genotypic diversity (Jang et al., 2017). Xing et al. (2019), for example, found longer survival times for an *E. coli* O157:H7 strain without virulence genes than for two *E. coli* O157:H7 strains with virulence genes in soil. Topp et al. (2003), in addition, demonstrated that while one of two *E. coli* strains isolated from swine manure slurry decreased in manured soil after six days of incubation in the laboratory, the other *E. coli* strain increased during that time.

As mentioned above, process management also influences the temperatures achieved during composting and the reduction in *E. coli*. Covering piles can help increase temperatures and protect piles from major rainfall events that may lead to moisture penetration and, consequently, to anaerobic conditions with lower temperatures. Turning of the piles raises the oxygen level in the inner parts of the piles and accelerates the temperature increase. It also

helps to mix particles, including bacteria, into the hotter interior of the piles, making inactivation possible (Patel et al., 2015).

Table 6. Factors influencing pathogen survival during manure treatment

Factors	Anaerobic digestion	Composting
Physical factors	Temperature (adjusted), time	Temperature (result of process), time
Chemical factors	pH, NH_3, VFAs	C/N ratio, MC, pH, NH_3, VFAs, oxygen
Operational factors	Operational system (continuous/batch system), HRT, temperature, OLR, substrate type and composition (substrate availability), process disturbance, accumulation of metabolites	Compost covers, turning, substrate type and composition (substrate availability, particle size)
Microbial factors	Bacterial species and strain, heat resistance, tolerance to chemical factors, microbial competition	Bacterial species and strain, heat resistance, tolerance to desiccation, tolerance to chemical factors, microbial competition
Environmental factors		Rainfall, solar radiation, ambient temperature

The anaerobic digestion process also influences the survival of bacteria and has the ability to inactivate pathogens (Liu et al., 2021; Weiland, 2010; Martens and Böhm, 2009). In a field study by Chiapetta et al. (2019), generic *E. coli* showed declines of 99% during mesophilic anaerobic digestion of dairy manure as the main substrate. Manyi-Loh et al. (2014) demonstrated an *E. coli* reduction to below the detection limit within 62 days of mesophilic anaerobic digestion in a balloon-type digester fed with dairy manure. In addition, Qi et al. (2019) could no longer detect *E. coli* in the digestion tanks of two full-scale mesophilic biogas plants running on dairy manure, with HRTs of 40 and 30 days, after the digestion process. In two laboratory-scale studies, D-values for *E. coli* were determined during anaerobic digestion of cow manure: Pandey and Soupir (2011) found D-values of 7 to 8 days and below 1 day at 37 °C and 52.5 °C, whereas Watcharasukarn et al. (2009) demonstrated even lower D-values

of 22.2 hours at 37 °C and 3.3 min at 55 °C. However, the number of studies focusing on ESBL-producing *E. coli* and on chicken manure as feedstock type is scarce. Iwasaki et al. (2019) reported the complete elimination of ESBL-producing *E. coli* during thermophilic anaerobic digestion of cow manure. In addition, ESBL genes were significantly reduced during anaerobic digestion of wastewater with an HRT of 6 to 10 hours (Yi et al., 2015) and in dairy manure after mesophilic and thermophilic digestion (Tran et al., 2021). In the latter study, a decreased potential of horizontal gene transfer was also reported.

Temperature and retention time are considered the most important factors influencing the survival of bacteria during anaerobic digestion (Ma et al., 2022; Jiang et al., 2020). Therefore, the decline in *E. coli* is faster the higher temperature is. Pandey and Soupir (2011), for example, found a 6 log reduction of *E. coli* in dairy manure during laboratory-scale anaerobic digestion at 37 °C after 41 days of incubation, whereas at 52.5 °C, a 7 log reduction was achieved within 3.5 days.

However, there are also studies that report an insufficient reduction in *E. coli* during anaerobic digestion and regrowth of these bacteria during storage and handling of the digestate (Schauss et al., 2016; Fu et al., 2014; Larsen et al., 1994). In particular, moderate and mesophilic temperatures are seen critically, since 37 °C is the optimum growth temperature for *E. coli* (Lang and Smith, 2008; Smith et al., 2005). Hence, not only temperature and retention time can play a role in the inactivation and survival of *E. coli* during anaerobic digestion, but also additional operational, microbial and chemical factors, such as the OLR, substrate type and composition, substrate availability, microbial competition, bacterial strain type, pH, and accumulation of process metabolites, such as VFAs and NH_3/NH_4+ (Ma et al., 2022; Lorine et al., 2021; Jiang et al., 2020; Nag et al., 2019; Jang et al., 2017; Orzi et al., 2015; Weiland, 2010) (Table 6). Many studies agree that the best sanitization is achieved by high thermophilic temperatures and long retention times. In batch digesters, it is clearly easier to achieve long and controlled retention times, whereas in continuously fed digesters, there is always the risk of shortcuts, which is why retention times might be prolonged for a sufficient reduction in bacteria (Jiang et al., 2020).

2.5 Aim of own work

Although it is common knowledge that both composting and anaerobic digestion have the potential to reduce pathogens and *E. coli* in the original substrate, little is known about the potential of these treatment methods concerning chicken manure, which is a challenging substrate. In addition, *E. coli* strains can differ in their resistance to heat and other factors,

such as tolerance to desiccation, pH, NH_3, and VFA levels, due to their high variability and shared core genome of only 20 to 30% (Xing et al., 2019; Jang et al., 2017; Topp et al., 2003). To date, only few studies have focused on the survival of ESBL-/AmpC-producing *E. coli* in the environment. Furthermore, it remains unclear to what extent ESBL-/AmpC-producing *E. coli* can be reduced by chicken manure treatment methods, how much time an efficient reduction requires, and which factors are the main ones that influence the survival of these bacteria. This information is necessary to adjust the common treatment procedures for chicken manure and to define critical process parameters. Since chicken manure is a valuable fertilizer available in massive amounts and the manure that is most contaminated by ESBL-producing *E. coli*, further research is of great public health interest.

Thus, the overall aim of this work was to obtain a better insight into the survival and reduction kinetics of ESBL-/AmpC-producing *E. coli* during different treatments of highly contaminated chicken manure. Special attention was devoted to the factors influencing the survival of *E. coli* to define critical process parameters.

To investigate the influence of temperature on the reduction kinetics of ESBL-/AmpC-producing *E. coli* during anaerobic digestion of chicken manure, laboratory-scale anaerobic digestion tests were performed at 37, 42 and 55 °C. Additionally, the process-related influences of the substrate-specific factors pH, VFAs, and NH_3 on the reduction kinetics of ESBL-/AmpC-producing *E. coli* were investigated (Chapter 3.1).

In a second laboratory study, the effects of the C/N ratio and MC on the survival of ESBL-producing *E. coli* during chicken manure composting were investigated. In addition, the influences of the temperature and the substrate-specific factors pH, VFAs, and ammoniacal nitrogen (NH_4-N) on the survival of ESBL-producing *E. coli* were investigated (Chapter 3.2).

In a third study, composting of chicken manure was performed under pilot-scale conditions to investigate the survival of *E. coli* in chicken manure. The objective was to determine the influences of three different management treatments for chicken manure, composting in (i) static piles, (ii) static piles covered with a compost fleece, and (iii) periodically turned piles, on the survival of a nonresistant *E. coli* strain at subsurface and center locations during summer and winter (Chapter 3.3).

3 Own Research Publications in Scientific Journals

3.1 Inactivation of ESBL-/AmpC-producing *Escherichia coli* during mesophilic and thermophilic anaerobic digestion of chicken manure

Authors: Thomas, C.; Idler, C.; Ammon, C.; Herrmann, C.; Amon, Thomas

Year: 2019

Journal: Waste Management

Bibliographic source: Thomas, C., Idler, C., Ammon, C., Herrmann, C., Amon, T., 2019. Inactivation of ESBL-/AmpC-producing *Escherichia coli* during mesophilic and thermophilic anaerobic digestion of chicken manure. Waste management 84, 74-82. https://doi.org/10.1016/j.wasman.2018.11.028

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This article can be purchased online.

3.2 Effects of the C/N ratio and moisture content on the survival of ESBL-producing *Escherichia coli* during chicken manure composting

Authors: Thomas, C.; Idler, C.; Ammon, C.; Amon, Thomas

Year: 2020

Journal: Waste Management

Bibliographic source: Thomas, C., Idler, C., Ammon, C., Amon, T., 2020. Effects of the C/N ratio and moisture content on the survival of ESBL-producing *Escherichia coli* during chicken manure composting. Waste management 105, 110-118. https://doi.org/10.1016/j.wasman.2020.01.031

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3.3 Survival of *Escherichia coli* during pilot-scale chicken manure composting in summer and winter

Authors: Thomas, C.; Idler, C.; Ammon, C.; Amon, Thomas

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Survival of *Escherichia coli* during pilot-scale chicken manure composting in summer and winter

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ABSTRACT

The presence of *Escherichia (E.) coli* in chicken manure can potentially lead to serious infections and foodborne diseases in humans and animals when spread on land as organic fertilizer. Therefore, it is essential to inactivate these bacteria before land application. The aim of the present study was to determine the influences of three different management treatments for chicken manure on the survival of *E. coli*: composting in (i) static piles, (ii) static piles covered with a compost fleece, and (iii) periodically turned piles during different seasons. In a summer and winter trial, chicken manure piles, each with a length of 5.5 m, a width of 3 m and a height of 1.5 m, were stacked, and the samples were inoculated with a nonpathogenic *E. coli* strain at levels of 10^7 cfu/g and placed at subsurface and center locations of the piles.

Within 24 h, *E. coli* were undetectable by direct count in all piles and at all sample locations. By day 28, all samples were also negative for *E. coli* by enrichment. Temperatures within all piles mainly exceeded 50 °C within the first 24 h, and this factor was critical to the inactivation of *E. coli*. Statistical analyses showed that the sample location and the total hours at temperatures ≥ 50 °C and ≥ 55 °C in the piles had significant influences on the survival of *E. coli* in the chicken manure compost. The season and manure treatment method had no significant effects on the presence of *E. coli*.

Key words: chicken manure, composting, *Escherichia coli*, inactivation

1. INTRODUCTION

Escherichia (E.) coli are often found in farm animals, manure and farm surroundings. These bacteria can be a threat to human and animal health since *E. coli* strains have the potential to cause serious infections as seen during some large outbreaks of foodborne diseases in recent years (EFSA and ECDC., 2021). Furthermore, even antibiotic-resistant extended-spectrum β-lactamase (ESBL)-producing *E. coli* are found at a high prevalence, in particular on European broiler farms (Blaak et al., 2015). Thus, using potentially contaminated chicken manure as organic fertilizer has been critically discussed as a source of contamination for vegetables and fruit, especially since studies have shown the prolonged survival of *E. coli* in manure (Chen and Jiang, 2014).

Appropriate manure treatment methods are necessary to reduce these bacteria before manure is applied to land. Composting is known to reduce *E. coli* effectively when certain guidelines are followed (Erickson et al., 2010; Wilkinson et al., 2011; Chen and Jiang, 2014). Hereby, temperature is considered to be the main inactivation factor for *E. coli* together with a sufficient exposure time (Chen and Jiang, 2014).

However, achieving high temperatures during composting depends on further factors such as the carbon/nitrogen (C/N) ratio, moisture content (MC), substrate availability, microbial activity and oxygen level during the aerobic process (Thomas et al., 2020). Composting material should generally have a C/N ratio between 20:1 and 40:1 and an MC between 40 and 65% to achieve high microbial activity and thus high temperatures (Rynk, 1992). Although several studies have reported rapid reductions in pathogens during composting in turned and static piles, there are also numerous studies showing that bacteria can survive for prolonged periods in manure compost, especially at subsurface and surface locations and other areas of the piles where only lower temperatures were achieved or when winter composting was performed (Erickson et al., 2010; Berry et al., 2013). Therefore, covers on compost could help increase

temperatures in the outer area of compost piles and protect against major rainfall events; turning piles can accelerate the temperature increase through aeration of the piles and the bacterial inactivation by mixing particles from the outside into the hotter inside (Patel et al., 2015). However, it is a common procedure in chicken manure waste management to only store chicken manure in piles behind barns, in halls or silos until used as fertilizer on fields. In addition, chicken manure often has a C/N ratio lower than required for good composting results, and the MC is often below 40% (Wilkinson et al., 2011; Thomas et al., 2020).

