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The impact of feed additives on prevalence and conjugation of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in poultry

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Meiner Familie

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List of abbreviations

AK	Amikacin
bla	Genes encoding for ESBL
C	Chloramphenicol
CF	Conjugation frequency
CF/D	Conjugation frequency = transconjugants/donor
CF/R	Conjugation frequency = transconjugants/recipient
cfu	Colony forming units
CL	Cephalexime
CN	Gentamycin
CPD	Cefpodoxime
CT	Colistin
CTX	Cefotaxime
DFM	Direct-fed microbials
E. coli	*Escherichia coli*
EMA	European Medicines Agency
EFSA	European Food Safety Authority
EFT	Ceftiofur
ENR	Enrofloxacin
ESBL	Extended-spectrum beta-lactamase
ESBL-PE	Extended-spectrum beta-lactamase producing *Enterobacteriaceae*
F	Nitrofurantoin
FAO	Food and Agriculture Organization of the United Nations
MAR	Marbofloxacin
OA	Organic acids
OM	Outer membrane
PB	Polymyxin
PBS	Phosphate-buffered saline
RD	Rifampicin
SIF	Stress impact factor
SXT	Sulfamethoxazole/Trimethoprim
TOB	Tobramycin
WHO	World Health Organization

1. General introduction

Multi-drug resistant bacteria have gained high political priority as they pose a major threat to public health and the global economy (Tang et al., 2017; Urumova, 2015). Animal husbandry is one of the factors responsible for the raise and spread of these 'superbugs' (Apata, 2009; Davies and Davies, 2010; Lazarus et al., 2015). Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* belong to the multi-drug resistant bacteria of high importance for public health (WHO, 2017). The highest prevalence of ESBL-producing *Enterobacteriaceae* in livestock was reported in poultry (Carmo et al., 2014; de Jong et al., 2014; Friese et al., 2013). Commonly, these bacteria are non-pathogenic *Escherichia coli* (*E. coli*) strains, resulting in intestinal colonization without causing diseases. It was proven that the genes encoding for ESBL (*bla*) are mainly located on conjugative plasmids, which can transfer to potentially pathogenic and/or human adapted *Enterobacteriaceae* (Saliu et al., 2017; Smet et al., 2011). The impact of feed additives on undesired and/or potentially pathogenic bacteria has been reported in various publications (Clavijo and Florez, 2018; Yadav and Jha, 2019). Also, a reduction of ESBL-producing *E. coli* was found in broilers fed with direct-fed microbials (DFM) (Ceccarelli et al., 2017; Methner et al., 2019; Nuotio et al., 2013). Still, the impact of these, other feed additives or nutrition related stress factors on conjugation has not yet been portrayed in poultry.

Thus, this study focused on identifying a suitable mating pair comprising an *E. coli* donor strain producing one of the most frequently observed ESBL types in poultry: CTX-M-1, CTX-M-15, SHV-12 or TEM-52 (Saliu et al., 2017). Intentionally, the recipient should be associated with public health concerns. The transfer rate of the plasmid should be as high as possible, to determine significant changes when exposed to stressors. *E. coli* ESBL10682 and *Salmonella* Typhimurium L1219-R32 crystalized as suitable donor and recipient strains, respectively. This mating pair was challenged with the stressors of varying pH levels, increasing osmolality and sub-inhibitory levels of zinc, copper, antibiotics (cefotaxime, sulfamethoxazole/trimethoprim, nitrofurantoin) and short-chain fatty acids (acetate, n-butyrate, D/L-lactate and propionate) while conjugation frequencies were determined. The impact of DFM (*Lactobacillus* spp.) and phytogenic feed additives on conjugation was also investigated in an *ex vivo* experiment. Additionally, the prevalence of ESBL-producing *Enterobacteriaceae* in the cecal content was recorded.

1.1. Aims and Objectives of the Thesis

The microbiota of broilers comprises a broad variety of bacterial species. Among these, the family of *Enterobacteriaceae* with non-pathogenic and potentially pathogenic representatives such as *E. coli*, *Salmonella* spp., *Klebsiella* spp. or *Enterobacter* spp. is well represented. These microorganisms constitute potential recipients for *bla*-carrying plasmids. Potentially pathogenic *Enterobacteriaceae* species may pose a hazard on animal health and food safety, which increases further when gaining antibiotic resistance through conjugation. Existing literature does not provide sufficient information about the impact of nutrition related stress factors on conjugation rates of ESBL-carrying plasmids. Thus, it was the aim of this thesis to:

I. Identify potential recipients for *bla*-carrying plasmids from bacteria belonging to the indigenous microbiota of broilers as well as potential pathogens.

II. Evaluate the impact of various nutrition related stress factors, direct-fed microbials and phytogenic feed additives on conjugation frequencies.

III. Identify means of nutritional intervention able to reduce or inhibit the conjugation of *bla*-carrying plasmids.

To comply with this approach, two *in vitro* and one *ex vivo/in vivo* experiments were designed, and the results summarized in three publications.

1.2. Hypothesis

It was the hypothesis of this thesis that feed additives and nutrition related stressors impact on conjugation involving *bla*-carrying plasmids.

2. Literature Review

2.1. Antibiotic resistance

2.1.1. Ancient development of antibacterial resistance

Recent developing antibacterial resistances are promoted by antibiotics used for humans, in agriculture and aquaculture, for pets, in the research industry and in hygiene and cleaning products. However, antibiotic resistance is not a new phenomenon (Gaze et al., 2013). Several studies have found evidence of the existence of antibiotic resistance genes and activity in samples dating thousands to millions of years back in time (Ponder et al., 2005; D'costa et al., 2011; Bhullar et al., 2012; Zhang et al., 2013; Petrova et al., 2014). This can be explained by the fact that bacteria have existed for more than 3.8 billion years (Wright and Poinar, 2012). Other organisms such as fungi and plants have coexisted and interacted with bacteria during the main part of this time period. To protect themselves, these organisms developed antibacterial substances long before humans started to use antibiotics (Wright and Poinar, 2012). In order to persist, bacteria started to develop mechanisms protecting themselves from, or inactivating these natural antibiotic substances. Thus, just as antibiotic substances have existed long before they were discovered by humans, antibiotic resistance has existed for a very long evolutionary time (Gaze et al., 2013). Therefore, many modern antibiotics are not only considered natural products, but the resistance mechanisms are also in terms of evolution 'natural' (Gaze et al., 2013; Wright and Poinar, 2012).

2.1.2. Discovery of antibiotics and its consequences for poultry farming

When penicillin was discovered by Alexander Fleming in 1928 and antibiotics were introduced in clinical usage in the early 1940's, infectious diseases could be treated effectively by humans for the very first time in history (Davies and Davies, 2010). Besides functioning as a valuable resource in treatment, it opened the door for a new era of preventive medicine, especially in surgery. The risk of post-operative infections was decreased enormously, allowing surgery to become more invasive and complicated (Wright, 2010). The discovery of antibiotics also had a major impact on animal production. Since the 1950s, antibiotics have been used worldwide as growth promoters and to prevent diseases in livestock and thereby also in poultry farming (Dibner and Richards, 2005). This contributed to a complete change of poultry farming systems. Before the 1950s, chickens were kept in smaller flocks for both egg and meat production. From the 1950s and onwards, intensive poultry farming instead became the main way to produce poultry products and two lines of poultry farming, and with it two kinds of chickens, developed. Broilers were specialized for the production of meat and laying hens were solely used for egg production (Barbato, 1999; Siegel, 2014). In both systems, stock density became much higher due to the common husbandry-systems, creating conditions for bacteria to spread easily between individual chickens. Due to the systematic use of antibiotics, these bacteria could be kept under control and large-scale outbreaks could be prevented (McEwen and Fedorka-Cray, 2002). It was then unknown that this created ideal preconditions for antibacterial resistance to rise due to selective pressure. However, today it is a well-known fact that the ample use of antibiotics, especially in subtherapeutic dosage, can trigger the development of antibacterial resistance (Barlow and Hall, 2002; Gniadkowski, 2008; Perry and Wright, 2014) and hence, animal production has been identified as playing a main role in the global rise of antibacterial resistance. Besides the therapeutic, preventative or performance promoting usage of antibiotics in farm animals, uncontrolled treatments or treatments without a secured diagnosis have also contributed to the development of antibiotic resistance (Wright, 2010).

2.1.3. Evolution of extended-spectrum beta-lactamases

Extended-spectrum beta-lactamases have existed for a long evolutionary time. By using Bayesian phylogeny, the age of class A beta-lactamases was estimated as 2.4 billion years (Hall and Barlow, 2004). Accordingly, the first horizontal transfer to Gram-positive bacteria is assumed to have occurred about 800 million years ago, while the separation of the enzymes TEM, SHV and CTX-M from each other possibly occurred around 400 (TEM and SHV) and 200 – 300 (CTX-M) million years ago (Hall and Barlow, 2004).

The derivatives of TEM and SHV, which are observed frequently in modern time, are speculated to have developed more recently under the selective pressure of antibiotics, with TEM-1 being the ancestor of the other TEM types (Barlow and Hall, 2002b). SHV-2 was observed for the first time in 1983, shortly after the introduction of third generation cephalosporins to the market in the early 1980s (Barlow and Hall, 2002b; Rawat and Nair, 2010). Equivalently, CMY-2 was identified as the ancestor of class C beta-lactamases, originating from a *Citrobacter freundii* chromosomal allele (Hall and Barlow, 2004). A thorough review of the evolution of ESBL and its consequences has been presented (Gniadkowski, 2008).

It is assumed that genes encoding for beta-lactamases have been transferred from chromosomes to plasmids and back for millions of years (Barlow and Hall, 2002a; Barlow and Hall, 2002b; Hall and Barlow, 2004). Nevertheless, the percentage of the occurrence of ESBL-producing bacteria in poultry increased significantly after the usage of beta-lactam antibiotics (Dierikx et al., 2013).

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Review

Types and prevalence of extended–spectrum beta–lactamase producing *Enterobacteriaceae* in poultry

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Abstract

For several billion years, bacteria have developed mechanisms to resist antibacterial substances. In modern time, antibiotics are frequently used in veterinary and human medicine for prevention and treatment of diseases, globally still also for their growth promoting effects as feed additives. This complex situation has evolved in accelerating development and prevalence of multi-drug resistant bacteria in livestock and people. Extended-spectrum beta-lactamase (ESBL) producing bacteria are resistant to a wide range of ß-lactam antibiotics. They are currently considered as one of the main threats for the treatment of infections in humans and animals. In livestock and animal products, poultry and poultry products show the highest prevalence of ESBL-producers with CTX-M-1, TEM-52 and SHV-12 being the most common ESBL-types in poultry. *Escherichia coli* and *Salmonella* spp. are the bacteria in poultry, which carry ESBL-genes most frequently. ESBL-producing bacteria are present at every level of the poultry production pyramid and can be detected even in the meconium of newly hatched chicks. The environment close to poultry barns shows high prevalence rates of these bacteria and contributes to an ongoing infection pressure with further ESBL-types. Probiotics have been shown to successfully reduce ESBL-producers in chicken, as well as ESBL-gene transfer. Other feed additives, such as zinc and copper, increase the prevalence of ESBL-producing bacteria when fed to animals. To our best knowledge, this is the first publication presenting a comparative overview of the prevalence of ESBL-types using data from different countries. To reduce the hazard for public health from poultry carrying high numbers of ESBL-producers, preventive measurements must include the surrounding environment and avoidance of antibiotic usage at all levels of the production pyramid. The first results, of the research on the impact of feed additives on the spread of ESBL-genes, indicate the diet as a further, possible magnitude of influence.

Keywords: broilers, ESBL, antibiotic resistance, feed additives.

Introduction

Usage of antibiotic growth promotors has been a common practice in European poultry farming in order to increase the performance until the phasing out in the year 2006. The raising awareness on the hazard subsequent to antibiotic feed additives in animal farming has led to the ban of such in European poultry production (Regulation (EC) No 1831/2003 chapter II article 11). Nevertheless, poultry meat still carries the highest contamination with ESBL-producing bacteria compared with other meat sources (Geser *et al.*, 2012; Carmo *et al.*, 2014; De Jong *et al.*, 2014). Cephalosporins belong to the ß-lactam antibiotics, which can be hydrolyzed by extended-spectrum ß-lactamases and thereby exert a selective pressure on ESBL-producing bacteria (Paterson and Bonomo, 2005). Despite that several countries, such as Sweden and Belgium, do not use cephalosporins for poultry, a high prevalence of

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ESBL-producing bacteria remains (Smet *et al.*, 2008; SVARM 2011, 2012). This suggests that there are additional sources for the contamination with ESBL-producing bacteria in poultry farming (Hiroi *et al.*, 2012a).

Worldwide, chicken was the second most common meat source (35.2%) in 2012, behind pork (36.3%) and followed by beef (22.2%) (http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html). Poultry is the meat source with the highest percentage rise between 1990 and 2012 (104.2%) according to the Food and Agriculture Organization of the United Nations (FAO) (http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html). ESBL-producing bacteria are able to transmit from poultry and poultry products to humans (Bertrand *et al.*, 2006; Leverstein-Van Hall *et al.*, 2011). The transmission from poultry to humans as food borne diseases or through the environment leads to a major hazard for public health (Apata, 2009; Davies and Davies, 2010). Infections with multi-drug resistant bacteria, such as ESBL-producing bacteria, are difficult to treat and cause high morbidity and increased mortality in humans (Davies and Davies, 2010). Trivially, the accountable bacteria are therefore often referred to as 'superbugs'. Examples for such bacterial species are *Escherichia coli, Salmonella enterica* and *Klebsiella pneumoniae*, all common bacteria in the gastrointestinal tract of poultry (Rehman *et al.*, 2007; Apata, 2009; Davies and Davies, 2010). Third-generation cephalosporins are frequently used to treat humans with difficult-to-cure infections caused by *Enterobacteriaceae* (Koga *et al.*, 2015). Currently, a rising occurrence of bacteria resistant to third-generation cephalosporins causes concern. In 2014, the national prevalence in Europe reached from 3.3 to 40.4% leading to an increase of the mean percentage from 9.6% (2011) to 12.0% (2014) in the EU/EEA (European Economic Area) (Ears-Net, 2015). A major part of these bacteria produces ESBL. The European Antimicrobial Resistance Surveillance System (EARSS) reported a national prevalence in ESBL-producing bacteria in the EU/EEA between 73.6 to 100% in 2013 and 71.1 to 100% in 2014 (Ears-Net, 2014, 2015). In the EU, the growing awareness on multi resistant bacteria and the impact of co- and cross selection has led to a ban on antibiotic feed additives (Regulation (EC) No 1831/2003 chapter II article 11) and new adjustments on recommended mineral contents in feed (EFSA: https://www.efsa.europa.eu/en/press/news/160809a.). According to the World Health organization, ESBL-producing *Enterobacteriaceae* belong to the most urgent health issues (World Health Organization, 2014). It is therefore crucial to identify and reduce the ESBL load in poultry farming.

To our best knowledge, this is the first review comparing types and prevalence of ESBLs common in poultry. This information is vital hence to the possible transmission of ESBL genes from commensals to pathogens and from poultry to humans.

Classification of ESBL genes

Due to the transmission of plasmids with genes encoding for ESBLs between different species (Apata, 2009) and the absence of correlation between ESBL-type and ESBL-producing bacteria, a categorization of ESBL by bacterial species is not considered useful. Hence, these bacteria are classified based on the amino acid sequences of the ESBL. They can be functionally classified into the group of Ambler's class A (TEM, SHV, CTX-M, PER, VEB, GES, TLA, BES) and D (OXA) lactamases according to Ambler *et al.* (1991) (Bush *et al.*, 1995; Gniadkowski, 2001). Worldwide, TEM (named after the patient Temoniera), SHV (sulphydryl reagent variable), and CTX-M (hydrolyses cefotaxime) are the most frequently detected ESBL-types (Canton *et al.*, 2008). The genes encoding for these enzymes are termed bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$. These also represent the major ESBL-types observed in poultry and poultry products. Within the groups, there are a number of subtypes.

ESBL-producing bacteria have the ability to interchange ESBL-genes within and across species (Apata, 2009). The Genes encoding for ESBLs are located on mobile elements (plasmids, integrons or transposons), but can also be found on the bacterial chromosome. An active transposition from plasmid to genome was suggested by Shahada *et al.* (2013). The ESBL-genes spread vertically through cell division or by gene transfer within one species, but also horizontally to other species and genera by conjugation (Händel *et al.*, 2015; Yamaichi *et al.*, 2015; Porse *et al.*, 2016). In poultry, ESBL-genes are found on a variety of plasmid types where IncI1 and IncFIB are examples for frequently detected types (Supplementary data). Beside ESBL genes, these plasmids often carry genes that encode resistance to other antibiotics or heavy metals and are therefore often co-selected, especially when located close to each other (Silver and Phung, 1996; Meunier *et al.*, 2006; Liu *et al.*, 2011; Seiler and Berendonk, 2012; Borjesson *et al.*, 2013)

Recent development of ESBL in poultry

Although antimicrobial resistance is an ancient phenomenon, prevalence of ESBL-producing bacteria in poultry became significantly higher after the usage of ß-lactam antibiotics (Dierikx *et al.*, 2013b). Besides mutations in genes encoding for ESBL, selective pressure influences other genes, promoters and the quantity of ESBL-genes enhancing the resistance against antibiotics. A change of porin proteins through mutation can e.g. alter the outer membrane permeability for antibiotics. The emergence of resistances against other groups of antibiotics can lead to co-selection and stronger promoters can increase the expression of ESBL-genes (Gniadkowski, 2001, 2008).

The exposure of bacteria to ß-lactam antibiotics in poultry farming through feed additives and clinical or preventive treatments leads to the elimination of sensitive strains but spares resistant bacteria. Merely these 'persisters' are able to multiply in the presence of ß-lactam antibiotics. They can increase tremendously due to selective pressures leading to an increase in resistance against ß-lactams (Apata, 2009; Poole, 2012). Furthermore, ESBL-producing bacteria may serve as a gene reservoir for other strains and species (Apata, 2009). Another possible way for bacteria to obtain ESBL-genes is through

interactions with environmental bacteria, which can hold both ancient or recently developed antibiotic resistance (Galan *et al.*, 2013).