Little is currently known about the respective influence of various treatment methods for chicken manure - especially under suboptimal composting conditions - on the survival of *E. coli* during different seasons. In addition, there is little evidence about the differences in temperature profiles during chicken manure composting with different methods, which is essential for the inactivation of *E. coli*.

Therefore, a better understanding of the survival of *E. coli* as well as the temperature development during different treatments of chicken manure under field conditions is essential to adjust and improve current composting procedures in terms of sanitization.

The objective of this study was to determine the influences of three different management treatments for chicken manure, composting in (i) static piles, (ii) static piles covered with a compost fleece, and (iii) periodically turned piles, on the survival of a nonpathogenic *E. coli* strain at subsurface and center locations during summer and winter.

2. MATERIALS AND METHODS

2.1. Study Design

Chicken manure was collected from a broiler farm in Germany on the day when the animals were removed. Both the summer and winter trials started one day later. On each starting day,

30 samples were collected from the chicken manure to determine the initial number of *E. coli*, the initial C/N ratio and the initial MC in the substrate.

Trials were conducted from July to August 2018 and from February to March 2019. For each trial, three compost piles, each with a length of 5.5 m, a width of 3 m and a height of 1.5 m, were stacked on even, impermeable ground at the test grounds in Potsdam, Germany.

A nonpathogenic *E. coli* strain (*E. coli* DSM 1116) was used as the test strain for inoculation of the compost mixtures. *E. coli* suspensions with concentrations of 10^9 cfu/mL were prepared as described previously (Thomas et al., 2020).

On each starting day, 10 kg of chicken manure was placed in a sanitized mixing bowl and sprayed with the *E. coli* suspension to obtain initial *E. coli* levels of 10^7 cfu/g in the mixture. After mixing by hand and 30 min of resting, five samples were taken for initial microbiological analyses. Then, 50 g samples of this mixture were added to bags that were permeable to water, gas and light. These bags were then placed in a larger bag of the same consistency with 200 g of original chicken manure for later chemical analyses. Then, the bags were placed in a firm net bag and attached to colored strings to enable identification of the samples. The sample bags were randomly arranged in the center of each chicken manure pile at a height of 0.75 m above the ground and 0.5 m from the surface and at 0.5 m height and only 0.15 m below the surface for the subsurface locations. For a period of 28 days, pile 1 was treated as a static pile, and pile 2 was additionally covered with a gas-permeable compost fleece (Toptex 200 g/m^2, TenCate Industrial Fabrics, Linz, Austria). Pile 3 was not covered but turned at days 2, 7, 14 and 21, and all subsurface samples were then placed in the center of the pile and vice versa.

Sampling was conducted on days 0, 1, 2, 4, 7, 14, 21 and 28. For each sampling day, three subsurface bags and three center bags of each pile were removed.

2.2. Microbiological and Chemical Analysis and Temperature Measurement

Samples for microbiological analyses were processed immediately; samples for chemical analyses were frozen at -25 °C and processed later. For microbiological analyses, 25 g samples were mixed in 225 mL Luria-Bertani (LB) broth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with a stomacher, and a serial dilution was performed in phosphate-buffered saline (PBS, pH= 7.3 to 7.5) (VWR International GmbH, Darmstadt, Germany). Each dilution was spread-plated on MacConkey No. 3 agar plates (Oxoid Deutschland GmbH, Wesel, Germany) and incubated for 24 h at 37 °C. In case no direct *E. coli* count was possible, 1:10 dilutions of each sample were incubated at 37 °C for 24 h, and a loop-full of this enrichment was streaked on MacConkey No. 3 agar plates to determine if the sample was still positive for *E. coli*. Characteristic *E. coli* colonies were randomly chosen throughout the experiment to confirm the species using MALDI-TOF-MS (AXIMA Confidence, Shimadzu Deutschland GmbH, Duisburg, Germany). In addition, on each sampling day, the MC of the samples and the C/N ratio were analyzed as described previously (Thomas et al., 2020). Inside each of the 18 sample bags from the last sample day, a temperature data logger (Tinytag Plus 2, TGP-4017, Gemini Data Loggers Ltd, Chichester, UK) measured the temperature every hour. In addition, weather data during the trials were obtained from the weather station at the test grounds.

2.3. Statistical Analyses

E. coli concentrations were converted to logarithmic values before statistical analysis. The value “99” was used for bacterial concentrations below the detection limit. The value “0” was used when the samples were negative by enrichment. Means and standard deviations of the data were determined. Either the MIXED or the GLIMMIX procedure of the SAS 9.4 software package (SAS Institute Inc., Cary, NC) was used for the analyses.

3. RESULTS AND DISCUSSION

3.1. Chemical Conditions within the Compost Piles

The initial C/N ratio of the chicken manure was 9.11 ± 0.36 and 11.35 ± 0.36, and the initial MC was 35.70 ± 1.95% and 26.01 ± 2.16% in summer and winter, respectively. In summer, the C/N ratios at the end of the experiment ranged from 8.14 to 8.90. The final MC ranged from 28.18% to 31.93%. In winter, the final C/N ratios ranged from 9.92 to 11.66, and the final MC ranged from 21.51 to 30.78% with the lowest C/N ratios and the highest MCs in the turned pile.

3.2. *Survival of E. coli during Composting of Chicken Manure*

E. coli numbers in the original chicken manure substrate used for preparation ranged from 0 to 10^3 cfu/g in summer and were approximately 10^3 cfu/g in winter.

Table 1 shows the numbers of *E. coli* during both composting trials. The mean initial levels of *E. coli* in the sample bags after spiking were 6.64 ± 0.12 cfu/g and 6.48 ± 0.26 cfu/g in summer and winter, respectively. Within 24 h, the number of *E. coli* at all sample locations was reduced significantly to below the detection limit (< 100 cfu/g) by direct count in both trials. In winter, however, *E. coli* were found again by direct count in one of the subsurface samples in the covered pile and in the turned pile on day 2. This result could be due to cold spots within the piles. By day 28 at the latest, all samples of both trials were also negative by enrichment.

Table 1

Detection of *E. coli* in subsurface and center samples and associated total hours (h_{total}) at a temperature ≥ 50 °C and ≥ 55 °C in static (pile 1), covered (pile 2) and turned (pile 3) piles of chicken manure composted in summer and winter

Day	Location	*E. coli* (log_{10} cfu/g [a] or positive/negative by enrichment, n=3)			h_{total} [b] ≥ 50 °C			≥ 55 °C		
		Pile 1	Pile 2	Pile 3	Pile 1	Pile 2	Pile 3	Pile 1	Pile 2	Pile 3
Summer										
0	Subsurface		6.64 ± 0.12		0	0	0	0	0	0
	Center				0	0	0	0	0	0
0.33 (8 h)	Subsurface		*Not tested*		1	0	0	0	0	0
	Center				0	0	0	0	0	0
1	Subsurface	+ - -	+ + -	+ - -	17	10	14	0	0	0
	Center	+ + -	+ + +	+ - -	12	13	10	0	0	0
2	Subsurface	+ + +	+ - -	+ + +	20	10	30	0	0	0
	Center	- - -	+ + -	+ + -	36	37	34	0	9	0
4	Subsurface	+ + -	+ - -	+ - -	58	49	63	11	0	10
	Center	- - -	+ - -	- - -	84	85	47	43	57	0
7	Subsurface	- - -	+ - -	- - -	130	121	135	83	0	74
	Center	- - -	- - -	- - -	156	157	119	115	129	58
14	Subsurface	- - -	+ + -	- - -	298	148	136	206	0	74
	Center	- - -	- - -	- - -	324	325	273	283	297	201
21	Subsurface	- - -	+ - -	- - -	390	148	296	206	0	122
	Center	- - -	- - -	- - -	492	493	425	451	417	339
28	Subsurface	- - -	- - -	- - -	390	148	297	206	0	122
	Center	- - -	- - -	- - -	660	591	566	509	417	463
Winter										
0	Subsurface		6.48 ± 0.26		0	0	0	0	0	0
	Center				0	0	0	0	0	0
0.33 (8 h)	Subsurface	+++	3.09 ± 1.88	2.95 ± 1.28	0	0	0	0	0	0
	Center	3.30 ± 1.03	3.05 ± 0.73	2.14 ± 0.25	0	0	0	0	0	0
1	Subsurface	+ - -	+ + -	+ + -	0	12	0	0	0	0
	Center	- - -	+ - -	- - -	10	4	9	4	0	2
2	Subsurface	- - -	3.34 + -	2.43 - -	0	19	0	0	0	0
	Center	+ - -	- - -	- - -	34	28	33	28	22	26
4	Subsurface	+ - -	- - -	- - -	0	19	1	0	0	1
	Center	- - -	- - -	- - -	82	76	63	76	70	45
7	Subsurface	- - -	- - -	+ + -	0	19	1	0	0	1
	Center	+ - -	- - -	- - -	154	148	135	97	142	117
14	Subsurface	- - -	+ - -	- - -	0	19	150	0	0	142
	Center	- - -	- - -	- - -	169	228	283	97	166	258
21	Subsurface	+ - -	- - -	- - -	0	19	263	0	0	221
	Center	- - -	- - -	- - -	169	228	434	97	166	407
28	Subsurface	- - -	- - -	- - -	0	19	383	0	0	317
	Center	- - -	- - -	- - -	169	228	581	97	166	549

[a] log_{10} cfu/g is the mean ± standard deviation

[b] total hours

These findings are in agreement with the results of previous laboratory experiments, where ESBL-producing *E. coli* were below the detection limit within two days when the C/N ratio of chicken manure compost was 10:1 (Thomas et al., 2020). This indicates that the results found in the laboratory environment are comparable to the results of the present field trials. In another field study, Erickson et al. (2010) also found no *E. coli* O157:H7 by direct count in subsurface samples of static chicken manure compost piles after two days, regardless of season. Wilkinson et al. (2011), on the other hand, found no significant change in *E. coli* numbers in the outer layers of both static and turned piles consisting of chicken manure over a 12 week trial period. This difference in results may be due to a higher MC because of rainfall and temperatures below 45 °C in the piles in this study.

Statistical analysis in the present study showed a significant influence of day and sample location on the reduction in *E. coli* during composting, whereas season and treatment method had no significant effect. Siller et al. (2020) also found a faster reduction in ESBL-producing and total *E. coli* for deep samples than for surface samples during a 96 h on farm storage of chicken manure in summer and winter. In addition, a seasonal influence was observed. However, in contrast to the results in Siller et al. (2020), no increase in *E. coli* on the surface of the piles in summer was found in the present study. This difference in results may be due to different environmental conditions during the experiments, as Siller et al. (2020) reported lower temperatures than those in the present study. However, the results of that study suggest that pile turning can help reduce the risk of survival and regrowth of bacteria in outer regions. Berry et al. (2013) also showed that in comparison to uncovered piles, covering cow manure compost piles results in faster *E. coli* O157:H7 reductions compared to uncovered piles. In the present study, however, no difference was seen between the covered and uncovered static piles and the turned pile due to the very fast reduction in *E. coli* in all three piles. The fast reductions in the subsurface areas may also have been due to desiccation, which also played a role during

laboratory experiments even though temperatures were not that high (Thomas et al., 2020). In addition, Thomas et al. (2020) also showed that a low C/N ratio can contribute to the reduction in *E. coli*, and this scenario might also be a reason for the fast reductions seen in the present study compared to those observed when composting other manure types.

Although the present study showed no significant benefit of turning or covering a pile, both treatment methods may become essential when the environmental conditions are different from those in the present study, e.g., more precipitation or lower temperatures during a treatment or a difference in the substrate, in the C/N ratio or in the microbial composition of the manure.

3.3. *Temperature Profile during Composting*

Fig. 1 shows the temperature profile within the compost piles, the ambient temperature and the precipitation during the trial periods. Temperatures within the piles increased rapidly within the first 24 h to above 50 °C in all pile locations in winter and summer, except for the subsurface location of the turned pile in winter. In both trials, in comparison to the subsurface areas, the center locations reached higher temperatures and maintained a longer high temperature phase in all piles with greater differences between both locations in the winter trial, likely due to the significantly lower ambient temperatures. This result is in agreement with those of Patel et al. (2015), who also found higher temperatures at internal loci than at interface loci of cow manure composts. In the summer trials, temperatures reached 70 °C and higher in all center locations, and the turned pile remained at temperatures above 60 °C until day 28, probably as a result of effective turning. In addition, the subsurface locations remained almost above 40 °C until the end of the trial. In winter, the highest temperatures were again reached in the turned pile in the center locations. In the uncovered and covered static piles, temperatures at both locations dropped faster during the winter trial than during the summer trial. However, throughout both

trials, the temperature at the subsurface and center locations remained above the ambient air temperature.

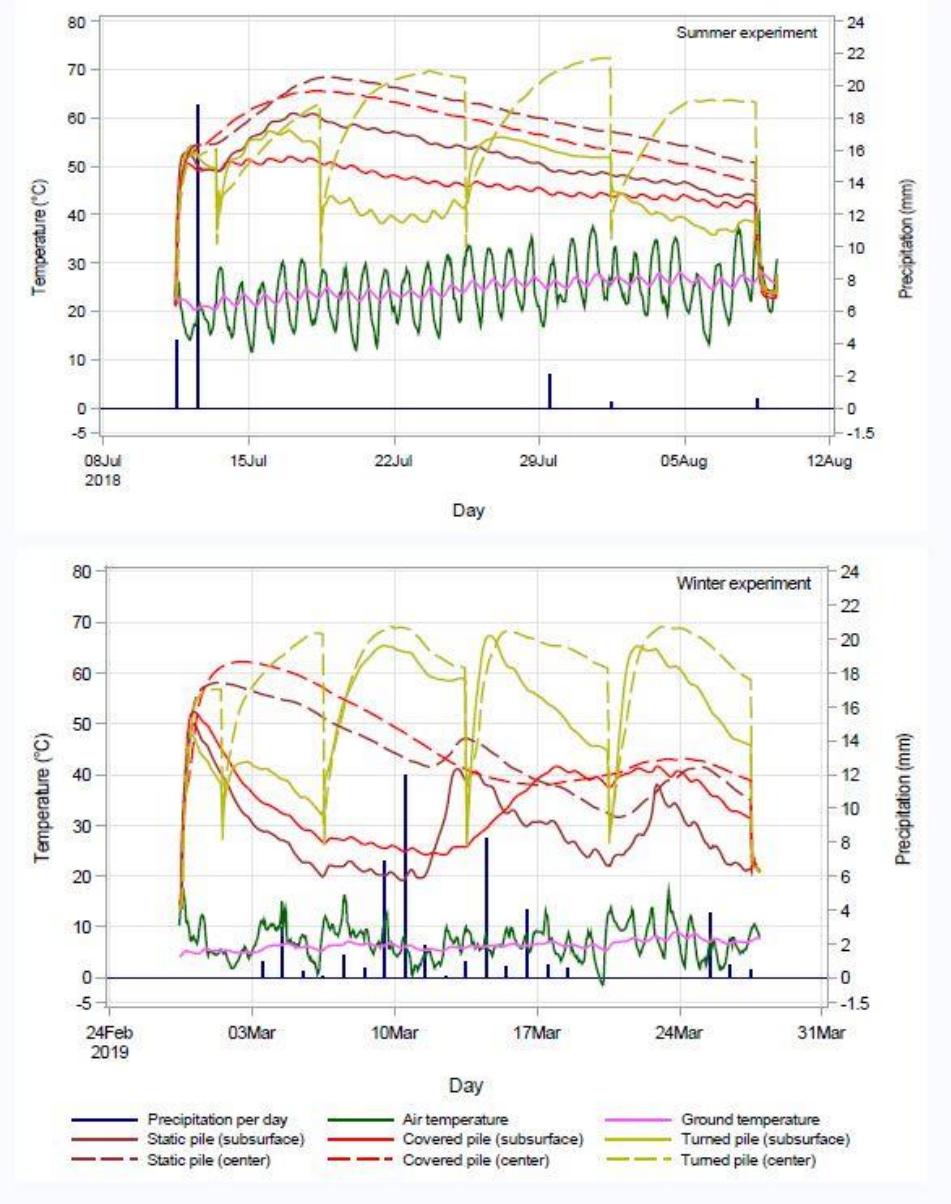

Fig. 1. Weather conditions and in-pile temperatures during composting of static, covered and turned chicken manure piles during summer and winter

The temperatures achieved in both trials were comparable to those measured in other field studies. Wilkinson et al. (2011), for example, observed temperatures above 65 °C in static and turned chicken manure windrows with consistently higher temperatures in the turned

windrow. Berry et al. (2013) also found higher temperatures in cow manure compost piles that were periodically turned than in static piles.

When comparing the reduction in *E. coli* with the pile temperature, especially the first 24 h, during which temperatures of 50 °C and more were reached, were critical to the inactivation of *E. coli* in the present study. Temperature is known to be a main inactivation factor, and laboratory experiments showed that temperatures above 50 °C were mainly sufficient to inactivate ESBL-producing *E. coli* in chicken manure substrate (Thomas et al., 2020). Therefore, Table 1 shows the total hours (h_{total}) at temperatures ≥ 50 and ≥ 55 °C at different locations in summer and winter. In summer, the highest h_{total} values were reached in the center locations of the uncovered, static pile with 660 and 509 h, respectively. In winter, the center locations of the turned pile showed the highest h_{total} with 581 and 549 h, respectively. Since 55 °C was mainly not reached before day 2 or 4 of the experiment, however, 50 °C seemed to be sufficient for inactivation of *E. coli* in chicken manure.

Chicken manure is a substrate with a typically low MC and C/N ratio, both of which are too low for optimal composting results (Rynk, 1992). However, as the present study showed, these characteristics still led to rapid and high-temperature development within the piles, even though composting management was not ideal. Since chicken manure is often stored behind barns or only minimally managed, it is important that temperatures still reach levels that inactivate bacteria such as *E. coli*.

Statistical analyses showed that season and sample location both significantly influenced h_{total} ≥ 50 and 55 °C. In summer and in the center locations, h_{total} ≥ 50 and 55 °C was higher than during winter and in the subsurface locations. In addition, both h_{total} ≥ 50 and 55 °C had a significant influence on the probability of finding *E. coli* in the sample. In winter, in comparison to the other two pile types, the turned pile also led to significantly more h at ≥ 50 and 55 °C. Considering the rather mild winter during the trial with the daily mean ambient

temperatures ranging from 3.4 to 10.6 °C, the differences in the results could be even more dominant when ambient temperatures are lower. Thus, turning might become essential to sufficient composting processes. Covering the pile had no positive effect on $h_{total} \geq 50$ and 55 °C in summer; in winter, however, covering led to an increased h_{total} in the center locations. Patel et al. (2015) also found an increased number of days at ≥ 55 °C when the windrows were covered with a 30 cm layer of finished compost.

The present study examined three different treatment methods on the survival of *E. coli* and the temperature profile within chicken manure compost piles under field conditions with natural weather conditions. Therefore, the study is a valuable addition to laboratory studies. The results are in agreement with those from a previous laboratory study (Thomas et al., 2020), showing even faster reductions in *E. coli* and higher temperature profiles. The results of the study also allow a better understanding of the temperature profile within chicken manure compost piles, which helps to evaluate the potential of composting in terms of sanitization for other bacterial groups.

In conclusion, composting chicken manure in static or turned piles effectively reduced the number of *E. coli* to below the detection limit within 24 h and to levels undetectable by enrichment within 28 days in summer and winter. Sample location and an $h_{total} \geq 50$ °C had a significant influence on the survival of *E. coli*, whereas the treatment method had no significant effect. However, to increase the likelihood that all parts of the compost are exposed to high temperatures, to accelerate temperatures and to achieve sufficient composting of the material, periodic turning should be considered, especially in winter. In addition, covering piles is a useful method to prevent odor and gas emissions and to protect piles against major rainfall events.

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4 Discussion

Coupled with the reported high prevalence of ESBL-/AmpC-producing *E. coli* in animal husbandry, the public concern rises that these resistant bacteria are spread to soils and food by contaminated manure used as fertilizer. In waste management, it is common knowledge that pathogens can be eliminated by very high temperatures, for example, during pasteurization at above 70 °C, steam sterilization at a temperature of 133 °C and a pressure of 3 bar, or also during incineration. For slaughterhouse wastes as so-called Category 3 material according to the Animal By-Products Regulation (EC 1069/2009), a pasteurization step ahead of use in biogas plants at 70 °C for 60 minutes is required. However, given the enormous amounts of manure, it is impossible to meet such requirements in routine manure management. Since such high temperatures are needed to sanitize waste against a variety of pathogens, including eggs of parasites and spore-forming bacteria and fungi, lower temperatures may be sufficient to eliminate heat-sensitive bacteria, such as *E. coli,* in manure. As *E. coli* strains also differ greatly in their resistance to heat and other factors, the present work aimed to obtain a better insight into the potential of common chicken manure treatment methods such as composting and anaerobic digestion to reduce ESBL-/AmpC-producing *E. coli* in the substrate. Hereby, a special focus was devoted to the possible roles of influencing physical and chemical factors such as temperature, C/N ratio, and MC, as well as on operational factors, such as turning or covering compost piles, and on environmental factors, such as season and rainfall events. In laboratory-scale studies, it was possible to determine the reduction kinetics of ESBL-/AmpC-producing *E. coli* strains both during anaerobic digestion and composting, and D-values were evaluated. In addition, the influences of temperature, the C/N ratio, and the MC on the survival of ESBL-/AmpC-producing *E. coli* were demonstrated. During a pilot-scale composting trial, the influences of season, covering, and turning could also be investigated. All investigated treatment methods showed strong effectiveness against ESBL-/AmpC-producing *E. coli*.

4.1 Choosing a laboratory setup for this work

In this work, we used two laboratory setups to determine the reduction kinetics in ESBL-/AmpC-producing *E. coli* during anaerobic digestion and composting of chicken manure. These setups were chosen for different reasons. On the one hand, laboratory setups provide an opportunity to evaluate distinct factors, such as different temperatures or different C/N ratios and MCs, in a controlled environment without the influence of additional environmental factors, such as ambient temperature, solar radiation, and precipitation, and without the influence of operational failures. On the other hand, laboratory setups are also more standardized. The

anaerobic digestion batch tests, for example, were performed according to VDI (2006). In addition, in the laboratory setup, it was possible to add the ESBL-/AmpC-producing *E. coli* strains at high concentrations of 7 log cfu/mL and cfu/g, respectively. This was important to understand the reduction kinetics of these bacteria during different treatments and to evaluate significant differences between treatments. As ESBL-/AmpC-producing *E. coli* belong to risk group 2 according to the German Biological Agents Ordinance (Biostoff-Verordnung, 2013) it is not permitted to add these bacteria to biomass during field trials.

However, laboratory-scale trials cannot fully simulate the complex biogas process in biogas plants or composting in large-sale composting facilities that operate with optimum control of operational factors, but also under the influences of further environmental and physical factors. In this sense, the results of laboratory trials must always be interpreted thoroughly and considered in context. It is likely that the reduction in ESBL-/AmpC-producing *E. coli* is even greater or faster in large-scale trials, since temperatures, microbial competition, and further environmental factors are certainly even more pronounced. It can also be concluded that differences in heat generation and *E. coli* reduction of different compost mixtures and differences in *E. coli* reductions of different anaerobic digestion temperatures are likely to be greater in large-scale systems. This assumption was confirmed by the results of the third study conducted at pilot-scale on the test grounds in Potsdam (Chapter 3.3). Here, the reduction in *E. coli* during composting was even faster than during the laboratory-scale composting trials, and the heat generation within the piles was greater.

However, in large-scale composting and anaerobic digestion systems it must also be considered that operational failures can lead to the survival of ESBL-/AmpC-producing *E. coli*, or at least to a lower reduction in *E. coli*. For example, cold spots within compost piles due to insufficient turning or at surface locations prolong *E. coli* survival. Major rainfall events can cause anaerobic spots within the compost mass that result in lower temperatures (Epstein, 2019). In biogas plants, shortcuts can lead to insufficient retention times, and process disturbance can occur during digestion, for example, by the accumulation of NH_3 (Weiland, 2010).

4.2 Reduction kinetics of ESBL-/AmpC-producing *E. coli* during composting and anaerobic digestion of chicken manure

To date, many studies have focused on the survival of different bacterial groups during composting and anaerobic digestion. Therefore, it had to be assumed that both treatment methods also have a negative effect on the survival of ESBL-/AmpC-producing *E. coli*.