Selective pressure, due to the usage of antibiotics, may enhance the emergence and rise of antibiotic resistance. In the case of ESBL-producing bacteria, ß-lactam antibiotics can be at the root of the resistance (Dierikx *et al.*, 2013b). Declining prevalence of ESBL-producing bacteria in broiler meat and intestinal content were observed subsequent to the ban of antibiotic growth promotors in Denmark, Sweden and the Netherlands (DANMAP, 2015; Swedress-Svarm, 2015; Veldman *et al.*, 2016). Correspondingly, the occurrence of bacteria resistant to cefotaxime[1] in fecal samples from broilers reduced significantly after a national ban on the usage of ceftiofur in Dutch hatcheries in 2010 from about 18% (2010) to under 10% (2011) (Koene *et al.*, 2012). These data show an immediate effect from antibiotic reduction measurements on the prevalence of antibiotic resistance. Nevertheless, there are several reports on an occurrence of ESBL-producing bacteria in broilers and their products superior to the incidence in other livestock. The high prevalence of ESBL in broilers is remarkable since many countries, such as Denmark, Belgium and Sweden, have banned the usage of cephalosporins for poultry but not for other livestock (Smet *et al.*, 2008; Bengtsson *et al.*, 2012; Kameyama *et al.*, 2013). This must lead to the assumption that other factors besides the usage of ß-lactam antibiotics affect the spread and prevalence of ESBL- producing bacteria in poultry.

As mentioned, antibiotic resistance against different types of antibiotics can be co-selected (Meunier *et al.*, 2006; Liu *et al.*, 2011). Examples for co-resistance against ß-lactams and heavy metals are silver and CTX-M-15 and CTX-M-14, mercury and SHV and TEM or mercury and ampicillin among others. Copper and zinc may promote multi resistant bacteria (Sutterlin *et al.*, 2014; Yazdankhah *et al.*, 2014; Vahjen *et al.*, 2015). Co-resistance also exists among different antibiotic classes, i.e. ESBL-producers may hold co-resistance to fluoroquinolone, tetracycline and/or trimethoprim (Shashwati *et al.*, 2014; Tacao *et al.*, 2014; Bajaj *et al.*, 2016). A high percentage (98%) of the ESBL-producing strains obtained from chicken carcasses in Brazil also expressed resistance to tetracycline (Koga *et al.*, 2015). ESBL-producing *E. coli* occurred with a high prevalence (34% in 2010 and 54% in 2011) in Swedish broiler flocks. Genes encoding for CTX-M were found on an IncI1 plasmid together with resistance against tetracycline and sulfamethoxazole, suggesting co-selection as a source for the high prevalence (Borjesson *et al.*, 2013). Nevertheless, low usage of not only cephalosporins but also other antibiotics (0.19% of all flocks) (Bengtsson *et al.*, 2012) must lead to the assumption that the resistance against ß-lactam antibiotics growing in poultry is also due to reasons other than antibiotic usage (Borjesson *et al.*, 2013). Especially when compared with the usage of antibiotics in other livestock, which show significantly lower contamination rates with ESBL-producing bacteria despite higher consumption of antibiotics (Bengtsson *et al.*, 2012; Borjesson *et al.*, 2013). The prevalence of ESBL-producing *E. coli* was compared in three flocks of broilers, one treated with antibiotics (not cephalosporins), one without and one fed antibiotics and kept in laboratories, which never housed poultry before. Surprisingly, the first two flocks showed a high occurrence of ESBL, while the flocks kept in the laboratories showed much lower contamination with ESBL-producing bacteria (Hiroi *et al.*, 2012a). This implies that the environment plays a major role, even more important than antibiotic co-selection. Correspondingly, high contamination levels with ESBL-producers were observed in the environment close to barns housing poultry (Blaak *et al.*, 2015). The bacteria may spread to the environment by waste products from animal production (Apata, 2009). The high prevalence of ESBL-producers in the environment may also explain the high prevalence of ESBL-producing bacteria in organic broiler flocks (Stuart *et al.*, 2012).

Antibiotic and heavy metal resistance are often observed simultaneously. Bacteria, which frequently carry plasmids with both genes encoding for biocide/metal resistance and antibiotic resistance genes, are *Staphylococcus* spp., *Klebsiella* spp., *Salmonella* spp., *Enterococcus* spp. and *Escherichia* spp. (Pal *et al.*, 2015). The prevalence of co-resistance plasmids is significantly higher in humans and domestic animals than in other environments such as wild animals, soil, plants or food (Pal *et al.*, 2015). ESBL-producing bacteria from a hospital environment showed co-resistance to cadmium, copper, mercury and lead, but not zinc (Touati *et al.*, 2010). Silver resistance was associated with CTX-M-15 and CTX-M-14 in humans but not in birds, while mercury resistance genes were found together with SHV and TEM genes in both human and avian samples (Sutterlin *et al.*, 2014). Hence to these common co- and cross-selection of antibiotic resistance and heavy metal resistance, it is to no surprise that mineral feed can influence the gut resistome (the gathered bacterial genetic pool of antibiotic resistance, regardless if pathogenic or not (Wright, 2007)). High levels of zinc and copper supplementation may promote multi resistant bacteria (resistant against three or more antibiotics) in animals and animal excretions (Yazdankhah *et al.*, 2014; Vahjen *et al.*, 2015). Zinc supplementation was associated with evaluated resistance against antibiotics such as ampicillin, piperacillin, doxycycline, penicillin, tetracycline and sulfonamide/trimethoprim in pigs. It also enhanced the prevalence of ESBL-producing *E. coli* and methicillin resistant *Staphylococcus aureus*, both causing difficult-to-treat infections in humans and animals (Aarestrup *et al.*, 2010; Holzel *et al.*, 2012; Bednorz *et al.*, 2013; Vahjen *et al.*, 2015). Copper supplementation led to increased resistance against macrolides, glycopeptides, ampicillin, amoxicillin/clavulanic acid and piperacillin (Hasman and Aarestrup, 2002; Holzel *et al.*, 2012). Feed supplements, which tend to reduce antibiotic resistance, are mercury (also reducing the prevalence of multi resistant bacteria) and, with less impact, lead. Co-resistance to cadmium and ß-lactams was detected irregularly in pigs (Hustavova *et al.*, 1994; Holzel *et al.*, 2012). Nickel and chrome had no impact on observed resistance in pigs' excretions (Holzel *et al.*, 2012). Besides from co-selection, an increased uptake of plasmids, due to mineral

[1]Cefotaxime is a third-generation cephalosporin commonly indicating ESBL-resistance in bacteria.

Table 1. Recent prevalence of ESBL in Enterobacteriaceae in poultry (2006–2011)

Prevalence (%)	Country of sample collection	Reference
93.0[1]	Denmark	Agerso *et al.* (2014)
27.0[2]		Agerso *et al.* (2014)
3.3–8.6[3]		Agerso *et al.* (2014)
94.5[3]	Finland	Lyhs *et al.* (2012).
43.9–88.6[3]	Germany	Kola *et al.* (2012), Lyhs *et al.* (2012), Reich *et al.* (2013), Belmar Campos *et al.* (2014)
81.0–85.5[2]		Laube *et al.* (2013), Blaak *et al.* (2015)
65.0[4]		Blaak *et al.* (2015)
57.7[3]	Japan	Kawamura *et al.* (2014)
94.0[3]	Netherlands	Leverstein-Van Hall *et al.* (2011)
84.0[5]–100.0[6]		Stuart *et al.* (2012)
85.0[2]		Dierikx *et al.* (2013a)
15.0–44.0[7]		Dierikx *et al.* (2013b)
0.3–5.8[1]		Dierikx *et al.* (2013b)
79.7[2]	Spain	Blanc *et al.* (2006)

[1]Broiler parent flocks.
[2]Broiler flocks.
[3]Poultry meat.
[4]Laying hens.
[5]Organic poultry farming.
[6]Conventional poultry farming.
[7]Broiler grandparent flocks poultry.

interaction, may be the reason for co-resistance as a consequence of mineral feed supplementation (Bednorz *et al.*, 2013).

ESBL-producing bacteria and resistance types in broilers

The majority of ESBL producing bacteria in poultry are *E. coli* and *Salmonella* spp. (Table 1). ESBL-producing *E. coli* belong to the phylogenic groups A, B1, B2, D and E (Table 2). While A and B1 are considered part of the commensal intestinal community, B2 and D are linked to pathogenic activity (Herzer *et al.*, 1990; Clermont *et al.*, 2000). The most frequent detected ESBL type in poultry is CTX-M (De Jong *et al.*, 2014; Tschudin-Sutter *et al.*, 2014; Valentin *et al.*, 2014) (Table 5). TEM and SHV are predominant in subclinical infections in poultry while TEM and CTX-M dominate samples taken from poultry with disease-associated symptoms (Olsen *et al.*, 2014).

ESBL producing *E. coli* were detected in the meconium of 1-day-old chickens showing a tendency of preservation of genotypes throughout the poultry production pyramid. This implies that ESBL genes are transmitted clonally and vertically throughout the entire poultry production pyramid even without antimicrobial selection pressure (Koene *et al.*, 2009; Dierikx *et al.*, 2013b; Laube *et al.*, 2013; Agerso *et al.*, 2014; Olsen *et al.*, 2014; Zurfluh *et al.*, 2014). In the Netherlands, ESBL-producing bacteria are suspected to be introduced to the poultry producing system through imported 1-day-old grandparent chicks. This could be a further explanation for the high prevalence of ESBL-producing bacteria in organic broiler flocks (Stuart *et al.*, 2012). The prevalence of ESBL-producing bacteria is hence much higher in 1-day-old grandparent chicks than in 1-day-old parent chicks, who in return show higher contamination than 1-day-old broiler chicks (Koene *et al.*, 2009; Dierikx *et al.*, 2013b). Combined with the fact that CTX-M producing *Enterobacteriaceae* cause food-borne diseases, which are difficult to treat, a hazard to human health arises from poultry production even without the usage of antimicrobial feed additives. Evidence that intraspecies transmission of ESBL encoding genes takes place in *Salmonella enterica* and *E. coli*, was found (Shahada *et al.*, 2013). Besides from the vertical transmission, the contaminated environment seems to be crucial for the infection of poultry. While bla_{CMY-2} was the only ß-lactamase gene found at the top of the poultry production pyramid, other types were found in older grandparent chickens and in 1-day old parent chicks and broilers. The same enzyme types were isolated in samples taken from the environment, suggesting this to be the source of the infection with the additional ESBL-types (Dierikx *et al.*, 2013b).

ESBL-producing bacteria and resistance types in poultry products and their transmission to humans

Poultry meat shows the highest contamination of ESBL-producing bacteria compared with other meat sources (Friese *et al.*, 2013). Different research groups independently found a high prevalence of ESBL-producing bacteria in products from poultry while meat origin from other livestock showed significantly lower contamination. Contamination of broiler feces and objects in close contact with broiler meat reached up to 100% (Table 1). In a Danish study carried out from 2009 to 2011, poultry meat carried the highest contamination of the examined meat sources (human exposure to ESBL-producing bacteria: 83.8% from broiler meat, 12.5% from pork and

Table 2. Recent prevalence of *E. coli* phylogenic groups in Enterobacteriaceae in poultry (2003–2013)

Phylogenic group	Prevalence (%)	Country of sample collection	Reference
A	31.5–34.0	Germany	Reich *et al.* (2013), Blaak *et al.* (2015)
	13.3–28	Netherlands	Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
	37.7	Not specified	Lyhs *et al.* (2012)
	3.85–36.5	Spain	Cortes *et al.* (2010), Egea *et al.* (2012)
B1	20.2–42.0	Germany	Reich *et al.* (2013), Blaak *et al.* (2015)
	20–38	Netherlands	Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
	28.8–38.6	Spain	Cortes *et al.* (2010), Egea *et al.* (2012)
B2	5.4–13.5	Germany	Reich *et al.* (2013), Blaak *et al.* (2015)
	2–8.9	Netherlands	Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
	7.7	Not specified	Lyhs *et al.* (2012)
	0.0–7.0	Spain	Cortes *et al.* (2010), Egea *et al.* (2012)
D	17.7–34.8	Germany	Reich *et al.* (2013), Blaak *et al.* (2015)
	4.4–32.2	Netherlands	Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
	50.7	Not specified	Lyhs *et al.* (2012)
	0–32.6	Spain	Cortes *et al.* (2010), Egea *et al.* (2012)
E	3	Netherlands	Kluytmans *et al.* (2013)

3.7% from beef) (Carmo *et al.*, 2014). Equally, poultry meat was found to be contaminated with ESBL-producing *Salmonella* spp. to a higher extent than pork meat (De Jong *et al.*, 2014). In Germany and Switzerland, feces were collected from healthy poultry, pigs and cattle. Again, samples from broilers showed the highest contamination rate (100% respectively, 63.4%) followed by cattle (60% respectively, 13.7%) and pigs (up to 56.3% respectively, 15.3%) by ESBL-producing *E. coli* (Geser *et al.*, 2012; Friese *et al.*, 2013). In Japan, broilers carry the highest contamination with ESBL-producing *E. coli* (broilers 60.0%, laying hens 5.9%, cattle 12.5% and pigs 3.0%) (Hiroi *et al.*, 2012b). In the Netherlands, ESBL-producing *E. coli* were detected in 79.8% of the examined chicken meat compared with 4.7% in beef and 1.8% in pork (Overdevest *et al.*, 2011). Within poultry, broilers are contaminated with ESBL-producing bacteria to a higher extent than laying hens (Hiroi *et al.*, 2012b; Blaak *et al.*, 2015; Evers *et al.*, 2016).

Interestingly, ESBL-producing bacteria show a high prevalence both in conventional and organic poultry farming and retail meat products (Stuart *et al.*, 2012) (Table 1). This is surprising considering the strict limitation of antibiotic usage in organic farming. However, when comparing chicken carcasses from free-ranged chicken with those of conventional raised poultry in different countries, the prevalence of extended-spectrum ß-lactamase producers depended on many factors where the production system is not decisive (Blaak *et al.*, 2015; Koga *et al.*, 2015; Mancabelli *et al.*, 2016). Important variables, such as animal housing, feed or the size of flocks of defined poultry production systems, differ between countries. This complicates the comparison of data on the prevalence of ESBL-producers in different production systems from studies carried out in different countries.

Because products from broilers have the highest ESBL contamination among all animal products, it is not surprising that, when it comes to the ESBL transmission from livestock products to people, poultry is considered as main source. The relationship between ESBL-producing *E. coli* isolates obtained from poultry and humans was closer than the one between ESBL-producing *E. coli* from pig and human origin (Cortes *et al.*, 2010). A high similarity was observed between plasmids encoding CTX-M-1 isolated from *E. coli* and *Salmonella* spp. strains of poultry origin. Conversely, they showed differences to CTX-M-1-encoding plasmids from other animal origins (Cloeckaert *et al.*, 2010). This led to the assumption that poultry might be the origin of plasmids encoding CTX-M-1.

The ability to transfer genes encoding for ESBL from an *E. coli* strain from poultry origin to an *E. coli* recipient with human origin has been frequently reported. The mechanism behind this is the transfer of plasmids by conjugation. Because these plasmids often carry additional genes encoding for other types of antibiotic resistance, they are transferred together with the resistance against ß-lactams (Meunier *et al.*, 2006; Liu *et al.*, 2011). *E. coli*-strains isolated from broiler feces may both proliferate and become part of the simulated human gastrointestinal tract. Simultaneously, plasmids encoding for ESBL can be transferred from the *E. coli* poultry strain to *E. coli* strains of human origin (Smet *et al.*, 2011). CTX-M-2 was identified in Belgium and France, samples containing this enzyme were chronologically obtained first from poultry flocks, then from poultry meat and finally from humans. This led to the assumption that poultry was the source of the infection in humans (Bertrand *et al.*, 2006). Comparing ESBL-genes, plasmids and strain genotypes in *E. coli* from poultry, chicken meat and human sources, a close relation between isolates from human and animal origin was observed (Leverstein-Van Hall *et al.*, 2011). This suggests that poultry might be a reservoir for ESBL producing bacteria (Girlich *et al.*, 2007). Beside food borne infection, flies may function as a possible vector for transmission of ESBL-producing *E. coli* from poultry to humans (Blaak *et al.*, 2014, 2015). A quantitative microbiological risk assessment investigated the exposure of humans to ESBL-producing *E. coli* originating from poultry at a worst-case scenario in 2013. Comparing chicken fillets and flies as possible sources for the transmission, the evaluation identified a higher public health risk due to ESBL-producing bacteria originating from chicken fillets than from flies (Evers *et al.*, 2016).

Although ESBL-producing bacteria are classified as food-borne pathogens originating from livestock products, the prevalence of different ESBL types can differ between people and livestock. CTX-M-1 was found the most common ESBL type in livestock (pigs, cattle and poultry) in Germany, followed by a combination of CTX-M-1 and TEM (Valentin *et al.*, 2014). In the same study, $bla_{CTX\text{-}M\text{-}15}$ was the main gene found in samples from humans. In the Netherlands, CTX-M-1 and SHV-12 were the most common types of ESBL in bacteria from both humans and poultry (Huijbers *et al.*, 2014). Further studies found CTX-M and TEM-52 to be the predominant enzymes in ESBL-producing *E. coli* from both humans and poultry (Leverstein-Van Hall *et al.*, 2011; Overdevest *et al.*, 2011). In a Dutch study, samples from humans and retail chicken meat were compared for their ESBL-producing *E. coli*. A high similarity in mobile resistance elements, virulence genes and genomic backbones were detected (Kluytmans *et al.*, 2013).

Whether poultry meat is the reason for human infection with ESBL-producing bacteria or only serves as a reservoir for ESBL-producing bacteria is currently under discussion. In humans, ESBL-producing bacteria are often associated with urinary-tract-infections or the nosocomial bacterial community (Dierikx *et al.*, 2013a; Huijbers *et al.*, 2014; Valentin *et al.*, 2014). A review of the infection with ESBL-producing bacteria through products of livestock origin identified poultry as a major source (Lazarus *et al.*, 2015). ESBL-producing bacteria from poultry may reach the environment with waste products, manure and excretions. They may proceed to humans through surface water, vegetables and fruits as well as when handling the animals. Poultry products, especially when treated with poor hygiene, are suggested to be another major source of human infection with ESBL-producing bacteria (Apata, 2009).