However, information about the survival of ESBL-/AmpC-producing *E. coli* in chicken manure in general and about the reduction kinetics of these bacteria during different treatment methods is scarce. To produce a safe fertilizer for the fields, however, it is important to know the time required for the elimination of ESBL-/AmpC-producing *E. coli* in chicken manure. One aim of this work was therefore to define the reduction kinetics of two ESBL-/AmpC-producing *E. coli* strains during anaerobic digestion and composting of chicken manure in the laboratory. The setup of both laboratory trials, including the use of the same ESBL-producing *E. coli* strain as the test strain and chicken manure from the same broiler farm, also allowed comparison of the two different treatment methods. Furthermore, we were able to generate D-values, which facilitate the comparison to other studies focusing on different treatment methods, different manure types, or different bacteria and bacterial strains. In the studies, D-values were also predicted for a temperature range between 37 and 55 °C during anaerobic digestion and for compost mixtures with C/N ratios between 10:1 and 40:1 and MCs between 20% and 60%. These results may help operators of biogas or composting plants to define individual retention times or ideal compost mixtures to reduce ESBL-/AmpC-producing *E. coli* to undetectable levels.

During the anaerobic digestion trials, the D-values ranged from 3 to 6 days at 37 °C, 1.5 days at 42 °C, and up to 48 min at 55 °C. After 35 days of anaerobic digestion, the initial *E. coli* counts of 7 log cfu/mL were below the detection limit at all temperatures; however, at 37 and 42 °C, ESBL-/AmpC-producing *E. coli* were still partially detectable by enrichment (Thomas et al., 2019). The reduction kinetics showed a strong dependency on temperature, the decline being faster the higher the temperature was. This is in agreement with studies by Watcharasukarn et al. (2009) and Pandey and Soupir (2011) and is explained by the low heat resistance of *E. coli* (Bell and Kyriakides, 1998). D-values for the reduction in *E. coli* varied in comparison to different studies, most likely due to the use of different substrates and different *E. coli* strains. For example, in a study by Pandey and Soupir (2011), the D-values for *E. coli* during anaerobic digestion of dairy manure at 37 and 52.5 °C were similar to the D-values of the present work at 7 to 8 days and below 1 day, respectively. Watcharasukarn et al. (2009), on the other hand, found notably lower D-values for *E. coli* of 22.2 hours at 37 °C and 3.3 min at 55 °C in cow manure. In addition, Russell et al. (2020) found lower D-values for *E. coli* O 157 during anaerobic incubation at 37 °C. Here, depending on the formulation food waste, bovine slurry, and mixtures of bovine slurry or grease trap waste, the D-values ranged from 1.5 to 2.8 days. However, both Watcharasukarn et al. (2009) and Russell et al. (2020) used only small amounts of test substrate (2 mL and 30 mL, respectively) instead of the 1.5 L used in the test series of the present work. Since anaerobic digestion is a complex system with many factors involved in the process and also in bacterial reduction, on such a small scale, a proper

anaerobic digestion process seems unlikely. In addition, the different substrates used in the compared studies also represent an explanation for the discrepancy in results.

The laboratory test series was conducted as batch tests. However, biogas plants mostly operate on a continuous or semicontinuous scale. Here, the feeding times per day and the HRT may be important factors influencing the risk of finding ESBL-/AmpC-producing *E. coli* in the output. Batch operation of biogas plants is therefore probably the safest way to eliminate ESBL-producing *E. coli* in the digestate. In a review by Jiang et al. (2020), for example, the survival times of *E. coli* were prolonged in continuous experiments, and also under thermophilic conditions, when compared to batch experiments. Reasons for this may be the continuous introduction of new *E. coli* to the digester and particles that do not truly meet the HRT due to shortcuts. These might also explain the finding of ESBL-producing *E. coli* in biogas effluents by Schauss et al. (2015), although the number was greatly reduced. However, field studies have also shown the great potential of continuous biogas plants to eliminate ESBL-producing *E. coli*. Iwasaki et al. (2019), for example, demonstrated that ESBL-producing *E. coli* was eliminated during thermophilic anaerobic digestion of dairy manure at 55 °C. Although the dairy manure substrate was contaminated with ESBL-producing *E. coli* at 10^4 cfu/g, no ESBL-producing *E. coli* were found in the digestate after anaerobic digestion at an HRT of 15 days.

In Germany, the HRT ranges from 1.6 to 140 days (Gemmeke et al., 2009), and most biogas plants run at mesophilic temperatures (Weiland, 2010). As the results of the present work show, longer HRTs and therefore fewer feeding times per day are recommended to eliminate ESBL-/AmpC-producing *E. coli* during mesophilic anaerobic digestion, depending on the initial *E. coli* level of the introduced substrate. At thermophilic temperatures, short HRTs seem sufficient for the elimination of *E. coli*. Batch fermenters are uncommon but would also help to reduce the risk of finding ESBL-/AmpC-producing *E. coli* in the output of biogas plants, especially at mesophilic temperatures. Clearly, operating biogas plants at high temperatures above 50 °C is the safest way to inactivate ESBL-/AmpC-producing *E. coli*. However, the thermophilic anaerobic digestion of chicken manure remains challenging because of its high N content and the resulting shift from NH_4+ to NH_3 during anaerobic digestion with increasing temperature (Bi et al., 2020; Niu et al., 2015). In recent years, new technologies to handle the challenging substrate chicken manure in anaerobic digestion have emerged and will hopefully lead to solutions for stable performance during thermophilic anaerobic digestion of chicken manure (Fuchs et al., 2018).

During composting of different chicken manure mixtures in the laboratory, the presented work demonstrated D-values in a range from 0.27 days to 4.82 days for ESBL-producing *E. coli*

(Thomas et al., 2020). Hutchison et al. (2004), for example, found a D-value of 1.38 days for *E. coli* O157 when composting laying hens' litter with an MC of 40% in a 5 m^3 heap. In a laboratory study with cow manure compost with an MC of 38%, D-values for *E. coli* O157:H7 were even lower: 135 min, 35.4 min, and 3.9 min when incubated at 50, 55, and 60 °C, respectively (Jiang et al., 2003). The difference in results between the present study and the latter one is mainly explained by the fixed temperatures from the beginning of the experiment in the study by Jiang et al. (2003) and by the use of different substrates and different *E. coli* strains. In the present work, typical chicken manure mixtures with a low C/N ratio of 10:1 showed the fastest reductions in ESBL-producing *E. coli*, reaching the detection limit of 100 cfu/g within two days and being undetectable by enrichment within four days. In these mixtures, maximum temperatures of 54.3 to 66.4 °C were measured. By day ten at the latest, no ESBL-producing *E. coli* was detectable by enrichment in any mixtures except those with a C/N ratio of 40:1 and the mixture with a C/N ratio of 20:1 and 20% MC. These results are in agreement with a study by Wilkinson et al. (2011), who found significant reductions in *E. coli* in chicken manure compost mixtures at 35, 45, 55, and 65 °C in laboratory trials. In all treatments, *E. coli* was reduced by 2 $\log_{10}$ after eight hours; at 55 and 65 °C, *E. coli* was reduced by 2 $\log_{10}$ even after one hour.

In the pilot-scale composting trials, it was shown that the results were not only comparable to those found in the laboratory environment, but that field conditions even led to faster reductions and undetectable numbers of *E. coli* within 24 hours by direct count and levels undetectable by enrichment within 28 days in summer and winter. Here, especially the higher temperatures within the composting mass seem responsible for the faster decay in *E. coli*. The importance of temperature was also shown by the faster reductions in *E. coli* in the center locations of the compost piles compared to the subsurface locations. Siller et al. (2020) conducted a short-term storage trial with chicken manure and tested for ESBL-producing *E. coli* naturally occurring in the manure. The authors also found limited detection times for ESBL-producing *E. coli* of only 36 hours in summer and 72 hours in winter. However, prolonged survival times were again found at surface locations and at low ambient temperatures, which is in agreement with the results of the present study.

When comparing both the composting and anaerobic digestion trials, it must be mentioned that both treatment methods led to significant reductions in ESBL-producing *E. coli* in chicken manure. In both treatments, the extent of this reduction was mainly dependent on the process temperatures and dependent to a lesser extent on further influencing chemical, operational, and microbial factors, which is discussed further in the Sections 4.3.2 and 4.3.4. Thermophilic anaerobic digestion is to be rated as sufficient, as is the composting of chicken manure in its

typical characteristic form with a low C/N ratio, both of which result in D-values of 0.02 days and 0.27 to 0.6 days, respectively.

One advantage of composting is that it is easier to perform by small producers and farms without biogas plants. Even unmanaged chicken manure compost heaps lead to temperatures higher than those during thermophilic anaerobic digestion. No further addition of straw or water is necessary to produce a safe fertilizer. Nevertheless, the periodic turning to allow an aerobic process and to avoid cold spots within and at the surface of compost heaps is a sensible measure. Anaerobic digestion, especially at thermophilic temperatures, is a sensitive process that is susceptible to disturbance and necessitates proper management. In particular, nitrogen-rich materials such as chicken manure can challenge a thermophilic anaerobic digestion system. One advantage of anaerobic digestion, on the other hand, is the easier control of critical process parameters: once the system runs without disturbance, with a set HRT, a defined quantity and quality of the feedstock and at a defined temperature, there are no further external influences, especially environmental influences, to be feared, since the anaerobic digestion process takes place in a closed system. Temperature is hereby set to a certain level, and all particles, including bacteria, are exposed to that temperature. This is an advantage, since temperature was found to be the most important inactivation factor for ESBL-/AmpC-producing *E. coli*. During composting, on the other hand, temperature is the result of many other factors, including the chemical (C/N ratio and MC) and microbial composition of the substrate, the environmental conditions, such as ambient temperature, and rainfall, and operational factors, such as turning and covering. Here, the temperature varies with each composting process depending on environmental influences and cannot be set to a specific level. However, the composting studies of the present work showed that temperatures in chicken manure compost will almost certainly reach levels above 50 °C, even in suboptimal mixtures. Nevertheless, temperature differences can occur within the compost mass, for example, at surface locations, and therefore make it advisable to turn the compost mass to mix the outer particles into the hotter inside. In addition, a reduction in ESBL-/AmpC-producing *E. coli* not only to below the detection limit, but also to undetectable levels by enrichment, should be aimed at in all cases, independent of treatment method, since there are studies showing the potential of regrowth of *E. coli* during the storage of biogas effluents and the storage of compost (Kim and Jiang, 2010; Larsen et al., 1994).

In the studies of the present work, the focus was on cultivable ESBL-/AmpC-producing *E. coli* and strains with analyzed resistance genes were used. This approach helped to define reduction kinetics and factors influencing the survival of these bacteria. Nonetheless, the presence or absence of antibiotic resistance genes (ARGs) in biogas digestate or compost is

also discussed in the literature, with varying conclusions. Overall, studies mostly demonstrate a decrease in ARGs but no complete elimination. Yi et al. (2015), for example, could no longer detect CTX-M genes in digested sludge after wastewater treatment, whereas oxacillinase (OXA), SHV and TEM genes were still detectable. Tran et al. (2021) additionally noticed a decrease in horizontal gene transfer of ESBL genes by mesophilic/thermophilic anaerobic digestion of dairy manure. Resende et al. (2014) found a reduction in TEM-1 genes after 60 days of mesophilic anaerobic digestion of dairy manure. Similar results were observed during composting experiments. Riaz et al. (2020), for example, demonstrated a decline in CTX-M genes in mature compost after industrial composting. Interestingly, Qian et al. (2018) additionally found a greater effect of ARG reduction in chicken manure compared to bovine and pig manure after industrial composting, mentioning mobile genetic elements, heavy metals, and total N as possible reasons for these differences among manure types. The results of all of these studies show that ESBL genes can also be effectively reduced during composting and anaerobic digestion. However, the persistence of some ARGs seems likely.

4.3 Factors influencing the reduction in ESBL-/AmpC-producing *E. coli* during chicken manure treatment

4.3.1 Temperature and time

Temperature and time are known to be important factors in regard to the survival of bacteria (Jiang et al., 2020; Pandey and Soupir, 2011; Wilkinson et al., 2011; Guan et al., 2007). *E. coli*, in particular, is relatively sensitive to heat, with an optimum growth temperature between 35 and 40 °C and a maximum limit of 44 to 46 °C (Bell and Kyriakides, 1998). However, even when a certain temperature is known to inactivate a bacterium, the time needed for the bacterium to be eliminated from the substrate is crucial for a safe final product. This time also depends on the initial level of the bacterium in the substrate. Time is also a relevant factor when temperatures are low, since many studies report a decrease in bacteria even at low temperature, for example, in slurry storage tanks, due to a decrease in nutrient availability and further influencing chemical and environmental factors (Vinnerås, 2013). Therefore, time – temperature guidelines are often established when it comes to the sanitization of products. The United States Environmental Protection Agency (USEPA), for example, has developed time – temperature regulations that require that all parts of a compost pile should maintain temperatures of 55 °C for three consecutive days to produce a pathogen-free end-product (Boczek et al., 2023). However, a review by Wichuk and McCartney (2007) concluded that these regulations may not be sufficient to ensure pathogen inactivation. Thus, the factors temperature and time were further investigated during the present work, and the reduction in

ESBL-/AmpC-producing *E. coli* over time was described both for anaerobic digestion of chicken manure and for chicken manure composting.

During the anaerobic digestion trials, it was shown that the higher the fermentation temperature, the faster was the decay in *E. coli*. The calculated D-values demonstrated a clear increase in the inactivation rate with increasing temperature. This is in agreement with a study by Smith et al. (2005), who investigated the survival of *E. coli* in sludge at 35, 55, and 70 °C. Pandey and Soupir (2011) also demonstrated D-values of 9 to 10 days, 7 to 8 days, and less than 1 day for *E. coli* during batch anaerobic digestion experiments with dairy manure feedstocks at 25, 37, and 52.5 °C, respectively. Since *E. coli* has an optimum growth temperature of 37 °C, it seems surprising that during anaerobic digestion at 37 °C, *E. coli* is not only not growing but even decaying. Here, temperature is unlikely to be the main inactivation factor. In addition, the anaerobic conditions themselves are also unlikely to be a reason for the decay in *E. coli*, since *E. coli* is a facultative anaerobic bacterium. Wagner et al. (2009), Semenov et al. (2011), and Pandey et al. (2015) even reported prolonged survival times for *E. coli* when comparing anaerobic to aerobic treatments. The decay in ESBL-/AmpC-producing *E. coli* at 37 °C is therefore more likely due to substrate limitations, microbial competition, and chemical composition (Lang and Smith, 2008; Smith et al., 2005). In addition, the study showed that the two chosen *E. coli* strains showed significantly different reduction kinetics at 37 °C, with D-values of 6.16 and 3.66 for the CTX-M15-strain and the CMY-2-strain, respectively. Here, the different adaptability of the two strains to chemical factors seems to be a likely explanation.

Although temperature was certainly not the main inactivation factor at 37 °C, the test series at 37 °C can serve as a control, the test series at 42 and 55 °C can be compared to since the substrate, strains, and experimental setup were generally the same in all three test series. Since temperature was the only factor that was changed, it clearly exerted a significant influence on the reduction kinetics of both *E. coli* strains at 42 and 55 °C. Temperature, however, not only directly influences *E. coli* survival but also influences the anaerobic digestion process in general, resulting in varying process-related chemical and microbial conditions, e.g., differences in pH, VFAs, and NH_3 concentrations and microbial competition, within digesters. These changes within the reactors might also add to the factors influencing ESBL-/AmpC-producing *E. coli* survival during the biogas process. A suboptimal chemical or microbial environment can therefore additionally stress *E. coli* and can reduce its heat tolerance. This was also discussed by Smith et al. (2005), who demonstrated longer survival times for *E. coli* in nutrient broth compared to sludge. This fact also indicates that different manure types will lead to different results in regard to bacterial survival during anaerobic digestion. In addition,

the results of the anaerobic digestion tests also indicated that the higher the process temperature was, the less important were other factors, such as substrate source or *E. coli* strain, as the reduction was fast and effective because of the high temperature.

The conducted composting trials, both in the laboratory and at the pilot-scale, also showed the significant influence of temperature on the reduction kinetics of *E. coli*. In the laboratory trials, temperatures above 50 °C were reached in most of the tested mixtures. Similar results were observed in a study by Guan et al. (2007), who showed that temperatures above 50 °C in chicken manure led to the elimination of *E. coli* and mobile plasmids carrying ARGs, whereas at 23 °C, *E. coli* survived for at least 27 days. In another study by Wilkinson et al. (2011), a faster reduction in *E. coli* in chicken manure at high temperatures compared to lower temperatures was also demonstrated; however, 65 °C was not more effective than 55 °C. As expected, temperatures in the pilot-scale composting trials rose faster and to higher levels than in the laboratory. Within 24 hours, the temperatures in the piles reached 50 °C and above in most locations during summer and winter and reached temperatures above 60 or even 70 °C in the following days. Since *E. coli* were mainly no longer detectable after 24 hours in direct count, irrespective of the treatment method, 50 °C seems to be a sufficient temperature to effectively reduce *E. coli* in chicken manure.

In the literature, temperature is often discussed as the main inactivation factor for bacteria (Jiang et al., 2020; Pandey and Soupir, 2011; Wilkinson et al., 2011), which is in agreement with the results of the three conducted studies. Depending on the temperature, a different amount of time is needed to reach the detection limit of ESBL-/AmpC-producing *E. coli* during anaerobic digestion or composting. Therefore, it is necessary to allow sufficient time depending on the process temperature to gain an ESBL-producing *E. coli*-free product. Time–temperature guidelines seem to be a useful tool to achieve a safe chicken manure product after both anaerobic digestion and composting. However, it must be considered that temperature is not the only factor influencing the survival of *E. coli*. Especially at mesophilic temperatures, complex interactions of different factors add to the survival of these bacteria. The heat resistance of different *E. coli* strains can therefore vary depending on the substrate used, the chemical composition, or the microbial competition, making simple time–temperature regulation nearly impossible, unless temperatures are very high, as found, for example, in a pasteurization process with temperatures of 70 °C and above. In addition, when using substrates or strains other than those in the present work, a difference in D-values must be expected, especially at mesophilic temperatures. The results of the present work, however, suggest that temperatures of 55 °C and above will most certainly lead to a rapid and satisfying

reduction in ESBL-/AmpC-producing *E. coli* in chicken manure during anaerobic digestion and composting, with further factors playing only a subordinate role.

4.3.2 C/N ratio and MC

In regard to composting, the C/N ratio and MC are important factors influencing the success of the composting process. Both factors affect the microbial activity within the compost mass and, consequently, the temperatures reached (Section 2.3.2). Since temperature is an important factor influencing the survival of *E. coli*, it is worth considering the effects of the C/N ratio and the MC on the survival of ESBL-producing *E. coli* as well. In general, compost guidelines advise a C/N ratio between 20:1 and 40:1 and an MC between 40 and 65% for good composting results with high microbial activity and high temperatures (Epstein, 2019; Rynk, 1992). In addition, previous studies showed that different C/N ratios can have a direct influence on the reduction in enterobacteria. Chicken manure compost mixtures with a C/N ratio of only 20:1, for example, reduced *Salmonella* spp. faster than mixtures with C/N ratios of 30:1 or 40:1 (Erickson et al., 2014). Singh et al. (2012), on the other hand, demonstrated a faster reduction in *Salmonella* spp. in chicken manure compost mixtures with an MC of 50% than with an MC of 40% during thermophilic composting. Knowledge about the effects of the C/N ratio and MC on the survival of ESBL-producing *E. coli*, however, is scarce. In addition, chicken manure from broiler farms often exhibits lower C/N ratios and MCs than considered sufficient for composting (Siller et al., 2020; Wiesler et al., 2016; Wilkinson et al., 2011). While large composting facilities adjust the C/N ratio and the MC by adding a carbon-rich source, such as straw and water, chicken manure is often stored in static compost piles or is only minimally managed behind producers' barns until land application is possible. Therefore, in the laboratory composting study, we also included suboptimal chicken manure compost mixtures to gain further insights into the reduction kinetics of ESBL-producing *E. coli* during chicken manure composting under ideal and suboptimal composting conditions. Interestingly, the study demonstrated the fastest decays in ESBL-producing *E. coli* in all mixtures with a C/N ratio of 10:1 with D-values of less than one day. This is in agreement with Erickson et al. (2014), who found a faster reduction in *Salmonella* spp. when chicken manure mixtures with a C/N ratio of 20:1 were used than in mixtures with a C/N ratio of 30:1 or 40:1. In that study, the authors discussed the possible effects of VFA and NH_3 concentrations on the reduction in *Salmonella* spp. since the amounts of VFAs and NH_3 were significantly higher in the mixtures with a C/N ratio of 20:1 than in the other two mixture types. Both VFAs and NH_3 are known for their antimicrobial potential (Singh et al., 2012; Erickson et al., 2009a, b; Himathongkham et al., 1999). In the laboratory composting study, however, the mixtures with a C/N ratio of 10:1 did not have the highest VFA concentrations, and no direct correlation between VFA concentrations and *E. coli* reduction

could be ascertained. The NH_4-N level, on the other hand, was clearly higher when the C/N ratio was lower, indicating that this might also have a bactericidal effect on ESBL-producing *E. coli*. In addition, the C/N ratio not only had significant effects on the reduction kinetics of ESBL-producing *E. coli* and on the content of NH_4-N, but also on the pH value and on the temperature development within the bioreactors. Hereby, mixtures with a C/N ratio of 10:1 on average led to higher temperatures than mixtures with a C/N ratio of 20:1 or 40:1 at each MC, which is also an explanation for the fastest decays in ESBL-producing *E. coli* in these mixtures. However, this was surprising, since a C/N ratio below 20:1 is not considered optimal for composting, and Erickson et al. (2014), for example, found no difference in heat generation during laboratory composting of chicken manure with C/N ratios of 20:1, 30:1, and 40:1 and an MC of 60%. Another study by Erickson et al. (2009a) even demonstrated a lower temperature increase and less heat generation for cow manure mixtures with a C/N ratio of 20:1 than for mixtures with a C/N ratio of 40:1. However, different manure types result in different substrate characteristics and in different substrate availability and can therefore lead to differences in microbial activity during composting. The conducted pilot-scale composting trials in summer and winter confirmed that chicken manure compost with a C/N ratio of 10:1 led to a strong and fast temperature rise to temperatures above 60 °C within the compost mass, although the C/N ratio was not considered acceptable for composting.

In the laboratory composting trials, we also demonstrated the significant influences of the MC on the reduction kinetics of ESBL-producing *E. coli* and on the temperature development within the bioreactors. Dry mixtures with an MC of 20% and a C/N ratio of either 10:1 or 40:1 resulted in similar or even lower D-values than the moist mixtures did, despite lower maximum temperatures within the bioreactors. Compost mixtures should have an MC between 40 and 65% to provide good composting results with high microbial activity (Rynk, 1992). This explains why in the laboratory tests, compost mixtures with an MC of only 20% led to lower maximum temperatures than mixtures with an MC of 40 or 60% when considering one C/N group, since microbial activity is reduced under dry conditions. Therefore, temperature is unlikely to be the main inactivation factor for mixtures with an MC of 20%. Himathongkham and Riemann (1999), as well as Hutchison et al. (2005), discussed desiccation as a further inactivation factor for *E. coli*, which was assumed to be the reason for the rapid decay in *E. coli* in the investigated mixtures with 20% MC in the present study. Wilkinson et al. (2011) also found that *E. coli* decayed faster in mixtures with an MC of 30% than in mixtures with an MC of 65% when incubating chicken manure at 35 °C, concluding that desiccation plays an important role in pathogen inactivation at low MC. In addition, Kim and Jiang (2010) demonstrated the sensitivity of *E. coli* to MC. In that study, the growth potential of *E. coli* was significantly higher in dairy compost with an MC above 40% compared to an MC below 30%, where no growth occurred.

The results of the laboratory composting study therefore indicate that an MC in the range of 40 to 65% will probably not directly affect the survival of ESBL-producing *E. coli* during composting of chicken manure. However, MC has a direct effect on temperature development, which in turn is the main inactivation factor for *E. coli* during composting. In addition, the study showed that a low MC may also directly affect the survival of *E. coli* due to desiccation. This knowledge can be of crucial importance for i) compost materials such as chicken manure that originally had a very low MC and therefore probably a low microbial activity and for ii) composting processes or parts of composting masses that only achieve low temperatures, for example, during winter or at surface locations. Here, a low MC can help to reduce ESBL-producing *E. coli* in the compost mass nonetheless.