Prevalence of ESBL types in poultry farms and products

Plasmids carrying genes encoding for ESBL often carry genes for more than one ESBL-type (Supplementary data). The prevalence of ESBL-producing bacteria in poultry and their products differs between countries, years and products, reaching from 3.3% in retail broiler meat in Denmark (2009) to 100% in conventional farmed flocks (2010) in the Netherlands. In 10 out of 14 cases, ESBL-producing bacteria were observed in more than 50% of the collected samples (Table 1). Furthermore, the prevalence of different ESBL types varies between countries, years and products (Tables 3–5). TEM-52 is the most frequently detected TEM type in poultry (Table 3). SHV-2 and SHV-12 are frequently detected in poultry. In studies comparing the occurrence of SHV-2 and SHV-12, SHV-12 positive samples are usually observed with a higher prevalence (Table 4). CTX-M is widely spread in poultry production. It occurs with high prevalence of up to 100% (UK) (Wu *et al.*, 2013). Many different CTX-M-types have been identified in samples from poultry. CTX-M-1 demonstrates to be the most important type of CTX-M enzymes in poultry production. However, a high variation can be observed between different countries (Table 5). While there is a high prevalence for CTX-M in many European countries, it is comparably low in Japan and China. European countries, with a high prevalence for detected CTX-M-1 enzymes from poultry samples, are the UK, the Netherlands and Germany. Spain belongs to the European countries with a low CTX-M-1 prevalence. Conversely, CTX-M-2 was observed with a high prevalence in Japan (51.2%) and a low prevalence in Europe (0–9%). CTX-M-15, being the predominant CTX-M type in humans (Valentin *et al.*, 2014), shows a relatively low prevalence in poultry products. The prevalence reaches from 0% in studies from Germany, the Netherlands and Spain to 17% in a study on poultry products from the UK (Table 5).

Suggested strategies to combat ESBL in chicken

By feeding a commercial competitive exclusion product comprising a defined mixture of commercial bacteria, consisting of *E. coli* strains with susceptibility against antibiotics and other microorganisms, the ESBL-producing *E. coli* could be reduced in the cecal content of broilers (Nuotio *et al.*, 2013). To the best of our knowledge, this is the only publication about the influence of probiotics on ESBL-producing bacteria in poultry. The inhibiting effect of probiotic *Bifidobacterium* spp. strains on the spread of ESBL-genes was demonstrated in an in-vitro experiment and confirmed in gnotobiotic mice. While *Bifidobacterium bifidum* and *Bifidobacterium pseudocatendatum* declined SHV-5 and CTX-M-15 gene transfer from a donor to a recipient by around 3 logs, *Bifidobacterium longum* failed to reduce the transconjugation frequency. Hence to the constant quantities of donors and recipients, the effect was suggested to result from metabolites, inhibiting the transfer of plasmids, rather than from an antibacterial effect of the probiotic bacteria (Moubareck *et al.*, 2007). Detection of ß-lactamases (not defined) in the feces of children treated with ceftriaxone reduced from 60% to 30–40% when treated with different mixtures of *Bifidobacterium* spp. and *Lactobacillus* spp. (*B. bifidicum*, *Lactobacillus acidophilus* and others). Other probiotics were less effective or even increased ß-lactamases (*Saccharomyces boulardii* and *Lactobacillus cusei* ssp. *rhamnosus* GG). Lactulose, a prebiotic, had no impact on the prevalence of ß-lactamases in the examined samples (Zoppi *et al.*, 2001). These results demonstrate the importance of detailed research, hence the protective capacities may differ within one species. Regardless of the urgent need of further comprehensive research on this topic, this suggests that probiotic or synbiotic feed additives may reduce antibiotic resistance in poultry production successfully. Research on the impact of feed additives, such as probiotics, prebiotics and organic acids, on the gastrointestinal bacterial community in poultry has been versatile, specific and directed (Van Immerseel *et al.*, 2006; Williams, 2010; Zalan *et al.*, 2010; Huyghebaert *et al.*, 2011; Alloui *et al.*, 2013). However, research on their impact on antibiotic resistant bacteria has been very sparse.

The mechanisms responsible for the antagonistic effect of probiotics towards pathogens are versatile and often strain-

Table 3. Recent prevalence of TEM in Enterobacteriaceae in poultry (2006–2013)

Enzyme	Prevalence in (%)	Country	Reference
TEM	27.0	Germany	Reich *et al.* (2013)
	0.0–1.6	Spain	Cortes *et al.* (2010), Egea *et al.* (2012)
TEM-1	12.54	Germany	Laube *et al.* (2013)
	41.2	Belgium	Smet *et al.* (2008)
TEM-19	0.0	Netherlands	Kluytmans *et al.* (2013)
TEM-20	0.0[1]–3.0[2]	Netherlands	Stuart *et al.* (2012)
	1.0[3]–3.0[4]	Netherlands	Leverstein-Van Hall *et al.* (2011)
TEM-52	9.1–14.0	Netherlands	Overdevest *et al.* (2011), Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
	20.0[2]–42.0[1]	Netherlands	Stuart *et al.* (2012)
	26.0[3]–29.0[4]	Netherlands	Leverstein-Van Hall *et al.* (2011)
	8.6–10.0	Germany, Switzerland	Geser *et al.* (2012), Kola *et al.* (2012)
	3.4–28.0	Germany	Laube *et al.* (2013), Belmar Campos *et al.* (2014), Blaak *et al.* (2015)
	2.33	Japan	Kawamura *et al.* (2014)
	3.1	Spain, Catalonia	Blanc *et al.* (2006)
	13.7–43.1	Belgium	Smet *et al.* (2008), De Jong *et al.* (2014)
TEM-106	2.0	Belgium	Smet *et al.* (2008)

[1]Organic farming.
[2]Conventional farming.
[3]Chicken meat.
[4]Poultry.

Table 4. Recent prevalence of SHV in Enterobacteriaceae in poultry (2003–2013)

Enzyme	Prevalence (%)	Country of sample collection	Reference
SHV	11.0	Denmark	Agerso *et al.* (2014)
	0.0–47.0	Germany	Reich *et al.* (2013), Wu *et al.* (2013), Belmar Campos *et al.* (2014)
	2.33	Japan	Kawamura *et al.* (2014)
	1.0–6.0	Netherlands	Overdevest *et al.* (2011), Kluytmans *et al.* (2013), Wu *et al.* (2013)
	4.0[1]–11.0[2]	Netherlands	Leverstein-Van Hall *et al.* (2011)
	8.8	Spain	Cortes *et al.* (2010)
	0.0	UK	Wu *et al.* (2013)
SHV-2	0.5	Germany	Kola *et al.* (2012)
	0.0[3]–5.0[4]	Netherlands	Stuart *et al.* (2012)
SHV-2A	2.1	Germany	Kola *et al.* (2012)
SHV-12	5.2	Belgium	De Jong *et al.* (2014)
	12.0	Denmark, import	Carmo *et al.* (2014)
	13.2–43.9	Germany	Kola *et al.* (2012), Laube *et al.* (2013), Belmar Campos *et al.* (2014), Blaak *et al.* (2015)
	16.28	Japan	Kawamura *et al.* (2014)
	0.0[2]–16.0[1]	Netherlands	Leverstein-Van Hall *et al.* (2011)
	3.0[3]–23.0[4]	Netherlands	Stuart *et al.* (2012)
	13.0–17.0	Netherlands	Overdevest *et al.* (2011), Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
	7.8–82.7	Spain	Blanc *et al.* (2006), Egea *et al.* (2012)
	19.0	Switzerland	Geser *et al.* (2012)

[1]Chicken meat.
[2]Poultry.
[3]Organic farming.
[4]Conventional farming.

specific. Possible inhibitory effects on ESBL-producing bacteria are probably directed towards the bacteria, regardless the ability to produce ESBL or not. Nevertheless, antagonizing a bacterial family or genus like *E. coli*, which commonly harbor ESBL-genes, the prevalence of ESBL-producing bacteria might reduce subsequent to treatment with probiotics. This leads to the assumption, that antibacterial activity, competitive exclusion and the modulation of the immune system by probiotic strains may reduce ESBL-producing bacteria in broilers. Secretion of microbial substances such as organic acids, bacteriocins or hydrogen peroxide are examples for antibacterial activity. Organic acids, such as lactic acid, acetic acid and propionic acid, may contribute to a lower pH and thereby decrease the number of pathogenic bacteria (Williams, 2010; Alloui *et al.*,

Table 5. Recent prevalence of CTX-M in Enterobacteriaceae in poultry (2003–2013)

Enzyme	Prevalence (%)	Country of sample collection	Reference
CTX-M	10.59–89.0	Germany	Laube *et al.* (2013), Reich *et al.* (2013)
	12.34	China	Zheng *et al.* (2012)
CTX-M-1 group	5.3	Spain	Cortes *et al.* (2010)
CTX-M-1	19.6–44.8	Belgium	Smet *et al.* (2008), De Jong *et al.* (2014)
	5.7	China	Zheng *et al.* (2012)
	8.6–37.5	Denmark	Agerso *et al.* (2014), Carmo *et al.* (2014)
	18.0–69.0	Germany	Kola *et al.* (2012), Wu *et al.* (2013), Belmar Campos *et al.* (2014), Blaak *et al.* (2015)
	11.6	Japan	Kawamura *et al.* (2014)
	28.4–69.0	Netherlands	Kluytmans *et al.* (2013), Wu *et al.* (2013), Huijbers *et al.* (2014)
	42.0[1]–56.0[2]	Netherlands	Stuart *et al.* (2012)
	49.0[3], [4]	Netherlands	Leverstein-Van Hall *et al.* (2011)
	1.6–3.2	Spain	Blanc *et al.* (2006), Egea *et al.* (2012), Overdevest *et al.* (2014)
	71.0	Switzerland	Geser *et al.* (2012)
	73.0–100.0	UK	Toszeghy *et al.* (2012), Wu *et al.* (2013)
CTX-M-2	1.7–7.8	Belgium	Smet *et al.* (2008), De Jong *et al.* (2014)
	1.7	Denmark, import	Carmo *et al.* (2014)
	0.34–1.1	Germany	Kola *et al.* (2012), Belmar Campos *et al.* (2014), Blaak *et al.* (2015)
	51.2	Japan	Kawamura *et al.* (2014)
	0.0[2]–7.0[1]	Netherlands	Stuart *et al.* (2012)
	1.0–9.0	Netherlands	Leverstein-Van Hall *et al.* (2011), Overdevest *et al.* (2012), Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
CTX-M-3	0.63	China	Zheng *et al.* (2012)
	4.7	Japan	Kawamura *et al.* (2014)
	6.6	UK	Toszeghy *et al.* (2012)
CTX-M-8	20.9	Japan	Kawamura *et al.* (2014)
CTX-M-9 group	64.9	Spain	Cortes *et al.* (2010)
CTX-M-9	7.28	China	Zheng *et al.* (2012)
	1.7	Belgium	De Jong *et al.* (2014)
	0.0	Germany	Wu *et al.* (2013)
	1.0–5.0	Netherlands	Kluytmans *et al.* (2013), Wu *et al.* (2013)
	0.0–14.1	Spain	Blanc *et al.* (2006), Egea *et al.* (2012)
	0.0	UK	Wu *et al.* (2013)
CTX-M-14	3.48	China	Zheng *et al.* (2012)
	5.9	Belgium	Smet *et al.* (2008)
	0.34–5.7	Germany	Belmar Campos *et al.* (2014)
	1.0–2.3	Netherlands	Overdevest *et al.* (2012), Kluytmans *et al.* (2013), Huijbers *et al.* (2014)
	45.3	Spain	Blanc *et al.* (2006)
CTX-M-15	0.32	China	Zheng *et al.* (2012)
	2.0	Belgium	Smet *et al.* (2008)
	0.0–0.34	Germany	Belmar Campos *et al.* (2014), Blaak *et al.* (2015)
	11.6	Japan	Kawamura *et al.* (2014)
	0.0–1.2	Netherlands	Overdevest *et al.* (2011), Kluytmans *et al.* (2013)
	0.0–3.8	Spain	Egea *et al.* (2012)
	17.0	UK	Toszeghy *et al.* (2012)
CTX-M-24	0.95	China	Zheng *et al.* (2012)
CTX-M-27	0.32	China	Zheng *et al.* (2012)
	0.34	Germany	Blaak *et al.* (2015)
CTX-M-32	1.1	Netherlands	Huijbers *et al.* (2014)
	1.9–8.1	Spain	Blanc *et al.* (2006), Egea *et al.* (2012)
CTX-M-55	4.75	China	Zheng *et al.* (2012)
CTX-M-65	0.5	Germany	Kola *et al.* (2012)
CTX-M-65	1.9	China	Zheng *et al.* (2012)
CTX-M-84	0.0	Netherlands	Kluytmans *et al.* (2013)
CTX-M-98	0.0	China	Zheng *et al.* (2012)
CTX-M-102	0.32	China	Zheng *et al.* (2012)
CTX-M-104	0.32	China	Zheng *et al.* (2012)
CTX-M-NT	3.3	UK	Toszeghy *et al.* (2012)

[1]Conventional farming.
[2]Organic farming.
[3]Poultry.
[4]Chicken meat.

2013). By rivaling for nutrition and attaching to the intestinal mucosa, probiotic strains may counteract the advancement of pathogens in the gastrointestinal tract of poultry. Lactobacilli commonly apply this mechanism of competitive exclusion against pathogens like *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Williams, 2010; Alloui *et al.*, 2013). Increased production of antibodies and cytokines by immune cells as well as enhanced local immune response and morphologic changes in the intestines may contribute to the antagonistic effect by probiotics, enhancing the immune system (Smith, 2014). Treatment with lactobacilli might result in stabilized tight junctions and stimulate the expression of mucins, reducing the adherence of pathogens to the epithelial cells in the intestines. A lower permeability and an enhanced local barrier, subsequent to these morphological alterations, may antagonize the uptake of pathogens (Otte and Podolsky, 2004; Doron and Gorbach, 2006). Furthermore, goblet cells, liable for local defense and reparation of the epithelium, might increase in the presence of probiotics as well (Smith, 2014). Prebiotics and synbiotics, a combination of pro- and prebiotics, may enhance the antagonistic effects of probiotics even further (Awad *et al.*, 2009; Huyghebaert *et al.*, 2011; Alloui *et al.*, 2013). As these mechanisms to combat pathogens may implement potential lethal threats on ESBL-producing bacteria the consequence to such stress must be considered (Boor, 2006). Genes encoding for ESBL are frequently located on plasmids, which may be transferred to other *Enterobacteriacea* by conjugation (Händel *et al.*, 2015; Yamaichi *et al.*, 2015; Porse *et al.*, 2016). Whether the stress induced by potential reduction measurements may induce higher conjugation frequencies has yet to be investigated.

Due to the correlation between antibiotic, zinc and copper resistance, a reduction of zinc and copper contents in animal feed may help to combat the prevalence of ESBL-producing bacteria in poultry. Already, the European Food Safety Authority (EFSA) recommended lower maximum copper contents in piglet and cattle feed in August 2016 (https://www.efsa.europa.eu/en/press/news/160809a). Correspondingly, the Committee for Medicinal Products for Veterinary Use (CVMP) recommended to withdraw veterinary medicinal products containing zinc oxide from the market in December 2016 (http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2016/12/news_detail_002661.jsp&mid=WC0b01ac058004d5c1).

Nutrition has a major impact on the gastrointestinal composition and antibiotic resistance in people and animals. The diversity and quantity of genes encoding for antibiotic resistance obtained from intestinal bacteria from obese children decreased due to an alteration of the dominant microbial fermentation source from protein to carbohydrates. Especially mechanisms for target alteration and efflux pumps were affected (Wu *et al.*, 2016).

Besides from optimizing feed and feed additives, an ESBL-free environment is necessary to keep poultry flocks free from new infections. However, this is almost impossible due to the high prevalence and spread of ESBL-producing bacteria (Hiroi *et al.*, 2012a). Nevertheless, high biosecurity, a low number of persons entering the stables as well as thorough cleaning and disinfection, may reduce the prevalence of ESBL-producing bacteria in poultry farms. Limiting the number of chicks' suppliers may also reduce prevalence and diversity of ESBL types in poultry farms (Mo *et al.*, 2016). In order to avoid international carryover, traded animals must not carry ESBL-producers. Therefore, an ESBL-reduction strategy should include all levels of the poultry production pyramid to be successful (Stuart *et al.*, 2012). Producers of grandparent chicks should aim for ESBL-free flocks and regular controls.

Conclusions

CTX-M-1, TEM-52 and SHV-12 are the extended-spectrum ß-lactamases most frequently detected in poultry. The high prevalence of ESBL-producing bacteria in poultry provides a global challenge, which should be addressed with preventive reduction measurements on all levels of the poultry production system, the environment and dietary factors.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S1466252317000020.

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2.2. Conjugation of extended-spectrum beta-lactamase carrying plasmids in *Enterobacteriaceae*

Bacteria stand out from cells due to their unique ability to interchange genes vertically (within the species) and horizontally (across species borders). Genes spread not only vertically through cell division within one species but also horizontally to other species and genera. Genes transfer horizontally by conjugation, where plasmids spread from cell to cell via direct cell-to-cell contact, transduction, which is phage-assisted, and natural transformation, where cells take up free DNA from the environment (Gaze et al., 2013). The genetic elements involved in gene transfer are plasmids, genetic islands, integrons and bacteriophages (Davies and Davies, 2010; Gaze et al., 2013). Genes located on the chromosome can be transferred to plasmids and back and thereby transfer genes to other bacterial species and genera by conjugation (Gaze et al., 2013). Another way for gene transfer is cell to cell fusion, promoted in niches with high bacterial density and high diversity, such as biofilms (Davies and Davies, 2010).

Conjugation enables bacteria to exchange genes located on mobile genetic elements. Thereby, antibiotic resistance can spread between bacteria of different species and genera, even in the absence of selective pressure. ESBL encoding genes are frequently located on conjugative plasmids (Apata, 2009; Carattoli, 2013; Shahada et al., 2013). These plasmids can be transferred from the original host (donor) to a bacterium deficient of this specific plasmid (recipient) and thereby create resistant bacteria of the same or different species (transconjugant) (Norman et al., 2009). There is a common assumption that the acceptance of plasmids causes fitness costs to the recipient, which would discard the mobile genomic element, unless enhancing survival chances. Correspondingly, transconjugants of *Klebsiella pneumonia* and *E. coli* recipients and a *Klebsiella pneumonia* donor showed reduced growth rates after the uptake of an IncN plasmid (Porse et al., 2016). However, this statement is considered controversial as the reduction in growth might not be severe enough to cause the discard of the plasmid (Dahlberg and Chao, 2003; Koraimann and Wagner, 2014). The plasmids R1 and R4 remained in a bacterial community for the entire experiment, lasting approximately 1100 generations, in the absence of a selective pressure (Dahlberg and Chao, 2003). A review attending to this question argues that if the plasmid acquisition would cause such heavy fitness costs, their prevalence would be much lower in bacteria (Koraimann and Wagner, 2014). Accordingly, in the absence of antibiotics, 50 % of the transconjugants retained their IncN plasmid for 2.5 to 12.5 days (20 to 100 generations of bacterial cells) and 100 % of the *K. pneumoniae* donor remained positive for the entire experiment (35 days, 280 generations) (Porse et al., 2016). A cultivation without antibiotics, equivalent to 25 generations, did not cause a loss of the ESBL-carrying (TEM and CTX-M-15) IncI1 plasmid in an *E. coli* donor strain (Yamaichi et al., 2015). Also, no impact on growth, fitness or virulence of the host was detected after obtaining the *bla*-carrying plasmid pCT. Additionally, in the absence of antibiotics, the plasmids remained in the host and daughter cells for the entire examination period, approximately 70 generations (Cottell et al., 2012). This indicates that ESBL-producing bacteria inhabiting the intestinal tract may maintain their *bla*-carrying plasmids even when the animals do not have any contact with antibiotics.

Several studies were conducted to investigate the transfer of ESBL-carrying plasmids between *Enterobacteriaceae*, a summary is provided in Table 2.1. Several factors, such as strain, co-incubation time or cell density (donor and recipient), impact on conjugation events (Händel et al., 2015) (Table 2.1).

Besides the genetic spread across bacterial species and genera, transmission between animals was suggested. Hence, it was shown that ESBL-carrying plasmids can be transferred from avian to human *E. coli* strains (Sarowska et al., 2009; Smet et al., 2011). This indicates that reducing the usage of antibiotics in animal production will not be sufficient to battle the issue of antibiotic resistance, but that there might be other, more effective strategies to reduce the prevalence and spread of antimicrobial resistant genes via conjugation.

2.3. Impact of feed additives on ESBL-producing *Enterobacteriaceae*

As antibiotic resistance and with it ESBL-producing *Enterobacteriaceae*, gained high priority due to the threat it exerts on global public health and economy, means to reduce prevalence and horizontal transfer of resistance genes are requested (Tang et al., 2017; WHO, 2017; WHO, 2014). Feed additives are among the potential parameters discussed in this context.

2.3.1. Impact on prevalence

Feed additives are frequently used in poultry to reduce the number of undesired bacteria or potential pathogens (Huyghebaert et al., 2011; Smith, 2014; Yadav and Jha, 2019). Direct-fed microbials (DFM), prebiotics, phytobiotics, minerals and short chain fatty acids are examples of feed additives which have been associated with an altered bacterial gastrointestinal community in poultry (EMA and EFSA, 2017). Many studies report a positive impact of these supplements on the “beneficial microbiota” while “pathogenic bacteria” were reduced (Alloui et al., 2013; Huyghebaert et al., 2011; Williams, 2010). This classification represents a tremendous simplification and bears some major biases. Commonly, *Lactobacillus* spp. is considered as beneficial, while *E. coli* was classified potential pathogenic in various studies. Still, pathogenic features have been reported in *Lactobacillus* spp. and *E. coli* may also serve as a probiotic product (Beimfohr, 2016; Cannon et al., 2005; Harty et al., 1994). These are just two examples depicturing that conclusions based on changes on species level should be interpreted with caution. As DFM and essential oils were used in the *in vivo* trial of the present thesis, the impact of these feed additives is discussed below.

2.3.1.1. Direct-fed microbials

Probiotics, defined as “live microorganisms, which when consumed in adequate amounts, confer a health effect on the host” (FAO et al., 2006) are often referred to as beneficial microorganisms. Due to the above discussed regarding bacterial classification as good or bad species, it is advised to use the term ‘direct-fed microbials’ when it comes to bacterial feed additives (Flint and Garner, 2009). The mechanisms behind the effectiveness of DFM are manifold and were described in several reviews (Alloui et al., 2013; Clavijo and Florez, 2018; Huyghebaert et al., 2011; Yadav and Jha, 2019). Also, DFM may help to protect broilers from ESBL-producing bacteria. Likewise, by feeding a commercial competitive exclusion product, comprising a mixture of commensal *E. coli* strains, the number of ESBL-producing *E. coli* could be reduced in the cecal content of broilers (Nuotio et al., 2013). The prevalence of ESBL-producing *E. coli* and their transmission between animals was successfully reduced by a further commercial DFM product, comprising an undefined microbial community derived from a healthy hen (Ceccarelli et al., 2017). Similarly, the administration of a competitive exclusion culture to broiler chicks reduced the colonization of their ceca with ESBL-producing *E. coli* (Methner et al., 2019). None of these studies characterized the microbial composition of the DFM product quantitatively.

2.3.1.2. Phytogenic feed additives

Phytogenic feed additives, or botanicals, phytogenics or phytobiotics, as they are also frequently termed (Gheisar and Kim, 2018; Grashorn, 2010; Windisch et al., 2008b), can be classified into the four groups of “herbs (flowering, nonwoody, and nonpersistent plants), spices (herbs with an intensive smell or taste commonly added to human food), essential oils (volatile lipophilic compounds derived by cold expression or by steam alcohol distillation) or oleoresins (extracts derived by nonaqueous solvents)” (Windisch et al., 2008b). Of these feed additives, essential oils represent the most common use in poultry nutrition (Clavijo and Florez, 2018; Diaz-Sanchez et al., 2015; Gheisar and Kim, 2018; Windisch et al., 2008a). Differences in susceptibility to essential oils between reference strains (more susceptible) and multi drug resistance strains were reported (Afshar et al., 2016). Tea tree oil showed bactericidal activity against different Gram positive and negative multi drug resistant bacteria at concentrations of 0.25 – 2%. Simultaneously, synergistic effects with several antibiotics were observed, increasing the susceptibility against these drugs in the presence of tea tree oil (Oliva et al., 2018). Antibiotic activity against ESBL-producing *E. coli* was observed in oregano essential oils. Additionally, synergistic and additive effects occurred when combined with different kind

of antibiotics or silver nanoparticles (Scandorieiro et al., 2016; Si et al., 2008). The screening of 8 essential oils and their components on 11 ESBL-producing *Klebsiella pneumoniae* isolates showed higher (7/11) or equal (4/11) minimum inhibitory concentrations than the compared antibiotic substance for these compounds (Orhan et al., 2011). Also, differences between different multi drug resistant strains were reported. Compared to multi drug resistant *Klebsiella* spp., multi drug resistant *E. coli* strains were less susceptible to essential oils derived from oregano, thyme and sage (Fournomiti et al., 2015). Reviews, describing additional, synergistic and indifferent effects of essential oils (phytogenic source and major constituent provided) and antibiotics on various bacterial strains, as well as the mechanisms behind these effects were provided by Yap et al. (2013) and Aelenei et al. (2016).

2.3.2. Impact on conjugation

Strategies to combat bacterial conjugation have been studied since the 1970s (Cabezon et al., 2017). Traditionally, results were presented and interpreted on the basis of conjugation frequency (CF) calculated as transconjugants/donor (CF/D) or transconjugants/recipients (CF/R) (Table 2.1). An observed increase or decrease was evaluated as a positive or negative impact on plasmid transfer. The change of bacterial growth was frequently neglected, which led to a bias in the evaluation as observed effects often can be explained by the influence of the feed additive on bacterial growth rather than a change in plasmid transfer rate (Cabezon et al., 2017; Lopatkin et al., 2016a). While the impact of different stressors, such as antibiotics on conjugation including *bla*-carrying plasmids has been thoroughly investigated, the influence of feed additives was only evaluated in few studies. A negative effect of linalool, R-carvone, S-carvone, eugenol, borneol and thymol and eucalyptol (84 %, 67 %, 66 %, 64 %, 63 %, 51 % and 31 % reduction) on plasmid (pKM101) transfer in *E. coli* was described (Skalicka-Wozniak et al., 2018).

Genes encoding for heavy metal resistance and pathogenic features are frequently located on the same mobile elements as antibiotic resistance and are therefore often co-selected (Silver and Phung, 1996; Vahjen et al., 2015). Thus, minerals may enhance conjugation frequencies due to the selective pressure. On the contrary, within an incubation of 2 hours on stainless steel or copper, conjugation with ESBL-producing *E. coli* and *Klebsiella pneumoniae* donors and *E. coli* recipients declined or fell under detection limit respectively (Warnes et al., 2012). Metal concentrations causing 20 % or 50 % reduction in donor and recipient growth resulted in a decreased transfer of the pKJK5-plasmid from its *E. coli* donor to recipients of a soil matrix (Klumper et al., 2017). It should be noticed that the mentioned plasmid did not harbor any resistance genes against the used metals (As, Cu, Cd, Ni, Zn) and thereby no selective pressure influenced the results.

The impact of short chain fatty acids (lactate) on conjugation was described as a possible explanation for the negative effect of the direct-fed microbials *Streptococcus thermophilus*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus bulgaricus* on conjugation (Maisonneuve et al., 2000; Maisonneuve et al., 2001, 2002; Sabia et al., 2009; Tallmeister et al., 1977).

As the ceca provide a diverse microbial community with a high total amount of bacteria (Rehman et al., 2007; Yeoman et al., 2012), conjugation is likely to occur in this part of the intestinal tract. When evaluating the impact of feed additives, it is crucial to determine if these products reach the intestinal part they are attended for. In the case of short chain fatty acids, there is a high possibility that the substances are metabolized before reaching the hind gut (Hume et al., 1993).

Against the background depictured in the relevant literature, it became clear that poultry is an important factor in the battle against ESBL-producing *Enterobacteriaceae* and prevalence and resistance gene transfer must be considered simultaneously to control ESBL-producing *Enterobacteriaceae*. Also, it was hypothesized that

- stress may increase conjugation frequencies
- feed additives such as DFM and phytogenic additives may decrease conjugation frequencies and that synergistic effects of DFM and phytogenic additives may exist
- trace minerals may increase transfer and prevalence due to co-selection.

The impact of the stress posed on bacteria by feed additives, pH and osmolality has not been studied previously in poultry. Thus, this thesis was designed to investigate possible ‘side effects’ on conjugation frequencies by common measurements to reduce pathogens in the gastrointestinal tract by nutrition related interventions.

Table 2.1: Conjugation events in *Enterobacteriaceae* mating pairs

Donor	Recipient	Donor: recipient concentration	Incubation conditions	Conjugation frequency	ESBL type	Plasmid	Reference
E. coli O104	*E. coli* MKW278	1:1	5 h, 37 °C	5×10^{-2} CF/D	TEM, CTX-M-15	IncI1	(Yamaichi et al., 2015)
E. coli MKW278	*E. coli* MC1061	1:1	5 h, 37 °C	5×10^{-1} CF/D	TEM, CTX-M-15	IncI1	
E. coli MKW278	*E. coli* CAG18439	1:1	5 h, 37 °C	5×10^{-1} CF/D	TEM, CTX-M-15	IncI1	
E. coli MC1061	*E. coli* MKW278	1:1	5 h, 37 °C	10^{-2} CF/D	TEM, CTX-M-15	IncI1	
E. coli MC1061	*E. coli* CAG18439	1:1	5 h, 37 °C	2.5×10^{-2} CF/D	TEM, CTX-M-15	IncI1	
E. coli MC1061	*K. pneumoniae*	1:1	5 h, 37 °C	5×10^{-5} CF/D	TEM, CTX-M-15	IncI1	
E. coli TOP10	*K. pneumoniae* Kp08	1:1	2 h, 37 °C	$7 \times 10^{-14} – 1 \times 10^{-11}$	CTX-M-15, TEM-1, OXA-1	IncN,	(Porse et al., 2016)
E. coli TOP10	*E. coli* Ec37	1:1	2 h, 37 °C	$5 \times 10^{-14} – 5 \times 10^{-13}$	CTX-M-15, TEM-1, OXA-1	IncN	
E. coli TOP10	*E. coli* Ec38	1:1	2 h, 37 °C	$4 \times 10^{-13} – 1 \times 10^{-12}$	CTX-M-15, TEM-1, OXA-1	IncN	
E. coli TOP10	*K. pneumoniae* Kp33	1:1	2 h, 37 °C	$1 \times 10^{-11} – 2 \times 10^{-11}$	CTX-M-15, TEM-1, OXA-1	IncN	
E. coli ESBL242	*E. coli* MG1655 YFP	1:1, $3 \times 10^5 – 10^8$ cells/mL	1 h, 37 °C	≥ 7.5×10^4 transconjugants/mL	CTX-M-1	IncI1	(Händel et al., 2015)
E. coli[1]	*E. coli* K12 C600	1:1, $3 \times 10^5 – 10^8$ cells/mL		$2.3 \times 10^{-7} – 5.2 \times 10^{-1}$ CF/D	CTX-M	nd	(Franiczek and Krzyzanowska, 2014)
E. coli C159/11	*E. coli* DH5α	nd	3 h, 37 °C	$10^{-4} – 10^{-5}$ CF/D	ESBL+	pCT	(Cottell et al., 2014)
E. coli C159/11	*Salmonella* Typhimurium SL1344	nd	3 h, 37 °C	10^{-5} CF/D	ESBL+	pCT	
Enterobacter cloacae	*E. coli*	nd	nd	10^{-5} CF/D	CTX-M-15, SHV-12	IncHI2	(Nilsen et al., 2013)
E. coli NCTC 13441	*E. coli*	10:1	2 h, 21 °C	3×10^{-3} CF/D	CTX-M-15	pEK499	(Warnes et al., 2012)
K. pneumoniae NCTC 13443	*E. coli*	10:1, 5×10^8 cells	2 h, 21 °C	2×10^{-6} CF/D	NDM-1	nd	(Warnes et al., 2012)
E. coli DH5α	*E. coli* J53-2	nd	3 h, 37 °C	$1.7 \times 10^2 – 7.3 \times 10^8$ CF/R×R/D	CTX-M-14	pCT	(Cottell et al., 2012)
E. coli DH5α	*E. coli* 3950	nd	3 h, 37 °C	$1.4 \times 10^5 – 6.1 \times 10^5$ CF/R×R/D	CTX-M-14	pCT	

Donor	Recipient	Donor: recipient concentration	Incubation conditions	Conjugation frequency	ESBL type	Plasmid	Reference
E. coli DH5α	*Salmonella* Typhimurium	nd	3 h, 37 °C	$5.3 \times 10^{6} – 6.0 \times 10^{6}$ CF/R×R/D	CTX-M-14	pCT	
E. coli[2]	*E. coli* AB 1157	1:1	24 h, 36 °C	$3 – 4 \times 10^{-5}$ CF/D	ESBL+	nd	(Vaidya, 2011)
E. coli B1-54 (avian)	*E. coli* (human)	nd	2 d, 37 °C	2.5×10^{-3} CF/R	TEM-52	IncI1	(Smet et al., 2011)
E. coli B1-54 (avian)	*E. coli* (human)	nd	3 d, 37 °C	2.5×10^{-5} CF/R	TEM-52	IncI1	
E. coli	*E. coli*	1:1, 10^{9} cfu	24 h	10^{-10} CF/R	CTX-M-27	nd	(Oguri et al., 2011)
K. pneumoniae ATCC 700603 or *K. pneumonia* 20	*Salmonella enterica* serovar Enteritidis	1:1	24 h, 37°C	$10^{-6} – 10^{-3}$ CF/D	SHV-18	nd	(Sarowska et al., 2009)
K. pneumoniae ATCC 700603 or *K. pneumonia* 20	*Salmonella enterica* serovar Typhimurium	1:1	24 h, 37°C	nd	SHV-18	nd	
K. pneumoniae ATCC 700603 or *K. pneumonia* 20	*Salmonella enterica* serovar Hardar	1:1	24 h, 37°C	10^{-8} CF/D	SHV-18	nd	(Sarowska et al., 2009)
E. coli[3]	*E. coli* K12 C600	1:1, 10^{9} cfu/mL	24 h, 37°C	$1.5 \times 10^{-5} – 4.5 \times 10^{-1}$ CF/D	ESBL+	nd	(Franiczek et al., 2007)
K. pneumoniae	*E. coli* K12 C600	1:1, 10^{9} cfu/mL	24 h, 37°C	$1.6 \times 10^{-5} – 5.8 \times 10^{-1}$ CF/D	ESBL+	nd	
Klebsiella oxyta	*E. coli* K12 C600	1:1, 10^{9} cfu/mL	24 h, 37°C	$9.3 \times 10^{-3} – 7.7 \times 10^{-2}$ CF/D	ESBL+	nd	
Citrobacter amaloniaticus VA-1340/03	*E. coli* MKD-135	nd	nd	7.3×10^{-2} CF/R	CTX-M-1	nd	(Mugnaioli et al., 2005)
E. coli VA-1339/03	*E. coli* MKD-135	nd	nd	1.3×10^{-1} CF/R	CTX-M-1, TEM-1	nd	
E. coli VA-1341/03	*E. coli* MKD-135	nd	nd	4.6×10^{-2} CF/R	CTX-M-1	nd	
M. morganii VA-1340/03	*E. coli* MKD-135	nd	nd	9.6×10^{-3} CF/R	CTX-M-1	nd	
K. pneumoniae 283	*Citrobacter freundii* Cf-1	nd	24 h, 37°C	3.2×10^{-2} CF/R	CTX-M, TEM, SHV	nd	(Sanchez et al., 2006)
K. pneumoniae 283	*Salmonella* Typhimurium Sal-21	nd	24 h, 37°C	6.5×10^{-4} CF/R	CTX-M, TEM, SHV	nd	
K. pneumoniae 283	*Serratia marcescens* S-25	nd	24 h, 37°C	4.9×10^{-3} CF/R	CTX-M, TEM, SHV	nd	

Donor	Recipient	Donor: recipient concentration	Incubation conditions	Conjugation frequency	ESBL type	Plasmid	Reference
K. pneumoniae 283	*E. coli* Ec-151	nd	24 h, 37°C	2.0×10^{-2} CF/R	CTX-M, TEM, SHV	nd	
K. pneumoniae 295	*Citrobacter freundii* Cf-1	nd	24 h, 37°C	2.7×10^{-2} CF/R	CTX-M, TEM, SHV	nd	
K. pneumoniae 295	*Salmonella* Typhimurium Sal-21	nd	24 h, 37°C	4.4×10^{-5} CF/R	CTX-M, TEM, SHV	nd	
K. pneumoniae 295	*Serratia marcescens* S-25	nd	24 h, 37°C	8.1×10^{-5} CF/R	CTX-M, TEM, SHV	nd	(Sanchez et al., 2006)
K. pneumoniae 295	*E. coli* Ec-151	nd	24 h, 37°C	9.7×10^{-3} CF/R	CTX-M, TEM, SHV	nd	
K. pneumoniae 329	*Citrobacter freundii* Cf-1	nd	24 h, 37°C	4.2×10^{-2} CF/R	CTX-M, TEM	nd	
K. pneumoniae 329	*Salmonella* Typhimurium Sal-21	nd	24 h, 37°C	7.8×10^{-7} CF/R	CTX-M, TEM	nd	
K. pneumoniae 329	*Serratia marcescens* S-25	nd	24 h, 37°C	9.3×10^{-5} CF/R	CTX-M, TEM	nd	
K. pneumoniae 329	*E. coli* Ec-151	nd	24 h, 37°C	5.1×10^{-4} CF/R	CTX-M, TEM	nd	

nd: not determined, [1]48 clinical isolates, [2]70 clinical isolates, [3]32 clinical isolates, [4]17 clinical isolates, [5]2 clinical isolates, ESBL+: plasmid carries *bla* genes, CF/D: conjugation frequency= transconjugants/donor, CF/R×R/D: conjugation frequency= transconjugants/(recipients×(initial donor : recipients)), CF/R: conjugation frequency= transconjugants/recipient

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In vitro conjugation kinetics of AmpC, broad spectrum and extended-spectrum beta-lactamase-producing *Escherichia coli* donors and various *Enterobacteriaceae* recipients

Eva-Maria Saliu*, Jürgen Zentek and Wilfried Vahjen

Abstract

Background: Extended spectrum beta-lactamase (ESBL)-producing enterobacteria pose a major hazard to public health. Due to the possibility of genetic transfer, ESBL genes might spread to pathogenic enterobacterial strains. Thus, information on possible genetic transfer between enterobacteria is of high interest. It was therefore the aim of this in vitro study to screen the capacity of a wide range of *Enterobacteriaceae* for differences in conjugation at different time points with five ESBL-producing *Escherichia coli* strains.