4.3.3 Season, turning and compost covers

Composting is a process that is influenced not only by the different composting substrates, and therefore different chemical and microbial compositions, but also by environmental factors such as ambient temperatures and rainfall events and by operational factors such as periodic turning of the compost mass, forced aeration, and covering of the piles. Therefore, it is likely that these factors also influence the survival of *E. coli* in the compost mass. This seems especially important when temperatures in the compost mass are lower, for example, at subsurface or surface locations and during winter. Here, studies reported prolonged survival times of bacteria in manure compost (Siller et al., 2020; Berry et al., 2013; Erickson et al., 2010). Covers on compost piles can therefore have insulating characteristics when layers of straw or finished compost are used (Patel et al., 2015; Shepherd et al., 2011) or serve as shelters from major rainfall events that would wet the compost mass. When the MC of the compost mass becomes too high, anaerobic spots within the mass may occur, which can lead to reduced temperatures (Epstein, 2019). Turning helps to aerate the compost mass, accelerating temperature increase, and also helps to mix outer parts into the hotter inside, including pathogens (Patel et al., 2015).

In the pilot-scale composting trials of the present work, we investigated the influences of different seasons, of a gas-permeable but water-repellent compost fleece cover, and of periodic turning. Despite high initial levels of *E. coli* of more than 6 cfu/g, *E. coli* were below the detection limit within 24 hours at all sample locations during summer and winter. The statistical analyses validated the significant influences of day and sample location on the survival of *E. coli*, while season and treatment methods had no significant effects. This is in contrast to a study by Siller et al. (2020), who found faster reductions in ESBL-producing *E.*

coli during 96 hours of farm storage of chicken manure in summer than in winter. Erickson et al. (2010) also demonstrated a seasonal influence on the reduction in *E. coli* in chicken manure compost. In addition, Shepherd et al. (2011) observed faster *E. coli* O157:H7 reduction in cow manure heaps covered with finished compost than in heaps left uncovered. However, one reason for the difference in results when compared to the present work is certainly the very rapid decay in *E. coli* in this study. Therefore, it was impossible to find differences between different treatment methods or seasons. In addition, the compositions of the chicken manure used in the trials with low MC values of 35.70 ± 1.95% and 26.01 ± 2.16% in summer and winter, respectively, might have also influenced the results in the present study compared to other studies. As demonstrated in the laboratory trials, a low MC can lead to the desiccation of *E. coli* even at colder spots within compost piles. The seasonal influence found in the study by Siller et al. (2020), who used chicken manure with a similar composition to that in the present study, might be primarily explained by a very cold winter trial with a daily mean ambient temperature ranging from -7.2 to 4.4 °C during the five trial days, while the daily mean ambient temperature ranged from 3.4 to 10.6 °C in the winter trial of the present work. It therefore must be noted that the winter trial was performed during rather warm weather conditions, with an average ambient temperature of 7 °C over all trial days. Within the last 20 years, the mean winter temperature in Germany ranged from -1.72 to 4.38 °C (Statista, 2022). Therefore, a seasonal influence seems possible when temperatures become colder than throughout the conducted trials, for example, in cold winters in Germany and especially in other northern countries. Although the conducted study could not validate a significant influence of turning of a pile on the survival of *E. coli*, the study demonstrated significantly longer survival times for subsurface than for surface locations, which is in agreement with studies by Siller et al. (2020) and Patel et al. (2015). Therefore, turning is recommended to ensure the exposure of all parts of the compost to the higher temperatures within the piles. This becomes even more important during winter composting.

The influences of the season and the treatment methods on the temperature development within the piles are also of importance for the potential to reduce *E. coli*. Here, the pilot-scale study demonstrated the significant influences of sample location and season on the total hours (h_{total}) at temperatures ≥ 50 and 55 °C. The h_{total} values at ≥ 50 and 55 °C were significantly higher during the summer trial than during the winter trial and higher at center locations than subsurface locations. This is in agreement with Siller et al. (2020), who also measured higher temperatures in deep sample locations than surface locations and higher temperatures within chicken manure piles in summer than in winter. In addition, turning led to increased h_{total} ≥ 50 and 55 °C in winter. In the summer trial, covering had no positive effect on h_{total} ≥ 50 and 55 °C, whereas in winter, covering led to increased h_{total} in the center locations of the compost pile.

This is in agreement with Patel et al. (2015), who also observed an increased number of days at ≥ 55 °C when windrows consisting of dairy manure were covered with a 30 cm layer of finished compost.

Although the pilot-scale trial results showed no significant benefit of turning or covering a pile during chicken manure composting concerning the survival of *E. coli,* turning resulted in more hours with temperatures at 50 and 55 °C or above and undeniably helps to mix pathogens and colder spots into the hotter inside. This becomes especially important when the environmental conditions are different from those during the present trials, e.g., more rainfall or lower temperatures, or the conditions of the compost mass itself, e.g., a difference in substrate, in the C/N ratio and MC, and in the microbial composition, as well as when pathogens more resistant than *E. coli* are considered. Covering, in addition, seems to help to increase temperatures in the center of the piles under colder weather conditions and may also be an additional tool when turning is already performed. Furthermore, covering becomes more important in regions with higher precipitation rates, and it is also a useful method for use against odor emissions.

4.3.4 VFAs, NH_3 and pH

In addition to the factors mentioned above, there are many further factors that have been discussed in studies as influencing factors concerning bacterial reduction. As described in Chapter 2.4 influencing factors can be divided into physical, chemical, operational, microbial and environmental factors. In the conducted studies, it was demonstrated that temperature was the main inactivation factor for ESBL-/AmpC-producing *E. coli* during composting and anaerobic digestion, and that also C/N ratio and MC had a significant influence during composting. Nonetheless, the influences of the chemical factors VFAs, NH_3, and pH on the reduction kinetics of ESBL-/AmpC-producing *E. coli* were also investigated. Those possible influences were an interesting aspect of this work, since chicken manure is a nitrogen-rich material with the NH_3 content and the pH value most likely being higher than during treatment of other animal manures.

VFAs are important metabolites of the anaerobic digestion process and have the potential to reduce pathogens in the digesters (Jiang et al., 2020). In the anaerobic digestion trials, VFA levels increased with increasing temperature and were the highest at 55 °C with a maximum level of 1.94 g/L. However, no direct correlation between VFA levels and the reduction in ESBL-/AmpC-producing *E. coli* was observed. This might be explained by the fact that VFA toxicity depends on further factors, such as pH and process temperature. At low pH, the level of

undissociated VFAs increases, which are considered to be more toxic to bacteria than ionized VFAs (Jiang et al., 2020). Kunte et al. (2000), for example, showed that *Shigella dysenteriae* could not be inhibited by VFA concentrations of 3,000 mg/L at a pH of 7, while growth of these bacteria was inhibited by 1,000 mg/L and 10 mg/L at the pH of 6 and 5, respectively. The pH during the trials of the present work, however, remained above 7.5 in all test series, and at 55°C, it even remained above 8 for several days, mainly due to the nitrogen-rich chicken manure substrate.

In addition, the NH_3 content was measured during the anaerobic digestion trials. With increasing temperature and pH, the NH_3 concentration also increased, which was to be expected since a shift from NH_4^+ to NH_3 occurs (Theuerl et al., 2019). Therefore, thermophilic anaerobic digestion led to significantly higher NH_3 concentrations with a maximum of 445.4 mg/kg, while at 37 and 42 °C, the maximum NH_3 concentrations were 137.1 and 99.8 mg/kg, respectively. NH_3 is known to affect pathogen survival by its effect on the membrane potential and its potential to destroy cell proteins, and it may also lead to a alkalinization of the cytoplasm; however, the mechanism is not fully understood yet (Vinnerås, 2013). Nevertheless, no direct correlation could be found between NH_3 concentration and the reduction in ESBL-/AmpC-producing *E. coli* in the anaerobic digestion trials. This might be explained by the NH_3 concentrations measured in the trials. In a review by Theuerl et al. (2019), it was stated that maximum NH_4^+/ NH_3 levels in a range from 3 to 5 g/L and from 80 to 450 mg/L for NH_4^+ and NH_3, respectively, are considered acceptable for a stable anaerobic digestion process. In addition, in a study by Park and Diez-Gonzalez (2003), a NH_3-N level of 2,520 mg/L (180 mmol/L) was required for a > 5 log reduction in *E. coli* O157:H7 after 6 hours. The authors also demonstrated that a lot lower NH_3-N level of 560 mg/L (40 mmol/L) was required for a >6 log reduction in *Salmonella* Typhimurium after 6 hours indicating that *E. coli* might be more resistant to high NH_3 levels than other bacterial species. Therefore, the NH_3 concentrations reached in the conducted test series may not have been high enough to inhibit ESBL-/AmpC-producing *E. coli*.

As mentioned above, the pH in the anaerobic digestion trials was alkaline throughout the experiments. A direct influence of the pH value on the reduction kinetics of ESBL-/AmpC-producing *E. coli* could not be shown. This is in agreement with a study by Park and Diez-Gonzalez (2003), who demonstrated that a pH of 8.5 to 9.5 could not inactivate *E. coli* O157:H7 within 24 hours. However, since pH influences both the balance of undissociated/dissociated VFAs and the balance of NH_4^+/NH_3 (Jiang et al., 2020), pH might have been indirectly involved in ESBL-/AmpC-producing *E. coli* reduction.

Although no direct influence of the individual factors pH, VFAs or NH_3 on the reduction kinetics of ESBL-/AmpC-producing *E. coli* could be shown, the test series at 37 °C demonstrated that at 37 °C, temperature is unlikely to be the main inactivation factor, since 37 °C is within the optimum growth temperature of *E. coli*. Here, the – possibly combined – effects of NH_3, VFAs, pH, and further factors, such as substrate limitations and microbial competition, play roles in inactivating ESBL-/AmpC-producing *E. coli*. In addition, at 55 °C, the inactivation of ESBL-/AmpC-producing *E. coli* occurred so rapidly, reaching the detection limit within two hours that the influence of further factors other than temperature remained difficult to demonstrate. Furthermore, with temperature being the main inactivation factor, a suboptimal environment during anaerobic digestion with alkaline pH and high levels of VFAs and NH_3 can additionally stress ESBL-/AmpC-producing *E. coli* and reduce their heat resistance. The pH, VFAs, and NH_3 are furthermore process-related and dependent on the biogas process itself, the substrates used, the temperature as well as HRT and OLR (Lorine et al., 2021).

In the composting trials, the different C/N ratios and MCs led to differences in the chemical composition: logically, the lower the C/N ratio, the higher were the NH_4-N and pH values. The mean VFA concentrations for all days of composting ranged from 1.57 g/L in the mixture with a C/N ratio of 40:1 and 40% MC to 4.73 g/L in the mixture with a C/N ratio of 20:1 and 40% MC. In contrast to Erickson et al. (2014), who also performed chicken manure composting trials, mixtures with a C/N ratio of 20:1 and 60% MC did not result in more VFAs than in 40:1 mixtures and 60% MC. In addition, Erickson et al. (2014) demonstrated a significant influence of VFAs on the level of *E. coli*, which was not found in the present trials. While Erickson et al. (2014) concluded that VFAs had antimicrobial activity, in the present trials, the mixture with the highest production of VFAs was the mixture with one of the slowest declines in ESBL-producing *E. coli*. Again, as mentioned above, the pH value might have been responsible for this discrepancy in results: in the present trials, the pH values remained above 7 in all the mixtures, throughout the trials. Only the mixture with a C/N ratio of 40.1 and an MC of 60% turned slightly acidic and remained acidic until day 5. Erickson et al. (2014), on the other hand, observed a drop in pH from above 7 to 6.3 and below in all three mixture types (20:1, 30:1 and 40:1) on day 1. The mixture with a C/N ratio of 20:1 remained acidic until day 3, the other two mixture types until day 2. Since undissociated VFAs are considered to be more toxic to bacteria and occur more frequently at acidic pH, this might be an explanation.

As mentioned for anaerobic digestion, it is also known for composting that a high N content affects pathogen survival (Singh et al., 2012; Himathongkham et al., 2000; Himathongkham and Riemann, 1999). In the laboratory composting trials, the mean NH_4-N levels in the mixtures for all days of composting ranged from 1,240.6 to 6,091.9 mg/kg being the higher the lower the

C/N ratio was. However, no significant influence on the number of ESBL-producing *E. coli* could be observed. Nevertheless, the NH_4-N contents of 4,669.5 to 6,091.6 mg/kg have to be seen critically concerning *E. coli* survival, and may have contributed to the rapid decay in ESBL-producing *E. coli* in the mixtures with a C/N ratio of 10:1.