Results: Conjugation frequencies for five potential *E. coli* donor strains producing the enzymes CTX-M-1, CTX-M-15, SHV-12, TEM-1, TEM-52 and CMY-2, and six potential recipient strains commonly detected in the gastrointestinal tract of poultry (*E. coli, Serratia marcescens* subsp. *marcescens*, *Enterobacter cloacae, Salmonella* (*S.*) *enterica* serovar Typhimurium and *Proteus mirabilis*) were obtained. Different combinations of donor and recipient strains were co-incubated for between 0 and 22 h and spread on selective agar. Conjugation frequencies were calculated as transconjugants per donor.
Some donor and recipient strain combinations did not perform plasmid transfer within 22 h. Hence, the recipient *Proteus mirabilis* did not accept plasmids from any of the given donors and the *E. coli* ESBL10716 donor was unable to transfer its plasmid to any recipient. *Enterobacter cloacae* only accepted the plasmids from the donors *E. coli* ESBL10708 and *E. coli* ESBL10716 while *E. coli* ESBL10708 did not transfer its plasmid to *Serratia marcescens* subsp. *marcescens*. *E. coli* IMT11716 on the other hand did not perform conjugation with the donor *E. coli* ESBL10689. The remaining mating pairs differed in conjugation frequency, ranging from 10^{-5} to 10^{-9} transconjugants/donor. The earliest conjugation events were detected after 4 h. However, some mating pairs turned positive only after 22 h of coincubation.

Conclusion: A suitable mating pair for future in vivo studies to combat transfer of antibiotic resistance to pathogenic bacteria in broiler chicken was determined. The results of this study also suggest that the kinetic of conjugation differs between mating pairs and is independent of species origin. This should be considered when performing conjugation experiments.

Keywords: ESBL, Extended-spectrum ß-lactamases, Horizontal gene transfer, Antibiotic resistance, Poultry

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Background

Consequential to the global increase of multidrug resistant bacteria, severe economic and public health related costs have been predicted to rise significantly in the near future [1]. In this context, extended-spectrum ß-lactamase (ESBL)-producing *Enterobacteriaceae* were identified as one of the antibiotic resistant bacterial groups currently posing the highest threat to public health [2]. These bacteria have been detected in humans and animals equally. Within livestock, the highest prevalence of ESBL-producing bacteria was observed in poultry [3]. ESBL-producing enterobacteria have been detected ubiquitous in poultry droppings and meat as well as the environment surrounding poultry [4, 5]. CTX-M-1, SHV-12 and TEM-52 are the most frequently detected ESBL-types in European chicken with *Escherichia coli* and *Salmonella* spp. as the most common bacterial hosts [3]. Often, these enterobacteria are associated with the commensal bacterial populations in animals. As ESBL-producing enterobacteria are most often non-pathogenic, no clinical signs or impact on the performance are observed [3].

ESBL encoding genes are generally located on plasmids, which can be transferred between bacterial strains and species, including pathogenic strains [4, 6, 7]. Thereby, harmless, unnoticed colonizations with ESBL-producing bacteria can lead to diseases, which are hard to cure with antibiotics, if the recipient also carries pathogenic traits. For some ESBL-carrying plasmids, conjugation is not linked to fitness costs for the recipient and may be passed on for generations, even in the absence of antibiotics [7–10].

A transmission of ESBL-carrying bacteria from animals to humans, were the animals constitute a reservoir for human infections, has been suggested [11–13]. Antibiotic resistant bacteria may spread to humans via direct or indirect contact with animals, animal food products, fecal matter or manure [4, 14, 15]. Correspondingly, the introduction of a TEM-52-carrying *E. coli* from poultry to the microbial community of a human stool sample resulted in the establishment of the strain and plasmid transfer to an *E. coli* of human origin [16]. Both donor and transconjugants were present at a lower concentration than the human bacterial strains. A simulated treatment with a selective antibiotic substance (cefotaxime) shifted the balance to the benefit of the resistant strains, which remained at high concentration, equal to the indigenous microbiota, even days after the termination of the treatment [16]. The aforementioned highlights a potential pathway for resistant bacteria from animal origin to persistently colonize the human gastrointestinal tract. Nevertheless, this transmission from animals to humans, and especially to the general population, seems to appear at a low rate [17].

The present study was undertaken to investigate the changes of conjugation events at different time points as a first step to estimate the possible transfer frequencies of ESBL genes in the intestinal tract and in the environment. Conjugation kinetics of ESBL-carrying *Enterobacteriaceae* strains commonly detected in poultry were obtained in vitro within a 22 h timeframe.

Results

Antibiotic resistance screening of recipient strains

The results obtained from the agar disc diffusion tests (supplementary data: Table S1) identified 6 potential recipients, 5 potential donors and 24 donor-recipient combinations for the screening assay (supplementary data: Fig. S1). Suitable antibiotic concentrations for the preparation of the double antibiotic agars were obtained from the broth dilution (supplementary data: Table S2). The levels of antibiotic supplementation inhibited the growth of the donor, while the recipients growth was not affected and vice versa. This led to the usage of 2 or 8 µg CTX/mL agar, 25 µg colistin (CT)/mL agar, 25 or 100 µg chloramphenicol (C)/mL agar, 25 µg sulfamethoxazole/trimethoprim (SXT)/mL agar and 30 µg nitrofurantoin (F)/mL agar.

Screening for suitable mating pairs

The results from the screening for conjugation are presented in Table 1. *Proteus mirabilis* DSM 4479 was excluded from further trials, because this strain showed no

Table 1 Screening for conjugation

Donor Recipient	***E. coli*** ESBL10682 (CTX-M-1)	***E. coli*** ESBL10689 (TEM-52)	***E. coli*** ESBL10708 (SHV-12)	***E. coli*** ESBL10716 (CTX-M-15)	***E. coli*** ESBL10717 (CMY-2, TEM-1)
***E. coli* IMT 20751/402**	+	+	nd	nd	+
***E. coli* IMT11716 (APEC)**	+	–	nd	nd	+
***S. enterica* serovar Typhimurium L1219-R32**	+	nd	+	nd	nd
***Serratia marcescens* subsp. *marcescens* DSM 30122**	+	+	–	–	nd
***Enterobacter cloacae* DSM 30060**	–	–	+	–	+
***Proteus mirabilis* DSM 4479**	–	–	–	–	–

Nd not determined, + = bacterial growth, – = no growth/< 5 colonies

conjugation with any donor used. The same applied to the potential donor *E. coli* 10716 which did not transfer plasmids to the three potential recipients *Serratia marcescens* subsp. *marcescens* DSM 30122, *Enterobacter cloacae* DSM 30060 and *Proteus mirabilis* DSM 4479. As the remaining 4 donors and 5 recipients proved the ability to produce transconjugants, they were further studied for the kinetic study.

Kinetic assay

Varying conjugation frequencies at different time points were observed for different donor and recipient pairs within the 22 h incubation period (Table 2). The earliest conjugation events were observed after 4 h, while transconjugants for other mating pairs only appeared after 22 h incubation. Also, the highest conjugation frequency was detected after 4 h incubation of donor *E. coli* 10682 and the recipient strain *S. enterica* serovar Typhimurium L1219-R32. While some mating pairs remained comparable conjugation frequencies throughout all measured time points, others differed depending on the incubation time. Interestingly, the development of conjugation frequencies with time differed for recipients and donors when mating with other strains.

In summary, observed conjugation frequencies were within the range of 10^{-9} – 10^{-5} transconjugants/donor. The highest conjugation frequency was observed for the *S. enterica* serovar Typhimurium recipient strain and a CTX-M-1 carrying plasmid. For the majority of the investigated strains, no transconjugants were observed within 8 h coincubation. For 4 of the mating pairs, transconjugants were observed within 4 h of co-cultivation. Differences in conjugation frequency and incubation period until first detection of transconjugants were observed depending on bacteria genera, species and strain.

Discussion

The present in vitro study investigated the conjugation kinetics between ESBL-producing *E. coli* donors and various *Enterobacteriaceae* recipients. Donors were cultivated on agar or in broth containing relatively high concentrations of 8 μg CTX/mL, according to the results from the initial resistance screening. Surprisingly, the same donors were inhibited by 30 μg CTX discs in the agar diffusion assay. The Clinical Laboratory Standards Institute (CLSI) suggests this disc type for screening for ESBL-producing bacteria or 1 μg CTX/mL for broth microdilution [18]. Hence, the donors would not have been identified in the recommended agar disc diffusion test but easily be recognized as ESBL-producers in the broth microdilution test.

The propensity of ESBL-producing donors to transfer their plasmids to various recipients differed significantly between strains from the same species in the present study. *E. coli* ESBL10716 did not transfer its plasmid to any of the recipients provided and the *Proteus mirabilis* recipient did not mate with any of the given donors. Plausible reasons for the absence of conjugation events are that the recipients may already harbor plasmids with the same replicon [19] or that the *bla* genes were located on the chromosome or a non-conjugative plasmid in this donor [20]. Furthermore, the incubation time might have been too short, or the initial concentration of donors and recipients was too low for conjugations to be detected [21]. The latter is unlikely, as the initial concentration of 10^5 cfu/mL is high compared to other studies [22] and the long incubation time of 22 h in media additionally increases cell concentrations. Also, higher concentrations and longer incubation times are unlikely to occur in the intestinal tract of poultry and were therefore not within the focus of this study. The transconjugants could also have been under detection limit. Here, the detection limit was 3 cfu transconjugants/mL, which makes this option rather unlikely at the given bacterial concentrations and incubation time. Finally, the recipient may harbor specific endonucleases which destroy the plasmids after uptake and thereby prevent the formation of transconjugants [23, 24]. However, as the aim of this

Table 2 Conjugation frequencies of selected donor- and recipient strains [$\log_{10}$transconjugants/donor]

Donor	***E. coli*** ESBL10682				***E. coli*** ESBL10689		***E. coli*** ESBL10708		***E. coli*** ESBL10717		
Recipient	***E. coli*** IMT 20751/402	***E. coli*** IMT11716	***S. enterica*** serovar Typhimurium L1219-R32	***Serratia marcescens*** subsp. ***marcescens*** DSM 31022	***E. coli*** IMT 20751/402	***Serratia marcescens*** subsp. ***marcescens*** DSM 31022	***Enterobacter cloacae*** DSM 30060	***S. enterica*** serovar Typhimurium L1219-R32	***E. coli*** IMT 20751/402	***E. coli*** IMT 11716	***Enterobacter cloacae*** DSM 30060
0 h	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
2 h	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
4 h	−7.00	NT	−4.98	−5.74	−7.26	NT	NT	NT	NT	NT	NT
6 h	−6.62	NT	−6.27	−7.05	−7.30	NT	NT	NT	NT	NT	NT
8 h	−6.70	NT	−5.91	−6.71	−8.48	− 6.91	NT	NT	NT	NT	NT
22 h	−6.78	−6.52	−5.92	−7.26	− 6.45	−6.98	− 5.86	−5.70	−6.80	− 6.25	−6.49

NT no transconjugants; experiments were performed in duplicates and the results were reported as their average (coefficients of variation < 10.6%)

study was to identify suitable mating pairs for in vitro experiments of this setup, the impact of the experimental conditions on the identification of mating pairs was not further investigated.

Conjugation frequencies differed between various donor and recipient strains in the employed in vitro assay. Genera and strain depending variations in conjugation frequency have been described previously [20, 25]. Some studies suggested that conjugation occurs more frequently with donor and recipients from different genera [20, 26]. Other studies described higher conjugation frequencies for mating pairs of the same species than interspecies donor/recipient combinations [10]. This corresponds with our findings for the *E. coli* ESBL10689 donor, which showed the highest conjugation frequency with a *Salmonella* recipient. This study cannot confirm a general conclusion in either direction, but rather suggests strain specific differences.

The relatively high conjugation frequencies reported in the literature compared to the results reported in the present study may depend on the usage of different strains. While some studies used different strains as donors and a consistent *E. coli* recipient [20, 26], the present study used varying donor and recipient strains. In the mentioned studies, especially *Citrobacter freundii*, a strain not investigated in the present study, revealed high conjugation frequencies, while *S. marcescens* donors led to comparatively low conjugation frequencies, similar to the results of this study. In another study, *Enterobacter* spp. donors reached an average of 10^{-5} transconjugants/donor when co-cultivated with *E. coli* recipient strains [27], compared to 10^{-6} transconjugants/donor when used as a recipient for *E. coli* donors in the present study. No information on incubation time or cell concentrations was provided. In this study, the highest conjugation frequency of 1.04×10^{-5} transconjugants/donor occurred when *E. coli* 10,682 was co-incubated for 4 h with the pathogen *S. enterica* serovar Typhimurium L1219-R32. This frequency corresponds with results obtained from conjugation trials with *Klebsiella* spp. donors and *Salmonella* spp. recipients with 24 h coincubation [25].

Higher initial concentrations of donors and recipients used in studies such as Franiczek et al. [20] or Franiczek and Krzyzanowska [26] (10^9 cells/mL compared to 10^5 cells/mL in this study) may explain the differences in conjugation frequencies. It must be mentioned that high conjugation frequencies may also be observed in experiments with low initial concentrations [22] and that there are large differences between strains. The reason for the comparatively low initial bacterial concentration in the present study was that cell numbers were chosen according to realistic amounts present in the gastrointestinal tract [28–30]. The detection limit must thus be considered when evaluating the time frame for the first observed conjugation event. The impact of the initial concentration of the mating pair on the number of transconjugants after a given time of coincubation and thereby the detection limit of conjugation was previously described [21].

The time period until detection of transconjugants differed significantly between donors with the same recipients. Conjugation kinetics for ESBL-carrying plasmids have previously been studied, at similar time points [22], but mainly with longer intervals [16]. The present study also found that both the time period and the number of conjugation events differed between different strains. Some strains revealed a higher conjugation frequency early during incubation with declining conjugation frequencies, while other strains increased in conjugation frequency at later time points. These results suggest that the most severe differences occur within the first day and therefore short time intervals should be chosen when investigating conjugation kinetics.

When co-cultivated, the recipient strains *S. marcescens* and *S. enterica* serovar Typhimurium revealed lower growth rates than the donor strains (supplementary data: Table S3). Hence, the donor/recipient ratio and subsequent conjugation frequencies were shifted towards the donor. This effect should be considered when evaluating conjugation events as conjugation frequencies per recipient cfu would have been higher than conjugation frequencies per donor cfu. Thus, calculation of conjugation events per donor only show a simplified picture and other methods of calculation may provide different results [22, 31, 32]. However, as the present study was designed to find model strains to study conjugation kinetics in detail, addressing calculation methods was not the focus of the research and thus, the calculation of transconjugants/donor was sufficient to compare the different mating pairs.

The aim of this study was to identify mating pairs suitable for future in vivo studies in poultry. These mating pairs should comprise a donor producing an ESBL type which is frequently detected in broilers [3] and perform conjugation at bacterial concentrations commonly observed in the hindgut [28, 30]. If a chicken acquires an ESBL-producing *E. coli* from the environment, this strain may establish in the GIT [33] or simply pass through. In both cases, it may transfer plasmids to indigenous bacteria of the fowl microbiota. To address both possibilities, mating pairs should transfer the plasmid within the passage time of the ingesta. Thus, the mating pairs that fulfilled these requirements were *E. coli* ESBL10682/*E. coli* IMT 20751/402, *E. coli* ESBL10682/*S. enterica* serovar Typhimurium L1219-R32, *E. coli* ESBL10682/*Serratia marcescens* subsp. *marcescens* DSM 30122 and *E. coli* ESBL10689/*E. coli* IMT 20751/402 due to their formation of transconjugants after a relative short incubation

period. To enhance the probability of detecting the conjugation events, high transfer frequencies are preferred [21]. Hence, the mating pair *E. coli* ESBL10682/*S. enterica* serovar Typhimurium L1219-R32 complied best with these requirements. Also, *S. enterica* serovar Typhimurium is a common pathogen of importance for public health. Thus, the chosen mating pair could be utilized to address research questions focusing on this topic as well. In this study, broth mating and equal volumes of donor and recipient strains were chosen to mimic the conditions in the gastrointestinal tract of broilers and to be able to investigate the impact of stress factors on both donor and recipient strains in a follow up study. Future in vitro experiments could compare these results to filter mating and non-equal volumes of donor and recipient. Conjugation frequencies are commonly obtained from in vitro trials. To understand the impact of the complex system in the intestinal tract ex vivo and in vivo trials should follow these studies.

Conclusion

Different ESBL-carrying plasmids were transferred to recipients of the *Enterobacteriaceae* family at frequencies of 10^{-9} – 10^{-5} transconjugants/donor within 22 h with earliest events after 4 h of coincubation. This finding suggests that genetic transfer may occur within a short time period. However, differences between mating pairs and first detection of transconjugants should be considered when performing conjugation experiments. The differences in conjugation frequency did not arise from the assignment of the mating pairs to the same or different species. Finally, this study has developed a suitable mating pair for future studies investigating the impact of stress factors on the transfer of ESBL genes to pathogenic bacteria. Still, the results suggest that conclusions drawn from experiments using specific mating pairs are strain specific rather than general.

Methods

Strains and cultivation conditions

A selection of *Enterobacteriaceae* strains (supplementary data: Table S4) commonly detected in the gastrointestinal tract of poultry [28] were screened for potential recipients. The ESBL-types of the potential donors (Table 3) had previously been identified in another project [34] and comprised CTX-M-1, CTX-M-15, SHV-12, and TEM-52 as well as the ampC ß-lactamase CMY-2 and the broad spectrum lactamase TEM-1 (Table 1). The donors were chosen to match the most common ESBL-types in poultry in Europe [3]. Also, TEM-1 and CMY-2 were included to match with other parts of the EsRAM project. These enzymes were also confirmed by real time qPCR at our institute within another study. All donor strains were isolated from broilers samples within the RESET project [34]. From this large number of bacterial strains, a screening for potential donors and recipients and a creation of mating pairs was performed based on the strains' antibiotic susceptibility profiles. The mating pairs, that performed conjugation within 22 h, were then further analyzed in conjugation kinetic experiments.

The bacterial strains were stored in cryo stocks at − 80 °C and cultivated aerobically overnight at 37 °C in Mueller-Hinton broth (MHB) (Carl Roth GmbH + Co. KG, Germany) with or without antibiotic supplementation, depending on the experiment. The pH value of the medium was 7.5 at room temperature. MacConkey agar (Carl Roth GmbH + Co. KG, Germany) was applied for all plates, except for an agar disc diffusion assay, where Müller Hinton agar (Carl Roth GmbH + Co. KG, Germany) was applied, and incubated aerobically overnight at 37 °C.

Antibiotic resistance screening

To perform conjugation trials, potential donors and recipients were chosen based on mismatching antibiotic resistance profiles. Resistance and sensitivity to 20 different antibiotic substances were determined for the potential recipients ($n = 32$) and the potential donors ($n = 5$) by agar disc diffusion tests (supplementary data: Table S1). In short, the investigated strain was smeared on 25 mL Müller Hinton agar plates and 6 antibiotic containing discs were equally distributed on the surface. The plates were incubated aerobically overnight at 37 °C and inhibition zones were determined. To qualify as potential recipients, strains had to reveal an inhibition zone around the 5 μg/mL cefotaxime disc (CTX) and be resistant to an additional antibiotic substance, which inhibited the growth of at least one potential donor. This enabled the detection of transconjugants on double antibiotic MacConkey agar. Mating pairs were created from donor-recipient combinations with non-overlapping antibiotic resistances.

Specification of antibiotic resistance and susceptibility

Suitable antibiotic dosages for the inhibition of the strains were determined by examination of growth kinetics in broth microdilution tests during 24 h at 37 °C.

In short, strains were pre-cultured from cryo stocks overnight in MHB without antibiotics and subsequently washed twice in phosphate-buffered saline (PBS, Sigma-Aldrich, Chemie GmbH, Germany). The cells were re-suspended in MHB without antibiotics and diluted to 10^5 cells/mL. Minimal inhibitory concentrations (MIC) were obtained for the relevant antibiotics by broth microdilution in duplicates. Turbidity was measured at 690 nm every 10 min for 24 h in a microtiter plate reader (Infinite200Pro, Tecan Austria GmbH, Austria). Non-inoculated media served as negative control, while

Table 3 Antibiotic resistance and susceptibility of the potential donor and recipient strains from disc diffusion test

Bacteria	Indication	AK 30 μg	CN 30 μg	TOB 10 μg	ENR 5 μg	MAR 50 μg	F 300 μg	C 30 μg	CT 25 μg	PB300 IE	RD 30 μg	SXT 25 μg	CTX30 μg	CEFT 30 μg	CL 30 μg	CPD 10 μg	CTX 5 μg	IPM 10 μg	MEM 10 μg	CIP 5 μg	FOS 50 μg	TGC 15 μg
Escherichia coli	IMT 20751/402	S	S	(R)	R	R	S	R	S	S	S	R	S	S	R	R	x	S	S	S	S	S
Escherichia coli APEC	IMT11716	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	x	S	S	S	S	S
S. enterica serovar Typhimurium	L1219-R32	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	x	S	S	S	S	S
Serratia marcescens subsp. *marcescens*	DSM 30122	S	S	S	S	S	S	S	R	R	S	S	S	S	R	S	x	S	S	S	S	S
ESBL *Escherichia coli* (CTX-M-1)	ESBL 10682	S	S	S	S	S	S	S	S	S	S	S	S	?	R	R	x	x	x	x	x	x
ESBL *Escherichia coli* (TEM-52)	ESBL 10689	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	x	x	x	x	x	x
ESBL *Escherichia coli* (SHV-12)	ESBL 10708	S	S	S	S	S	S	(R)	S	S	S	S	S	S	R	R	x	x	x	x	x	x
Enterobacter cloacae	DSM 30060	S	S	S	S	S	(R)	S	S	S	S	S	x	x	x	x	S	S	S	S	S	S
ESBL *Escherichia coli* (CTX-M-15)	ESBL 10716	S	R	R	R	R	S	R	S	S	x	R	R	R	R	R	R	S	S	R	S	S
ESBL *Escherichia coli* (CMY-2, TEM-1)	ESBL 10717	S	S	S	S	S	I	S	R	R	x	S	R	R	R	R	R	S	S	S	S	S

Abbreviations: *S* sensitive, *R* resistant, *x* not tested

Antibiotic substances and concentrations: Amikacin (AK) 30 μg, Chloramphenicol (C) 30 μg, Cefitofur (CEFT) 30 μg, Ciprofloxacin (CIP) 5 μg, Cephalexime (CL) 30 μg, Gentamycin (CN) 30 μg, Cefpodoxime (CPD) 10 μg, Colistin (CT) 25 μg, Cefotaxime (CTX) 30 μg, Cefotaxime (CTX) 5 μg, Enrofloxacin (ENR) 5 μg, Nitrofurantoin (F) 300 μg, Fosfomycin (FOS) 50 μg, Imipenem (IPM) 10 μg, Marbofloxacin (MAR) 5 μg, Meropenem (MEM) 10 μg, Polymyxin (PB) 300 IE, Rifampicin (RD) 30 μg, Sulfamethoxazol/ Trimethoprim (SXT) 25 μg, Tigecycline (TGC) 15 μg, Tobramycin (TOB) 10 μg

inoculated MHB without antibiotics provided the positive control. The MIC was defined as the concentration, at which no growth was observed within the 24 h measurement period. According to the MIC and growth curves, antibiotic concentrations for agar plates for the conjugation trials were chosen.

Screening for conjugation

In order to identify mating pairs, the potential donor and recipient strains were co-cultivated in duplicates for 22 h (supplementary data: Fig. S1). To obtain viable and antibiotic resistant bacterial cells in their log-phase, bacteria strains were pre-cultured twice in MHB with antibiotics (same antibiotic concentration as in agar plates), against which the strain was resistant, and once in MHB without antibiotics. Subsequently, the precultures were washed twice in PBS and diluted in MHB without antibiotics to 10^6 cells/mL. Equal volumes (100 μL) of donor and recipient suspensions were added to 800 μL MHB without antibiotics. Thus, in the coincubation suspensions, both donors and recipients were initially present at a concentration of 10^5 cells/mL and the initial total bacterial concentration was 2×10^5 cells/mL. Single strain donor and recipient dilutions served as control. The screening was implemented in duplicates.

All samples were incubated for 22 h and subsequently spread on double antibiotic agar plates at different dilutions. The antibiotic combinations for different donor and recipient strains are displayed in the supplementary data (supplementary data: Table S2). Positive and negative controls were obtained by spreading control suspensions on double (negative) and single (positive) antibiotic agar plates. All plates were incubated overnight, and conjugation events were identified as colony growth on double antibiotic agar plates. Colony forming units (cfu)/mL were obtained to estimate useful dilution levels for the 22 h kinetic assay.

Kinetic assay

A kinetic assay was designed to obtain conjugation frequencies for the different mating pairs at 6 time points (supplementary data: Fig. S2). Precultures were obtained by incubating donor and recipient strains in MHB with antibiotics (supplementary data: Table S2). The cells were washed and dilutions of 10^6 cells/mL were prepared as described in the chapter Screening for conjugation. Each 1 mL of the donor and the recipient suspensions were inoculated in 8 mL MHB without antibiotics, mixed thoroughly, dispensed to 1.4 mL aliquots and incubated for 2, 4, 6, 8 and 22 h, respectively in MHB with antibiotics (supplementary data: Fig. S2, Table S2). Inocula (1 mL, 10^5 cells/mL) with only one bacterial strain (donor or recipient) served as controls.

Immediately after the inoculation, 300 μL of the suspension were plated on two double antibiotic agar plates (supplementary data: Table S2), to identify transconjugants present at hour 0. Simultaneously, dilution series of the same sample were spread on MacConkey agar plates without antibiotics, to obtain the total cell count. This procedure was repeated after 2, 4, 6, 8 and 22 h coincubation with suitable dilutions. The single-strain suspensions were plated on the corresponding double and single antibiotic agars for negative and positive controls, respectively. The plates were incubated overnight, and the conjugation frequency was calculated as transconjugants/donors. The kinetic assay was performed with duplicates of mating pairs.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-020-01787-7.

Additional file 1.

Additional file 2.

Abbreviation

APEC: Avian pathogenic *E. coli*; BfR: German Federal Institute for risk assessment; C: Chloramphenicol; cfu: Colony forming units; CLSI: Clinical Laboratory Standards Institute; CT: Colistin; CTX: Cefotaxime; *E. coli*: *Escherichia coli*; ESBL: Extended-spectrum beta-lactamase; DSMZ: German Collection of Microorganisms and Cell Cultures, Leibnitz Institute; F: Nitrofurantoin; h: Hours; IAN: Institute of Animal Nutrition, Freie Universität Berlin; IMT: Institute of Microbiology and Epizootics, Freie Universität Berlin; MHB: Mueller Hinton Broth; MIC: Minimal inhibitory concentrations; Nd: Not determined; NT: No transconjugants; PBS: Phosphor buffered saline; *S. marcescens*: *Serratia marcescens*; SXT: Sulfamethoxazole/trimethoprim; WHO: World Health Organization

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Authors contributions

W.V. and E.S. planned the experiment, which was carried out by E.S. who also wrote the manuscript with input from all authors. J.Z. helped supervise the project. W.V. and J.Z. conceived the original idea. W.V. supervised the project. All authors discussed the results and commented on the manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Nutrition Related Stress Factors Reduce the Transfer of Extended-Spectrum Beta-Lactamase Resistance Genes between an *Escherichia coli* Donor and a *Salmonella* Typhimurium Recipient In Vitro

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Abstract: The transfer of extended spectrum β-lactamase (ESBL)-genes occurs frequently between different bacteria species. The aim of this study was to investigate the impact of nutrition related stress factors on this transfer. Thus, an *Escherichia coli* donor and a *Salmonella* Typhimurium recipient were co-incubated for 4 h in media containing different levels of the stress factors' pH, osmolality, copper, zinc and acetic, propionic, lactic, and n-butyric acid, as well as subtherapeutic levels of cefotaxime, sulfamethoxazole/trimethoprim, and nitrofurantoin. Conjugation frequencies were calculated as transconjugants per donor, recipient, and total bacterial count. A correction factor for the stress impact on bacterial growth was used. Acetic, lactic, and n-butyric, acid, as well as pH, showed no significant impact. In contrast, increasing concentrations of propionate, zinc, copper, and nitrofurantoin, as well as increased osmolality reduced conjugation frequencies. Sulfamethoxazole/trimethoprim and cefotaxime showed increased transconjugants per donor, which decreased after correction for stress. This study showed, for the model mating pair, that conjugation frequencies decreased under different physiological stress conditions, and, thus, the hypothesis that stress factors may enhance conjugation should be viewed with caution. Furthermore, for studies on in vitro gene transfer, it is vital to consider the impact of studied stressors on bacterial growth.

Keywords: extended-spectrum β-lactamases; horizontal gene transfer; minerals; short-chain fatty acids; organic acids; feed additives; osmolarity; bacterial conjugation

1. Introduction

As extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* (ESBL-PE) pose a major hazard on public health, the development of methods to reduce their occurrence has gained high priority [1,2]. In livestock, the highest ESBL-PE prevalence was observed in poultry with *Escherichia coli* being the most common species [3]. Besides comprising various pathogenic isolates, nonpathogenic *E. coli* isolates may also inhabit the intestinal tract of broilers as part of the commensal microbiota [4,5], and ESBL-production is not correlated with virulence. Thus, normally no symptoms are observed in animals colonized by ESBL-producing *E. coli* (ESBL-EC). This indicates that ESBL-EC may be harmless and does not require antibiotic treatment. However, as ESBL-genes are frequently located on plasmids, which are commonly transmitted between En*terobacteriaceae* [6], these plasmids can easily be transferred to pathogenic *Enterobacteriaceae*. This can induce the spread of antibiotic resistant pathogens in livestock.

Thus, animals may develop a disease, which is difficult to treat by antibiotics. Furthermore, treatment with β-lactam antibiotics may additionally increase the ESBL-PE load by selective pressure [5,7,8]. ESBL-PE transfer from livestock to humans and companion animals may occur

via direct contact, through animal products or the environment [9–11]. In this way, humans may obtain harmless ESBL-PE, which then could transfer their ESBL-carrying plasmids to human pathogens in the hosts' intestine and lead to serious, difficult to treat infections. It has been shown that conjugation occurs and transconjugants persist for many generations independent of the selective pressure by antibiotics [12].

Bacteria, which transfer mobile genetic elements to other bacteria, are referred to as donors, while bacteria capable of acquiring the genetic information are termed recipients. Once the plasmid is successfully transferred, the recipient, harboring the 'new' plasmid, will be termed transconjugant [13]. As the number of transconjugants is negligible compared to donor and recipient counts, it is not differentiated between 'conjugated' or 'nonconjugated' recipients. When quantifying and comparing conjugation events, one frequently refers to conjugation frequency (CF) [14]. There are different ways to calculate CF using the donor or recipient count as reference:

$$\text{Conjugation frequency based on donor count } (\text{CF}(\text{D})) \; = \; \frac{Transconjugants/mL}{Donors/mL}, \tag{1}$$

[15–17].

$$\text{Conjugation frequency based on to recipient count } (\text{CF}(\text{R})) \; = \; \frac{Transconjugants/mL}{Recipients/mL}, \tag{2}$$

[18,19]. It must be considered for studies on stress response that these methods of calculation neglect the impact of the used stressor on the growth of the different participants. This effect may give a false impression that conjugation was influenced by supplementation while the change in conjugation frequency just mirrors a change in bacterial growth. This bias may lead to wrong assumptions [14]. To avoid this misinterpretation, a different method to calculate the conjugation efficiency (η) can be applied as

$$\eta \approx \frac{T}{RD\Delta t}, \tag{3}$$

where T stands for the number of transconjugants/mL, R for the number of recipients/mL, D for the number of donors/mL, and Δt represents the conjugation time in hours [14]. The impact of time on conjugation must not be underestimated, since conjugation frequencies may change dramatically within hours [20]. When calculating conjugation efficiency, the impact of Δt on η can be neglected due to the tremendous numeric difference between time and transconjugants, recipients, and donors. While T, D, and R will range between 10^2 and 10^9, in most experiments the time will stay below 100. Thus, mathematically, time differences will not be large enough to affect the result. Additionally, these results are not easily compared with the results calculated with the more traditional approach of dividing transconjugants by donor or recipient counts. Furthermore, a prerequisite for this equation is that donor and recipient concentrations remain rather constant during the period of time. This assumption does not apply for the intestinal microbiota. In the intestinal tract, bacteria undergo dynamic growth states with a constant change of the composition [21,22]. In this way, both pathogens and nonpathogenic bacteria, donors, and recipients share a timely and spatially dynamic habitat in animals. As they may interact, their growth and total amount will be influenced by environmental factors in different ways [5].

Strain dependent differences are frequently observed when conjugation trials are performed with various donor and recipient strains, as the environment and their interaction influences them differently [23]. In this sense, stress can be defined as a potential threat to the survival of the bacterial cell [24]. The mechanisms explaining the influence of stress on conjugation are manifold. Hence, stressors may influence (1) the bacterial genome, (2) number of plasmids per cell, and/or (3) efficiency of the plasmid transfer [25]. Thus, stress reactions caused by sub-lethal concentrations of antibiotics, may originate from an induction of the bacterial conjugation machinery and/or the stimulation of the excision of transferable genes from the donors' chromosome [26,27]. It was previously hypothesized,

but not proven, that antibiotics, which affect the cell wall of bacteria, increase transfer rates [28]. Additionally, stress caused by extreme pH, starvation, and/or organic solvents among others may influence the uptake and release of plasmids [25]. Different kinds of stressors, such as pH, antibiotics, or nutrient starvation may induce stress and DNA mutations (SOS response) in bacteria, which can affect conjugation rates positively. Thus, conjugation under a given stress condition for one strain exemplifies the general possibility of gene transfer under these conditions.

As multi resistant bacteria rise as a problem and are a major health hazard, new reduction measurements are developed to reduce specific bacterial fractions of the intestinal microbiota in animals. Nutritional intervention steps have shown promising results to shift the microbiota towards a more desirable direction [29–34]. This may create stress for the suppressed bacteria, causing a change in their metabolic activity, including the transfer of genetic material [35–37]. Thus, modification of environmental conditions by certain feeds or feed additives can induce stress, which may threaten the survival of bacterial cells due to unfavorable conditions [36,37]. Therefore, conjugation rates may be influenced by specific feed additives such as copper and zinc, but also by bacterial metabolites (short chain fatty acids), as well as by factors defining the intestinal milieu (pH and osmolality), which may be changed by feed additives such as enzymes and pro- or prebiotics. Finally, it is known that antibiotic treatment has a tremendous impact on bacterial growth, providing a further stress factor investigated in this study [5,7].