The pH did not show any significant influence on the number of ESBL-producing *E. coli* in the laboratory composting study; however, as during anaerobic digestion, pH affects the levels of undissociated VFAs and NH_3. In addition, a lower pH is most likely due to accumulation of VFAs in the mass (Reinhardt, 2002), possibly through anaerobic spots within the piles. In addition, pH values below 7 in the mixture with a C/N ratio of 40:1 and an MC of 60% appeared to inhibit the temperature rise in the compost mass, thus, being responsible for the delay in *E. coli* inactivation in this mixture. Sundberg et al. (2004) also concluded that a low pH was responsible for a delayed transition from the mesophilic to the thermophilic phase during composting.

Although no direct correlation between the factors pH, VFAs or NH_3 and the number of ESBL-producing *E. coli* could be shown during the composting trials, these factors can stress *E. coli*, making it more susceptible to heat and desiccation, the two most important inactivation factors during composting as demonstrated by the present work.

Overall, the influences of many factors during anaerobic digestion and composting make it difficult to demonstrate the importance of a single factor due to the complexity of both processes. Factors often not only influence *E. coli* but also influence each other. Each composting or anaerobic digestion process is unique, making it difficult to compare results of studies, since a difference in substrate, operating system, microbial composition or, during composting, environmental conditions, will lead to differences in process-related factors, such as VFAs, pH and $NH_{3,}$, and to a difference in their – possibly combined – effect on ESBL-/AmpC-producing *E. coli*.

4.4 The importance of proper chicken manure management for the reduction in ESBL-/AmpC-producing *E. coli*

Proper manure management is important both for successful composting results and for high biogas yields during anaerobic digestion (Epstein, 2019; Weiland, 2010). Therefore, large-scale companies aim to find the optimum conditions for aerobic and anaerobic processes. Full-scale chicken manure composting should include precise control of rotting factors, such as the adjustment of the oxygen level, the MC, and the optimum C/N ratio. Only then can the

achievement of sufficient temperatures in the materials and the quality of the final compost be assured. Anaerobic digestion should be designed with regards to a stable process without disturbances and with consideration of high biogas yields.

However, as the results of the present work show, the survival of ESBL-/AmpC-producing *E. coli* is not solely dependent on optimum composting and anaerobic digestion processes. High temperature of 55 °C during anaerobic digestion reduced ESBL-/AmpC-producing *E. coli* within less than a few hours to undetectable levels. At that temperature, possible shortcuts or inhibition of the process because of operational failures will not necessarily lead to a lower reduction in ESBL-/AmpC-producing *E. coli* as long as the temperature remains high. At mesophilic temperatures, however, further influential factors play a role in the success of reduction in ESBL-/AmpC-producing *E. coli*. Therefore, it is essential to avoid process inhibition and shortcuts and to maintain sufficient retention times. The results of the present work also indicate that ESBL-producing *E. coli* can be efficiently reduced in chicken manure compost even if the compost guidelines are not followed closely or when chicken manure is only stored in piles without further management and without optimal control of rotting factors. Even then, temperatures within the chicken manure mass rise quickly to levels that are deadly for *E. coli*. This is important information in terms of chicken manure safety since many farmers do not properly compost their chicken manure, but only store it until it can be used on land. Therefore, it is reassuring that even when manure is only stored for one day or transported to another farm, the level of *E. coli* will decrease intensively during that time with no further intake. The chicken manure characteristics of a very low MC and a low C/N ratio seem to be reasons for the successful reduction in ESBL-producing *E. coli* even at suboptimal composting conditions: for example, in areas of compost heaps that do not reach temperatures above 50 °C, e.g., at surface sites or during winter. When examining other manure types, the results will most likely be less impressive. In general, it is important to ensure that new manure is not permanently added to the original substrate, as this procedure would lead to possible recontamination of the compost mass.

Although ESBL-producing *E. coli* can be sufficiently reduced even under suboptimal manure management conditions, composting should also always aim for the lowest possible emissions. This includes emissions of greenhouse gases, such as CH_4 and N_2O, as well as odor emissions (Rincón et al., 2019). Here, it is essential to allow an aerobic process with sufficient aeration, either by periodic turning of the compost mass or forced aeration to ensure an oxygen level above 5% (Epstein, 2019; Chowdhury et al., 2014; Amon et al., 2001). The covering of compost piles can help reduce odor emissions and is therefore a useful method even when it is not necessarily important for the reduction in ESBL-producing *E. coli*.

Concluding the results of the conducted studies, the most recommended ways to treat chicken manure in terms of a satisfying reduction in ESBL-/AmpC-producing *E. coli* are therefore either anaerobic digestion at temperatures above 50 °C or composting in a windrow system with periodic turning.

4.5 Relevance of chicken manure fertilizer as a source of dissemination of ESBL-/AmpC-producing *E. coli* into the environment

Annually, more than 500,000 tons of chicken manure are applied as fertilizer to arable land in Germany (Statistisches Bundesamt, 2021). Both consumers and scientists are concerned about the spread of ESBL-/AmpC-producing *E. coli* into the environment and the potential contamination of fruit, vegetables, crops, and water with these antibiotic-resistant bacteria. In the present work, two laboratory studies and one pilot-scale study were conducted to assess the risk of this dissemination during land application. In both laboratory trials, chicken manure treatment led to a significant reduction in ESBL-/AmpC-producing *E. coli*, minimizing the risk of spread into the environment. Furthermore, even storage of nonmanaged or only minimally managed chicken manure for more than one day led to an *E. coli* concentration below the detection limit and within 28 days at a maximum, the chicken manure compost was also negative by enrichment. Therefore, the risk of spreading ESBL-/AmpC-producing *E. coli* to the environment with chicken manure is considered low. This was also concluded by two studies recently conducted by Siller et al. (2021) and Thiel et al. (2020). Both authors investigated the airborne emission of ESBL-/AmpC-producing *E. coli* from chicken manure to arable land in the aerosolized state. For their studies, the authors used chicken manure that was highly contaminated with ESBL-producing *E. coli* when tested at the barns and found the manure to be below the detection limit after rapid transport to the test grounds. Therefore, the authors of both studies concluded that the airborne spread of these bacteria in the environment by wind erosion is rather unlikely. The main reason for these results may be the characteristics of chicken manure. Chicken manure is typically much drier than other manure types, with an MC often below 30% (Siller et al., 2020; Thomas et al., 2020). In the laboratory-scale composting study, it was shown that ESBL-producing *E. coli* were highly susceptible to desiccation. Furthermore, it can be assumed that the simple storage of chicken manure at broiler farms for a short amount of time, or even on transport vehicles, leads to a rapid and substantial rise in temperatures within the mass, which also results in a very rapid reduction in ESBL-producing *E. coli*, as shown in the studies of the present work.

5 Conclusion

With the conducted anaerobic digestion and composting trials, it was shown that ESBL-/AmpC-producing *E. coli* were quickly inactivated in chicken manure. Even suboptimal composting conditions led to a fast decay in ESBL-producing *E. coli*. Hence, the risk of spreading these resistant bacteria to land via contaminated chicken manure is considered low. The present work also demonstrated that the reasons for the reduction in *E. coli* during composting and anaerobic digestion are multifactorial. The main inactivation factor in all conducted trials was temperature. During composting, the influences of the C/N ratio and MC on ESBL-producing *E. coli* reduction were also shown. Further chemical, microbial, operational, and environmental factors were certainly also involved, but their single influences were difficult to demonstrate. Further studies are needed to assess the influences of additional factors, such as VFAs and NH_3, on the reduction kinetics of ESBL-/AmpC-producing *E. coli* both in the laboratory and in the field. Here, both the influences of single factors as well as the combined effects of different factors on ESBL-/AmpC-producing *E. coli* as well as on the microbial activity of the degradation process need to be further investigated. Regarding other manure types, to the best of our knowledge, information on the reduction kinetics of ESBL-/AmpC-producing *E. coli* during anaerobic digestion, storage, or composting is scarce and needs to be further investigated since differences in substrate lead to differences in the substrate-specific and process-related factors influencing *E. coli* survival.

Bearing in mind that manure management should aim both at an ESBL-/AmpC-producing *E. coli* free final substrate and at minimization of emissions during the process, composting of chicken manure in a windrow system with periodic turning and optimal control of rotting factors or anaerobic digestion of chicken manure at thermophilic temperatures is recommended.

6 Summary

Manure management measures to reduce the risk of spreading ESBL-/AmpC-producing *Escherichia coli* from chicken manure into the food chain

The prevalence of extended-spectrum beta-lactamase (ESBL)- and AmpC beta-lactamase-producing *Escherichia (E.) coli* in the broiler production chain is high. The finding of these resistant bacteria in high numbers in barns and in the environment of broiler farms raises concerns about the use of potentially contaminated chicken manure as fertilizer on fields. As it is assumed that ESBL-/AmpC-producing *E. coli* can enter the human food chain by contaminated water, vegetables, and fruit after land application of chicken manure, better insights into the survival and the reduction kinetics of these bacteria during storage and chicken manure treatments are needed. Thus, the present work aimed to determine the reduction kinetics of ESBL-/AmpC-producing *E. coli* during composting and anaerobic digestion with a special focus on potential inactivation factors, above all temperature, carbon/nitrogen (C/N) ratio, and moisture content (MC). To that aim, three studies were carried out.

In the first study, laboratory-scale anaerobic digestion tests were conducted at 37, 42 and 55 °C to investigate the influence of temperature on the reduction kinetics of ESBL-/AmpC-producing *E. coli* during anaerobic digestion of chicken manure. One ESBL- (CTX-M15) and one plasmid-mediated AmpC- (CMY-2) producing *E. coli* strain were added to the substrate to achieve an initial bacterial count of 7 $\log_{10}$ colony forming units (cfu) per mL. Both *E. coli* strains were below the detection limit of <100 cfu/mL after 35 days, with decimal reduction times (D-values) of 3-6 days at 37 °C, 1.5 days at 42 °C and 48 min at 55 °C. However, at 37 and 42 °C, ESBL-/AmpC-producing *E. coli* remained partially detectable by enrichment until the end of the trials after 35 days. Hence, the results showed a strong temperature dependency, whereas no direct correlation could be observed between pH, volatile fatty acids (VFAs) or ammonia (NH_3) and *E. coli* reduction.

The second study aimed to determine the effects of the C/N ratio and MC on the survival of ESBL-producing *E. coli* during laboratory-scale chicken manure composting in bioreactors. To that aim, a ESBL- (CTX-M15) producing *E. coli* strain was added to nine compost mixtures with different combinations of MC (20, 40, 60%) and C/N ratios (10:1, 20:1, 40:1) to obtain an initial bacterial count of 7 $\log_{10}$ cfu/g. The fastest decrease in *E. coli* was observed in all mixtures with a C/N ratio of 10:1 with D-values of 0.27 days to 0.60 days. Additionally, in dry mixtures with an MC of 20% and a C/N ratio of either 10:1 or 40:1, ESBL-producing *E. coli* were reduced faster than in the moist mixtures despite lower maximum temperatures within

the bioreactors. The highest D-value of 4.82 days was determined for a mixture with a C/N ratio of 40:1 and 40% MC. While temperature was the main inactivation factor, in dry mixtures, desiccation also played a significant role. Significant effects of the C/N ratio and MC on the number of ESBL-producing *E. coli* as well as on temperature development were shown. In addition, it was demonstrated that even suboptimal composting mixtures – typical for chicken manure – led to a rapid reduction in ESBL-producing *E. coli*.

To validate the results of the laboratory composting trials in a more practical environment, in a third study, pilot-scale composting of chicken manure was performed at the test grounds in Potsdam. Here, the influences of three different management treatments for chicken manure, composting in (i) static piles, (ii) static piles covered with a compost fleece, and (iii) periodically turned piles, were determined with respect to the survival of an artificially added, nonresistant *E. coli* strain (DSM 1116) during different environmental conditions (summer and winter). The results showed an even faster reduction in *E. coli* and higher temperatures within the compost mass than in the laboratory-scale composting trials. Within 24 hours, *E. coli* were no longer detectable by direct count in any piles in either center or subsurface locations. By day 28, *E. coli* were also no longer qualitatively detectable in any samples. Within these 24 hours, temperatures exceeded 50 °C in all piles, which was most likely responsible for the fast inactivation of *E. coli*. The statistical analysis revealed the significant influences of sample location (center, subsurface) and the total hours at temperatures ≥ 50 °C and ≥ 55 °C in the piles on the survival of *E. coli* in the chicken manure compost. Although no influences of season or manure treatment method were shown, periodical turning is recommended, especially in winter, to increase the likelihood of exposure of all parts of the compost, including possible ESBL-/AmpC-producing *E. coli*, to high temperatures.

Taken together, the results of the three studies showed the effectiveness of both composting and anaerobic digestion in terms of ESBL-/AmpC-producing *E. coli* reduction. The reasons for the reduction in ESBL-/AmpC-producing-*E. coli* were multifactorial, with temperature being the main inactivation factor. Most likely due to the chicken manure characteristics of a low MC and a low C/N ratio, even in stored, nonmanaged or only minimally managed chicken manure, ESBL-producing-*E. coli* levels were reduced below the detection limit after one day. Therefore, concluding the results of this work, the risk of spreading ESBL-/AmpC-producing *E. coli* to land by using chicken manure, chicken manure compost, or digestate from biogas plants running on chicken manure is considered low. Composting of chicken manure with periodic turning or anaerobic digestion at thermophilic temperatures are the safest methods in terms of ESBL-/AmpC-producing *E. coli* reduction to treat chicken manure before application to land and are therefore highly recommended.

7 Zusammenfassung

Maßnahmen im Wirtschaftsdüngermanagement zur Verringerung des Risikos einer Ausbreitung ESBL-/AmpC-bildender *Escherichia coli* von Hähnchenmist in die Nahrungskette

Die Prävalenz Extended-Spektrum Beta-Laktamase (ESBL) und AmpC-Beta-Laktamase bildender *Escherichia (E.) coli* in der Masthähnchenproduktionskette ist hoch. Das Vorkommen dieser resistenten Bakterien in hohen Konzentrationen in Ställen und der Umgebung von Masthähnchenbetrieben gibt Anlass zur Sorge vor der Verwendung von möglicherweise kontaminiertem Hähnchenmist als Dünger auf Feldern. Da angenommen wird, dass ESBL-/AmpC-bildende *E. coli* nach dem Ausbringen von Hähnchenmist auf Feldern über kontaminiertes Wasser, Gemüse und Obst in die menschliche Nahrungskette gelangen können, ist es erforderlich, einen besseren Einblick in das Überleben und die Reduktionskinetik dieser Bakterien während der Lagerung und der Behandlung von Hähnchenmist zu gewinnen. Die vorliegende Arbeit hatte daher zum Ziel, die Reduktionskinetik von ESBL-/AmpC-bildenden *E. coli* während der Kompostierung und der anaeroben Vergärung zu bestimmen. Dabei lag ein besonderer Fokus auf möglichen Inaktivierungsfaktoren, allen voran der Temperatur, dem Kohlenstoff/Stickstoff (C/N)-Verhältnis und dem Feuchtigkeitsgehalt (MC). Zu diesem Zweck wurden drei Studien durchgeführt.

In der ersten Studie wurden anaerobe Vergärungstests im Labormaßstab bei 37, 42 und 55 °C durchgeführt, um den Einfluss der Temperatur auf die Reduktionskinetik ESBL-/AmpC-bildender *E. coli* während der anaeroben Vergärung von Hähnchenmist zu untersuchen. Dabei wurden dem Substrat ein ESBL- (CTX-M15) und ein Plasmid-vermittelter AmpC- (CMY-2) bildender *E. coli*-Stamm zugegeben, um eine anfängliche Bakterienzahl von 7 $\log_{10}$ koloniebildenden Einheiten (KbE) pro mL zu erhalten. Beide *E. coli*-Stämme lagen nach 35 Tagen unter der Nachweisgrenze von <100 KbE/mL mit dezimalen Reduktionszeiten (D-Werten) von 3-6 Tagen bei 37 °C, 1,5 Tagen bei 42 °C und 48 min bei 55 °C. Bei 37 und 42 °C blieben ESBL-/AmpC-bildende *E. coli* jedoch teilweise bis zum Ende der Versuche nach 35 Tagen qualitativ nachweisbar. Die Ergebnisse zeigten daher eine starke Temperaturabhängigkeit, während keine direkte Korrelation zwischen pH, flüchtigen Fettsäuren (VFAs) oder Ammoniak (NH_3) und einer *E. coli*-Reduktion beobachtet werden konnte.

Die zweite Studie zielte darauf ab, die Auswirkungen des C/N-Verhältnisses und des MCs auf das Überleben ESBL-bildender *E. coli* während der Kompostierung von Hähnchenmist in Bioreaktoren im Labormaßstab zu bestimmen. Zu diesem Zweck wurde ein ESBL- (CTX-M15) bildender *E. coli*-Stamm zu neun Kompostmischungen mit unterschiedlichen Kombinationen aus MC (20, 40, 60 %) und C/N-Verhältnis (10:1, 20:1, 40:1) gegeben, um eine anfängliche Bakterienzahl von 7 $\log_{10}$ KbE/g zu erhalten. Die schnellste Abnahme von *E. coli* wurde in allen Mischungen mit einem C/N-Verhältnis von 10:1 mit D-Werten von 0,27 Tagen bis 0,60 Tagen beobachtet. Zusätzlich wurden in trockenen Mischungen mit einem MC von 20 % und einem C/N-Verhältnis von entweder 10:1 oder 40:1 ESBL-bildende *E. coli* schneller reduziert als in den feuchteren Mischungen, trotz niedrigerer Maximaltemperaturen innerhalb der Bioreaktoren. Der höchste D-Wert von 4,82 Tagen wurde für eine Mischung mit einem C/N-Verhältnis von 40:1 und 40 % MC bestimmt. Während die Temperatur der Hauptinaktivierungsfaktor war, spielte bei den trockenen Mischungen auch die Austrocknung eine bedeutende Rolle. Es konnte ein signifikanter Einfluss von C/N-Verhältnis und MC auf die Anzahl ESBL-bildender *E. coli* sowie auf die Temperaturentwicklung gezeigt werden. Darüber hinaus wurde gezeigt, dass selbst suboptimale Kompostmischungen – typisch für Hähnchenmist – zu einer schnellen Reduktion von ESBL-bildenden *E. coli* führten.

Um die Ergebnisse der Kompostierungsversuche im Labormaßstab in einem praxisnäheren Umfeld zu validieren, wurde in einer dritten Studie auf dem Versuchsgelände in Potsdam eine Kompostierung von Hähnchenmist im Pilotmaßstab durchgeführt. Hier wurde der Einfluss von drei verschiedenen Behandlungsverfahren für Hähnchenmist: Kompostierung in (i) statischen Mieten, (ii) mit einem Kompostvlies bedeckten statischen Mieten und (iii) regelmäßig umgesetzten Mieten während verschiedener Umweltbedingungen (Sommer und Winter) auf das Überleben eines künstlich zugesetzten, nicht resistenten *E. coli*-Stammes (DSM 1116) bestimmt. Die Ergebnisse zeigten eine noch schnellere Reduktion von *E. coli* und höhere Temperaturen innerhalb der Kompostmasse als bei den Kompostierungsversuchen im Labormaßstab. Innerhalb von 24 Stunden waren *E. coli* quantitativ in keiner Miete, weder im Zentrum noch unterhalb der Oberfläche, mehr nachweisbar. An Tag 28 waren *E. coli* auch qualitativ in keinen Proben mehr nachweisbar. Innerhalb dieser 24 Stunden überstiegen die Temperaturen in allen Mieten 50°C und waren höchstwahrscheinlich verantwortlich für die schnelle Inaktivierung von *E. coli*. Die statistische Analyse zeigte einen signifikanten Einfluss des Probenortes (Mitte, unter der Oberfläche) und der Gesamtstunden bei Temperaturen ≥ 50 °C und ≥ 55 °C in den Mieten auf das Überleben von *E. coli* im Hähnchenmist-Kompost. Obwohl kein Einfluss der Jahreszeit und der Behandlungsmethode gezeigt werden konnte, wird ein regelmäßiges Umsetzen, insbesondere im Winter, empfohlen, um die

Wahrscheinlichkeit einer Exposition aller Teile des Komposts, einschließlich möglicher ESBL-/AmpC-bildender *E. coli*, mit hohen Temperaturen zu erhöhen.

Zusammengenommen zeigten die Ergebnisse der drei Studien die Wirksamkeit von sowohl Kompostierung als auch anaerober Vergärung in Bezug auf die Reduktion ESBL-/AmpC-bildender *E. coli*. Dabei waren die Gründe für die Reduktion von ESBL-/AmpC-bildenden *E. coli* multifaktoriell, wobei die Temperatur der Hauptinaktivierungsfaktor war. Höchstwahrscheinlich wurden ESBL-bildende *E. coli* aufgrund der Eigenschaften von Hähnchenmist mit seinem niedrigen MC und C/N-Verhältnis selbst in gelagertem, nicht behandeltem oder nur minimal behandeltem Hähnchenmist bereits nach einem Tag unter die Nachweisgrenze reduziert. Aus diesem Grund wird als Schlussfolgerung aus den Ergebnisse dieser Arbeit das Risiko einer Verbreitung ESBL-/AmpC-bildender *E. coli* in der Umwelt durch die Verwendung von Hähnchenmist, Hähnchenmistkompost oder Gärresten aus Biogasanlagen, die mit Hähnchenmist betrieben werden, als gering eingeschätzt. Die Kompostierung von Hähnchenmist mit regelmäßigem Umsetzen oder die anaerobe Vergärung bei thermophilen Temperaturen sind die sichersten Methoden zur Düngebehandlung vor Ausbringung in Hinblick auf die Reduktion von ESBL-/AmpC-bildenden *E. coli* und werden daher dringend empfohlen.

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9 List of Own Publications

9.1 Research papers in peer-reviewed scientific journals

Thomas, C.; Idler, C.; Ammon, C.; Herrmann, C.; Amon, T. (2019) Inactivation of ESBL-/AmpC-producing *Escherichia coli* during mesophilic and thermophilic anaerobic digestion of chicken manure. Waste management, 84, 74-82.
https://doi.org/10.1016/j.wasman.2018.11.028

Thomas, C.; Idler, C.; Ammon, C.; Amon, T. (2020) Effects of the C/N ratio and moisture content on the survival of ESBL-producing *Escherichia coli* during chicken manure composting. Waste management, 105, 110-118.
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Thomas, C.; Idler, C.; Ammon, C.; Amon, T. Research note: Survival of *Escherichia coli* during pilot-scale chicken manure composting in summer and winter, submitted to Waste Management / rejected. (Original version as printed in dissertation, chapter 3.3)

Thomas, C.; Idler, C.; Ammon, C.; Amon, T. (2024) Applied research note: Survival of *Escherichia coli* and temperature development during composting of chicken manure with a typically low carbon/nitrogen ratio and moisture content. Journal of Applied Poultry Research, 33(2), 100402. (Revised version of chapter 3.3)
https://doi.org/10.1016/j.japr.2024.100402

9.2 Oral presentations

Thomas, C.; Idler, C.; Ammon, C.; Herrmann, C.; Amon, T. Inactivation of ESBL/AmpC-producing *Escherichia coli* in chicken manure during aeration and anaer-obic digestion. XIX International Congress of ISAH 2019, September 10-12 2019, Wroclow, Poland.

Thomas, C.; Idler, C.; Amon, T. Reduzierung ESBL-/AmpC-bildender *Escherichia coli* in Geflügelmist durch aerobe und anaerobe Behandlungsverfahren. Abschluss-Symposium des Verbundforschungsvorhabens EsRAM, June 13 2019, Berlin, Germany.

9.3 Poster presentations

Thomas, C.; Idler, C.; Ammon, C.; Amon, T. Effects of C/N ratio and moisture content on the survival of ESBL-producing *Escherichia coli* during chicken manure composting. 71. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e. V. (DGHM), February 26 2019. Göttingen, Germany.

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12 Conflict of Interest

In the context of this work, there are no conflicts of interest due to contributions from third parties.

Selbstständigkeitserklärung

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

Werder, den 09.10.2023 Corinna Thomas

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