From the considerations outlined above, this study was designed to investigate how different stress factors may affect the conjugation rates of an ESBL-producing *E. coli* donor strain and a *Salmonella* Typhimurium recipient strain. Special care was given to the analysis when referring to the donor, recipient, and total bacterial count, as well as addressing the impact of stress on bacterial growth.

2. Materials and Methods

2.1. Strains and Cultivation Conditions

A nonpathogenic *E. coli* isolate (ESBL10682, isolated from the excreta of one day old broilers within the RESET program), harboring the $bla_{CTX\text{-}M\text{-}1}$ gene and belonging to the B1 subgroup, was chosen as the donor. The strain *Salmonella* Typhimurium L1219-R32 (isolated from pigs) was chosen as the recipient strain. This mating pair was revealed to be the best fit for the study design in a previous study obtaining conjugation kinetics for five potential *E. coli* donor strains and six potential *Enterobacteriaceae* recipients every 2 h for 22 h [38]. From these results, the mating pair was known to result in a conjugation frequency of approximately 10^5 transconjugants/donor after 4 h of co-incubation (donor:recipient 1:1; 10^5 cells/mL starting conditions) [38]. All cultures were obtained from cryo-stocks and cultured in Mueller Hinton 2 broth (Sigma-Aldrich, Chemie GmbH, Darmstadt, Germany). Culturing of the strains was done in Mueller Hinton 2 broth, supplemented with 8 µg/mL cefotaxime (CTX) (Alfa Aesar, Thermo Fisher GmbH, Schwerte, Germany) for *E. coli* incubation or 300 µg/mL sulfamethoxazole/trimethoprim (SXT) (Sigma-Aldrich, Chemie GmbH, Darmstadt, Germany) for the *Salmonella* Typhimurium strain. All strains were incubated aerobically at 37 °C.

2.2. Experimental Design

After the second preculture in antibiotic supplemented medium, the bacterial strains were washed twice in Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Chemie GmbH, Darmstadt, Germany) and diluted to 5×10^8 cells/mL. Fifty µL of each donor and recipient strain were added to 800 µL media supplemented with stress factors, as described below. After vigorous vortexing, the samples were incubated aerobically for four hours at 37 °C. The suspensions were then placed on ice, serially diluted, and spread on a MacConkey agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) containing 8 µg cefotaxime/mL and 300 µg sulfamethoxazole/trimethoprim/mL to obtain transconjugants and on MacConkey agar without antibiotics to estimate the cell count of *E. coli* ESBL10682, *Salmonella* Typhimurium L1219-R32, and total bacterial count (TBC). In the set up with cefotaxime, MacConkey

agar plates containing 8 µg cefotaxime/mL, or 300 µg sulfamethoxazole/trimethoprim/mL were used to obtain the cell count of *E. coli* ESBL10682 and *Salmonella* Typhimurium L1219-R32, respectively. The conjugation frequency was calculated with respect to the donor, the recipient, and the total bacterial count by dividing the number of transconjugants/mL by the respective bacteria count:

$$\text{Conjugation frequency based on donor count (CF(D))} = \frac{Transconjugants/mL}{Donor\ cells/mL}, \quad (4)$$

$$\text{Conjugation frequency based on recipient count (CF(R))} = \frac{Transconjugants/mL}{Recipient\ cells/mL}, \quad (5)$$

$$\text{Conjugation frequency based on total bacterial count (CF(T))} = \frac{Transconjugants/mL}{Donor + Recipient\ cells/mL}. \quad (6)$$

All experiments were repeated three times with fresh cultures and with three replicates per repetition.

2.3. Stress Factors

For the challenge experiments, various stress factors were added to Mueller Hinton 2 broth in different concentrations. The studied stress factors were pH, osmolality, antibiotics at subtherapeutic concentrations, zinc, copper, and the short chain fatty acids acetic, propionic, and n-butyric acid and D/L-lactate.

2.3.1. pH

The impact of pH 4–7.5 on donor and recipient growth was determined by measuring turbidity during incubation in a micro titer plate reader (Infinite200Pro, Tecan Austria GmbH, Grödig, Austria) at 690 nm every 5 min over a time period of 4 h (data not shown). A pH adjustment to 5.0, 5.5, 6.0, and 6.5 was carried out in a double concentrated Mueller Hinton 2 broth using 1 M hydrochloric acid (HCl) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Equal volumes were achieved by adding ultrapure water to the solutions in volumetric flasks. The media were then sterile-filtered (0.2 µm, VWR International GmbH, Darmstadt, Germany). Mueller Hinton 2 broth exhibited a pH value of 7.5 and served as the control.

2.3.2. Osmolality

Sodium chloride (NaCl) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added to a 50 mL Mueller Hinton 2 Medium in Afnor bottles to obtain osmolalities of 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mOsm/kg and autoclaved. The correct osmolality was confirmed with a micro osmometer (type OM 806, Vogel Medizinische Technik und Elektronik, Fernwald, Germany), and the impact on bacterial growth was monitored by a turbidity measurement at 690 nm. Correlation between osmolality and CF was analyzed using the software IBM SPSS (Version 22, IBM Deutschland GmbH, Ehningen, Germany). The osmolality of 300 mOsm/kg served as the control, as it resembled the osmolality of the pure medium.

2.3.3. Antibiotics

Subtherapeutic levels of nitrofurantoin (F) (Sigma-Aldrich, Chemie GmbH, Darmstadt, Germany), cefotaxime, and sulfamethoxazole/trimethoprim were determined for the donor and recipient strains by studying their growth kinetics in the presence of different antibiotic concentrations. The impact on bacterial growth was monitored by turbidity measurement at 690 nm, measured for 4 h as described above. The antibiotics were added to Mueller Hinton 2 broth at each three different concentrations (0.4, 0.5, and 0.6 µg CTX/mL; 1.0, 2.5, and 5.0 µg SXT/mL; 2.0, 4.0, and 6.0 µg F/mL) while the Mueller Hinton 2 broth without antibiotics served as control.

2.3.4. Zinc and Copper

Saturated solutions of zinc from ZnO (Sigma-Aldrich, Chemie GmbH, Darmstadt, Germany) and copper from $CuSO_4(H_2O)_5$ (Merck KGaA, Darmstadt, Germany) were prepared according to Liedtke and Vahjen [39]. Atomic absorption spectroscopy (contrAA 700, Analytic Jena AG, Jena, Germany) was used to determine actual metal concentrations. The media were then serially diluted in Mueller Hinton 2 broth and donor and recipient growth was obtained by measuring turbidity at 690 nm for 4 h. The concentrations (zinc: 0, 10, 21, 42, 84, 167 µg/mL; copper: 0, 11, 22, 43, 87, 173 µg/mL) were chosen due to their ability to reduce, but not inhibit, bacterial growth. Pure Mueller Hinton 2 broth served as the control.

2.3.5. Short Chain Fatty Acids and Lactate

Acetic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), propionic acid (Merck KGaA, Darmstadt, Germany), D/L-lactic acid (D/L: equal volume units, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and n-butyric acid (Sigma-Aldrich, Chemie GmbH, Darmstadt, Germany) were added to double concentrated Mueller Hinton 2 Broth. The pH was adjusted to pH 7.5 using 5 M sodium hydroxide (NaOH) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The dilutions were sterile-filtered (0.2 µm) and the concentrations were confirmed by gas chromatography (Agilent 6890N, Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Four different concentrations were prepared by 1:2 serial dilutions in Mueller Hinton 2 Broth (acetate, propionate, n-butyrate: 0, 18.75, 37.50, 75.00, 150.00 mM; lactate: 0, 13.75, 27.50, 55.00, 110.00 mM) and the exact concentrations obtained by gas chromatography. The impact on donor and recipient growth was studied prior to the conjugation experiment). The control medium was nonsupplemented Mueller Hinton 2 Broth.

2.4. Calculation of Stress Impact Factor

Since stress does not only influence the conjugation but also the growth of the transconjugants, donors, and recipients, the results were corrected by a stress impact factor. This factor was defined as the percentage change of growth between the two concentrations/levels. At first, the stress impact factor (SIF), defined as the ratio between the mean colony forming units per mL of the control (cfu_{ctr}) and a certain level of supplementation with a stress factor (cfu_{stress}) was determined as

$$\mathrm{SIF} = \frac{\frac{mean\ cfu_{\mathrm{ctr}}}{mL}}{\frac{mean\ cfu_{\mathrm{stress}}}{mL}}. \quad (7)$$

Thus, SIF = 1 would indicate no impact on the growth, SIF > 1 shows a reduction in growth, and SIF < 1 designates an enhanced growth when exposed to the stressor. Secondly, the growth of donors, recipients, and transconjugants were corrected (cfu_{corr}) to the level of the controls. Thus, a condition without an impact of the stressor on bacterial growth was simulated by multiplying the cfu_{stress} with the SIF:

$$\mathrm{cfu_{corr}} = \mathrm{cfu_{stress}} \times \mathrm{SIF}. \quad (8)$$

In this way, cfu_{corr} was calculated for donor, recipient, total bacterial count, and transconjugants. For transconjugants, SIF was calculated according to recipient growth in all cases except when cefotaxime was supplemented, since the conjugation was assumed not to transfer growth benefits in the other cases. Conjugation frequencies were subsequently calculated as described above.

2.5. Statistics

All statistics were calculated with the software IBM SPSS (Version 22). Results are presented as mean values ± standard deviation. The nonparametric Kruskal–Wallis test and Mann–Whitney test were used to determine significant differences and subgroups, respectively. Differences were considered statistically significant at $p < 0.05$ and p-values between 0.05 and 0.1 were accepted as trends.

3. Results

3.1. pH

A preliminary screening of the donor and recipient growth kinetics at various pH values failed to detect a significant impact on the donor or recipient strains at pH levels of 5.0–7.5. The lowest numeric conjugation frequency was noted at an initial pH of 6.0 for all alternative calculations, but differences were only marginal (Table 1). Bacterial growth was not significantly affected between pH 5.0–7.5 for all incubations (Supplementary data, Figure S1, Table S2). Thus, CF values calculated acknowledging (CF(D)(SIF), CF(R)(SIF), CF(T)(SIF)) or neglecting (CF(D), CF(R), CF(T)) the stress impact factor are rather similar (Table 1).

Table 1. The impact of pH on the conjugation frequency [$\log_{10}$(CF[1])] of an *Escherichia coli* donor and a *Salmonella* Typhimurium recipient, calculated per donor, recipient, and total bacterial count corrected for the stress impact factor.

pH	CF(D)	CF(R)	CF(T)	CF(D) (SIF)	CF(R) (SIF)	CF(T) (SIF)
5.0	−5.0 ± 0.2	−4.6 ± 0.2	−5.2 ± 0.2	−5.0 ± 0.2	−4.6 ± 0.2	−5.2 ± 0.2
5.5	−5.0 ± 0.3	−4.4 ± 0.2	−5.1 ± 0.2	−5.2 ± 0.3	−4.4 ± 0.2	−5.2 ± 0.2
6.0	−5.2 ± 0.4	−4.6 ± 0.2	−5.3 ± 0.4	−5.3 ± 0.4	−4.6 ± 0.2	−5.4 ± 0.4
6.5	−5.1 ± 0.2	−4.6 ± 0.2	−5.2 ± 0.1	−5.1 ± 0.2	−4.6 ± 0.2	−5.2 ± 0.1
7.5	−5.0 ± 0.2	−4.6 ± 0.3	−5.2 ± 0.2	−5.0 ± 0.2	−4.6 ± 0.3	−5.2 ± 0.2
p-value	0.672	0.399	0.627	0.147	0.399	0.224

[1] CF = conjugation frequency; CF(D) = transconjugants/donor; CF(R) = transconjugants/recipients; CF(T) = transconjugants/total bacterial count; SIF = stress impact factor (Supplementary Data, Table S3); for each column, *p*-values were calculated comparing CF of different levels of exposure to the stressor using the nonparametric Kruskal–Wallis test and Mann–Whitney test. Raw data are provided in supplementary data, Table S1.

3.2. Osmolality

The growth of *Salmonella* Typhimurium declined with increasing osmolality, while the *E. coli* strain showed the highest number of colony forming units at 500 mOsm/L (Supplementary Data, Figure S2, Table S2). Conjugation frequencies declined exponentially with increasing osmolality (correlation analyses: single, 3 parameters; $R^2 = 0.97$, $R^2 = 0.48$ and $R^2 = 0.96$ respectively) (Table 2). After correcting for stress impact on growth, the correlation of CF(D) and CF(T) with osmolality became more linear ($R^2 = 0.48$, $R^2 = 0.51$). Significant differences in the CF of 0.4–0.5 log cfu/mL were observed for all three approaches when CF was corrected by SIF (Table 2).

Table 2. The impact of osmolality on the conjugation frequency [$\log_{10}$(CF[1])] of an *E. coli* donor and a *Salmonella* Typhimurium recipient, calculated per donor, recipient, and total bacterial count corrected for the stress impact factor. The osmolality of Mueller Hinton 2 broth (control) was 300 mOsm/kg.

Osmolality (mOsm/kg).	CF(D)	CF(R)	CF(T)	CF(D) (SIF)	CF(R) (SIF)	CF(T) (SIF)
200	−5.0 ± 0.2 ^{a}	−4.7 ± 0.3 ^{ab}	−5.2 ± 0.2 ^{ab}	−5.2 ± 0.2 ^{ab}	−4.7 ± 0.3 ^{ab}	−5.2 ± 0.2 ^{ab}
300	−5.1 ± 0.2 ^{a}	−4.6 ± 0.3 ^{b}	−5.2 ± 0.2 ^{a}	−5.1 ± 0.2 ^{a}	−4.6 ± 0.3 ^{b}	−5.2 ± 0.2 ^{a}
400	−5.3 ± 0.5 ^{ab}	−4.8 ± 0.6 ^{bc}	−5.4 ± 0.5 ^{ac}	−5.3 ± 0.5 ^{a}	−4.8 ± 0.6 ^{bc}	−5.4 ± 0.5 ^{ac}
500	−5.3 ± 0.5 ^{ab}	−4.7 ± 0.4 ^{bc}	−5.4 ± 0.5 ^{ac}	−5.16 ± 0.5 ^{ac}	−4.70 ± 0.4 ^{bc}	−5.3 ± 0.5 ^{acd}
600	−5.6 ± 0.7 ^{bc}	−5.3 ± 0.5 ^{a}	−5.8 ± 0.7 ^{b}	−5.62 ± 0.7 ^{bc}	−5.25 ± 0.5 ^{a}	−5.7 ± 0.7 ^{bcde}
700	−5.6 ± 0.1 ^{c}	−5.2 ± 0.4 ^{a}	−5.8 ± 0.1 ^{b}	−5.53 ± 0.2 ^{bd}	−5.20 ± 0.4 ^{a}	−5.7 ± 0.3 ^{e}
800	−5.8 ± 0.4 ^{c}	−4.9 ± 0.5 ^{abc}	−5.8 ± 0.4 ^{abc}	−5.52 ± 0.4 ^{bd}	−4.93 ± 0.5 ^{abc}	−5.7 ± 0.4 ^{bce}
900	−5.7 ± 0.2 ^{c}	−4.8 ± 0.1 ^{abc}	−5.8 ± 0.1 ^{abc}	−5.25 ± 0.2 ^{abc}	−4.76 ± 0.1 ^{abc}	−5.4 ± 0.2 ^{abcde}
1000	−5.7 ± 0.1 ^{c}	−5.0 ± 0.3 ^{bc}	−5.8 ± 0.1 ^{bc}	−5.57 ± 0.1 ^{bd}	−4.96 ± 0.3 ^{bc}	−5.7 ± 0.1 ^{be}
p-value	<0.001	0.019	<0.001	0.005	0.019	0.003

[1] Conjugation frequency (CF) calculated as CF/D = transconjugants/donor, CF/R = transconjugants/recipients or CF/T = transconjugants/total bacterial count; SIF = stress impact factor (Supplementary Data, Table S3); *p*-values were calculated comparing CF of different levels of exposure to the stressor using the nonparametric Kruskal–Wallis test and Mann–Whitney test. Significant differences ($p \leq 0.05$) between values are indicated by different superscript letters. Raw data are provided in supplementary data, Table S1.

3.3. Antibiotics

Cefotaxime had a relatively strong negative impact on the growth of the recipient strain, causing a reduction of 1.8 log cfu/mL, when no supplementation was compared to the highest CTX concentration of 0.6 µg/mL. The donor strain was less sensitive towards the substance, resulting in a reduction of 1.2 log cfu/mL from the control to 0.6 µg CTX/mL (Supplementary data, Figure S3, Table S2). When challenged with subtherapeutic concentrations of cefotaxime, the conjugation frequencies showed an increasing trend ($p = 0.06$), with increasing concentrations of CTX when calculated based on donor count. This effect was profound for CF(R) and CF(T) (Table 3). When corrected for the stress impact factor, a significant difference was observed for 0.4 µg CTX/mL supplementation in CF(D)(SIF) and CF(T)(SIF), while CF(R)(SIF) showed no significant differences.

Increasing concentrations of sulfamethoxazole/trimethoprim decreased the growth of the donor strain by 1.8 log, while the growth of the recipient strain only increased with 0.2 log from no to 5 µg SXT/mL supplementation (Supplementary data, Figure S4, Table S2). Furthermore, a strong impact on conjugation frequencies relating to donor, recipient, and total bacterial count was observed (Table 3). While the conjugation frequency increased significantly for 2.5 and 5.0 µg SXT/mL when based on the donor strain, it decreased significantly when referring to recipient and total bacterial cell growth. When corrected for the stress impact on bacterial growth, a significant decrease was observed for CF(D)(SIF), CF(R)(SIF), and CF(T)(SIF) between the control and the highest SXT concentration with 1.3, 0.9, and 1.2 log, respectively (Table 3).

Donor and recipient growth declined in a dose-dependent fashion at 0.7 and 0.8 log cfu/mL, respectively, with increasing concentrations of nitrofurantoin (Supplementary Data, Figure S5, Table S2). Similarly, conjugation frequencies (CF(D), CF(T), and CF(D)) were significantly affected by the supplementation of 6.0 µg F/mL (Table 3). This effect was slightly increased when the stress impact on bacterial growth was considered. Overall, the decrease in CF ranged from 1.3 to 1.5 log.

Table 3. The impact of subtherapeutic levels of antibiotics on conjugation frequency [$\log_{10}$(CF[1])] of an *E. c*oli donor and a *Salmonella* Typhimurium recipient, calculated per donor, recipient and total bacterial count corrected for the stress impact factor.

Antibiotic [2] (µg/mL)	CF(D)	CF(R)	CF(T)	CF(D) (SIF)	CF(R) (SIF)	CF(T) (SIF)
CTX						
0	−4.9 ± 0.5	−4.3 ± 0.6 ^{a}	−5.0 ± 0.5 ^{a}	−4.9 ± 0.5 ^{a}	−4.3 ± 0.6	−5.0 ± 0.7 ^{a}
0.4	−4.4 ± 0.7	−3.0 ± 0.6 ^{b}	−4.4 ± 0.7 ^{b}	−5.6 ± 0.5 ^{b}	−4. 9 ± 0.6	−5.7 ± 0.4 ^{b}
0.5	−4.1 ± 0.7	−2.9 ± 1.0 ^{b}	−4.1 ± 0.7 ^{b}	−5.0 ± 0.6 ^{a}	−5.0 ± 0.7	−5.3 ± 0.6 ^{ab}
0.6	−4.2 ± 0.5	−2.6 ± 0.9 ^{b}	−4.2 ± 0.5 ^{b}	−4.7 ± 0.5 ^{a}	−4.7 ± 0.6	−5.0 ± 0.5 ^{a}
p-value	0.060	0.002	0.002	0.011	0.163	0.021
SXT						
0	−4.9 ± 0.5 ^{ab}	−4.3 ± 0.6 ^{a}	−5.0 ± 0.5 ^{a}	−4.9 ± 0.5 ^{a}	−4.3 ± 0.6 ^{a}	−5.0 ± 0.5 ^{a}
1.0	−5.6 ± 0.9 ^{ac}	−5.9 ± 0.7 ^{b}	−6.1 ± 0.7 ^{b}	−6.9 ± 0. 9 ^{b}	−5.9 ± 0.7 ^{b}	−6.6 ± 0.8 ^{b}
2.5	−4.3 ± 0.3 ^{b}	−5.3 ± 0.4 ^{b}	−5.3 ± 0.4 ^{ab}	−5.7 ± 0.4 ^{ab}	−5.3 ± 0.4 ^{b}	−5.9 ± 0.4 ^{b}
5.0	−4.1 ± 0.4 ^{c}	−5.6 ± 0.5 ^{b}	−5.6 ± 0.5 ^{b}	−6.2 ± 0.4 ^{b}	−5.6 ± 0.5 ^{b}	−6.2 ± 0.5 ^{b}
p-value	0.001	0.001	0.017	<0.001	0.001	0.001
F						
0	−4.9 ± 0.5 ^{a}	−4.3 ± 0.6 ^{a}	−5.0 ± 0.7 ^{a}	−4.9 ± 0.5 ^{a}	−4.3 ± 0.6 ^{a}	−5.0 ± 0.5 ^{a}
2.0	−5.2 ± 0.5 ^{a}	−4.6 ± 0.5 ^{a}	−5.3 ± 0.4 ^{a}	−4.8 ± 0.7 ^{a}	−4.5 ± 0.5 ^{a}	−5.0 ± 0.6 ^{a}
4.0	−5.2 ± 0.5 ^{a}	−4.6 ± 0.3 ^{a}	−5.3 ± 0.5 ^{a}	−4.9 ± 0.3 ^{a}	−4.5 ± 0.4 ^{a}	−5.1 ± 0.3 ^{a}
6.0	−6.2 ± 0.7 ^{b}	−5.8 ± 0.8 ^{b}	−6.4 ± 0.7 ^{b}	−6.3 ± 0.6 ^{b}	−5.8 ± 0.8 ^{b}	−6.4 ± 0.7 ^{b}
p-value	0.002	0.001	0.001	0.001	0.001	0.001

[1] Conjugation frequency (CF) calculated as CF/D = transconjugants/donor, CF/R = transconjugants/recipients or CF/T = transconjugants/total bacterial count; SIF = stress impact factor (Supplementary Data, Table S3); 2CTX = cefotaxime; SXT = sulfamethoxazole/trimethoprim; F = nitrofurantoin; *p*-values were calculated comparing CF of different levels of exposure to the stressor using the nonparametric Kruskal–Wallis test and Mann–Whitney test. Significant differences ($p \leq 0.05$) between values are indicated by different superscript letters. Raw data are provided in supplementary data, Table S1.

3.4. Zinc and Copper

The normal growth of *E. coli* ESBL10682 was not affected significantly by zinc supplementation, while *Salmonella* Typhimurium L1219-R32 declined by 0.6 log cfu/mL at the highest investigated zinc concentration compared to the controls (Supplementary data, Figure S7, Table S2). The opposite occurred when copper was supplemented. While the recipient strain was not significantly influenced, the growth of the donor decreased with 1.5 log cfu/mL (Supplementary Data, Figure S6, Table S2). Values for the conjugation frequency declined with higher concentrations of zinc and copper. Zinc concentrations of 321 and 642 µM resulted in a slight but significant decrease when CF referred to recipient counts (Table 4). When corrected for the SIF, this effect was observed for CF(D)(SIF), CF(R)(SIF), as well as CF(T)(SIF). Copper supplementation decreased CF(D), CF(R), and CF(T) significantly by 1.1, 3.2, and 2.3 log cfu/mL, respectively. A correction for the stress impact on bacterial growth showed even more severe reductions of 3.1, 3.2, and 3.2 log cfu/mL, respectively (Table 4, Figure 1).

Table 4. The influence of zinc and copper on conjugation frequency [$\log_{10}$(CF[1])] of an *E.* coli donor and a *Salmonella* Typhimurium recipient, calculated per donor, recipient, and total bacterial count corrected for the stress impact factor.

Minerals[2] (µM)	CF(D)	CF(R)	CF(T)	CF(D) (SIF)	CF(R) (SIF)	CF(T) (SIF)
Zinc						
0	−5.3 ± 0.5	−4.2 ± 0.5 ^{a}	−5.4 ± 0.5	−5.3 ± 0.5 ^{a}	−4.2 ± 0.5 ^{a}	−5.4 ± 0.5 ^{a}
153	−5.2 ± 0.3	−4.5 ± 0.2 ^{ab}	−5.3 ± 0.3	−5.7 ± 0.3 ^{ab}	−4.5 ± 0.2 ^{ab}	−5.7 ± 0.3 ^{ab}
321	−5.7 ± 0.7	−5.0 ± 0.5 ^{bc}	−5.8 ± 0.6	−6.1 ± 0.7 ^{bc}	−5.0 ± 0.5 ^{bc}	−6.1 ± 0.6 ^{bcd}
642	−5.6 ± 0.4	−4.9 ± 0.5 ^{bc}	−5.7 ± 0.4	−6.1 ± 0.4 ^{bc}	−4.9 ± 0.5 ^{bc}	−6.2 ± 0.4 ^{c}
1285	−5.4 ± 0.4	−4.4 ± 0.1 ^{ab}	−5.5 ± 0.3	−5.7 ± 0.3 ^{abc}	−4.4 ± 0.1 ^{ab}	−5.6 ± 0.3 ^{abd}
2554	−5.5 ± 0.2	−4.4 ± 0.3 ^{ab}	−5.6 ± 0.2	−5.5 ± 0.2 ^{ab}	−4.4 ± 0.3 ^{ab}	−5.6 ± 0.2 ^{ab}
p-value	0.281	−0.015	−0.192	0.006	0.015	0.002
Copper						
0	−5.3 ± 0.5 ^{a}	−4.2 ± 0.5 ^{a}	−5.4 ± 0.5 ^{a}	−5.3 ± 0.5 ^{a}	−4.2 ± 0.5 ^{a}	−5.4 ± 0.5 ^{a}
173	−5.0 ± 0.2 ^{a}	−4.2 ± 0.2 ^{a}	−5.0 ± 0.2 ^{a}	−5.5 ± 0.2 ^{ab}	−4.2 ± 0.2 ^{a}	−5.5 ± 0.2 ^{ab}
346	−4.9 ± 0.6 ^{a}	−4.5 ± 0.8 ^{a}	−5.0 ± 0.6 ^{a}	−5.7 ± 0.6 ^{ab}	−4.5 ± 0.8 ^{a}	−5.7 ± 0.6 ^{ab}
677	−5.2 ± 0.4 ^{a}	−5.1 ± 0.8 ^{a}	−5.6 ± 0.5 ^{a}	−6.3 ± 0.4 ^{bc}	−5.1 ± 0.8 ^{a}	−6.4 ± 0.5 ^{bc}
1369	−6.4 ± 0.9 ^{b}	−6.9 ± 1.0 ^{b}	−7.2 ± 0.8 ^{b}	−8.0 ± 1.1 ^{cd}	−6.9 ± 1.0 ^{b}	−8.0 ± 1.0 ^{cd}
2722	−6.5 ± 0.7 ^{b}	−7.4 ± 0.9 ^{b}	−7.7 ± 0.7 ^{b}	−8.4 ± 0.9 ^{d}	−7.4 ± 0.9 ^{b}	−8.6 ± 0.9 ^{d}
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Conjugation frequency (CF) calculated as CF/D = transconjugants/donor, CF/R = transconjugants/recipients or CF/T = transconjugants/total bacterial count; SIF = stress impact factor, [2] concentrations are referring to elemental zinc and copper; *p*-values were calculated comparing CF of different levels of exposure to the stressor using the nonparametric Kruskal–Wallis test and Mann–Whitney test. Significant differences ($p \leq 0.05$) between values are indicated by different superscript letters. Raw data are provided in supplementary data, Table S1.

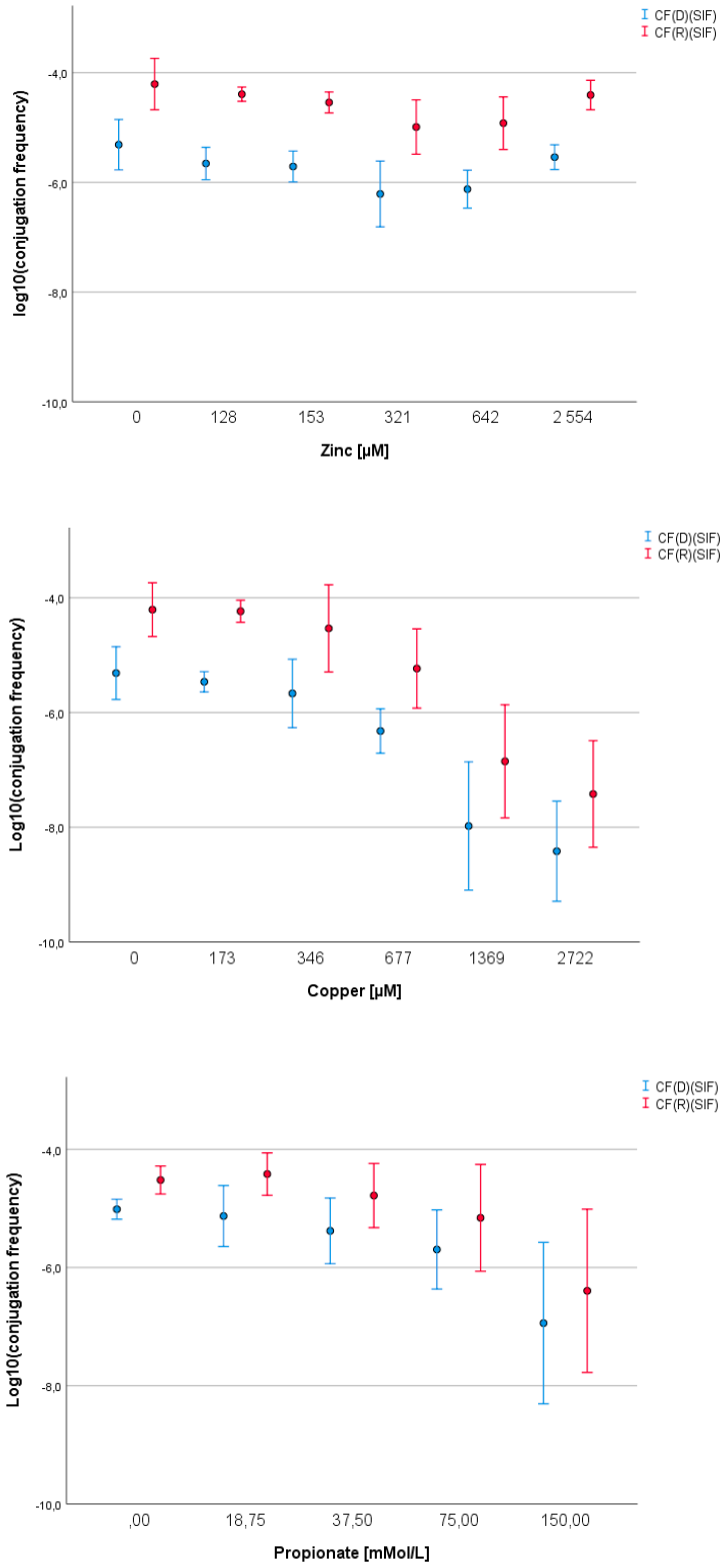

Figure 1. The impact of different concentrations of zinc, copper, and propionate on conjugation frequency of an *E. coli* donor and a *Salmonella* Typhimurium recipient after correction for the stress impact on bacterial growth. Mean values and standard deviations are displayed for conjugation frequencies (CF) calculated as CF(D) = transconjugants/donor or CF(R) = transconjugants/recipients; SIF = stress impact factor.

3.5. Short Chain Fatty Acids

The highest negative impact on the growth of both strains was observed in the presence of propionic acid with a decrease of 0.6 and 0.5 log units for the donor and recipient strains, respectively (Supplementary Data, Figure S10, Table S2). Acetic and n-butyric acid reduced bacterial growth by approximately 0.2 and 0.4 log units, respectively (Supplementary Data, Figure S8, Figure S11, Table S2). In the presence of lactic acid, the growth of both donor and recipient strains remained rather constant (Supplementary Data, Figure S9, Table S2). Similarly, n-butyric and lactic acid had no significant impact on conjugation frequencies (Table 5). However, supplementation of media with acetic or propionic acid led to a significantly negative impact on conjugation events. Thus, acetic acid showed significantly lower CF(D)-values, but after correction for SIF, this effect disappeared (Table 5). Propionic acid supplementation, on the other hand, led to decreasing conjugation frequencies for CF(D), CF(R), and CF(T) with 0.6, 0.5, and 0.7 log (Figure 1). A correction for SIF resulted in a sharper decrease with 0.8 log cfu/mL for both CF(D)(SIF) and CF(T)(SIF), considering the difference between control and highest propionic acid concentration (Table 5).

Table 5. Influence of bacterial metabolites on conjugation frequency [$\log_{10}$(CF[1])] of an *E. coli* donor and a *Salmonella* Typhimurium recipient, calculated per donor, recipient, and total bacterial count corrected for the stress impact factor.

Organic acid (mM)	CF(D)	CF(R)	CF(T)	CF(D) (SIF)	CF(R) (SIF)	CF(T) (SIF)
Acetate						
0	−5. ± 0.2 ^{a}	−4.5 ± 0.2	−5.1 ± 0.2	−5.0 ± 0.2	−4.5 ± 0.2	−5.1 ± 0.2
37	−5.0 ± 0.4 ^{ab}	−4.3 ± 0.5	−5.1 ± 0.4	−5.0 ± 0.4	−4.3 ± 0.5	−5.1 ± 0.4
74	−5.0 ± 0.2 ^{ac}	−4.2 ± 0.6	−5.1 ± 0.2	−4.9 ± 0.1	−4.2 ± 0.6	−5.0 ± 0.1
111	−5.5 ± 0.6 ^{b}	−4.6 ± 0.8	−5.6 ± 0.6	−5.4 ± 0.6	−4.6 ± 0.8	−5.5 ± 0.6
148	−5.5 ± 0.6 ^{bc}	−4.8 ± 0.3	−5.6 ± 0.5	−5.3 ± 0.6	−4.8 ± 0.3	−5.4 ± 0.5
p-value	0.036	0.231	0.069	0.239	0.231	0.168
Propionate						
0	−5.0 ± 0.2 ^{a}	−4.5 ± 0.3 ^{a}	−5.1 ± 0.2 ^{a}	−5.0 ± 0.2 ^{a}	−4.5 ± 0.3 ^{a}	−5.1 ± 0.2 ^{ab}
36	−5.1 ± 0.5 ^{a}	−4.4 ± 0.4 ^{a}	−5.1 ± 0.5 ^{a}	−5.1 ± 0.5 ^{a}	−4.4 ± 0.4 ^{a}	−5.0 ± 0.5 ^{b}
73	−5.5 ± 0.6 ^{ab}	−4.8 ± 0.5 ^{a}	−5.6 ± 0.5 ^{b}	−5.4 ± 0.6 ^{a}	−4.8 ± 0.5 ^{a}	−5.5 ± 0.5 ^{ac}
109	−5.6 ± 0.7 ^{ab}	−5.2 ± 0.9 ^{ab}	−5.7 ± 0.8 ^{ab}	−5.6 ± 0.7 ^{ab}	−5.2 ± 0.9 ^{ab}	−5.7 ± 0.8 ^{ac}
145	−6.8 ± 1.4 ^{b}	−6.2 ± 1.5 ^{b}	−6.9 ± 1.4 ^{b}	−6.9 ± 1.4 ^{b}	−6.2 ± 1.5 ^{b}	−7.1 ± 1.4 ^{c}
p-value	0.002	0.006	<0.001	<0.001	0.006	<0.001
D/L-Lactate						
0	−5.0 ± 0.2	−4.6 ± 0.2	−5.1 ± 0.2	−5.0 ± 0.2	−4.6 ± 0.2	−5.1 ± 0.2
29	−5.0 ± 0.3	−4.7 ± 0.2	−5.1 ± 0.4	−5.1 ± 0.3	−4.7 ± 0.2	−5.3 ± 0.2
57	−5.2 ± 0.4	−4.7 ± 0.4	−5.4 ± 0.4	−5.2 ± 0.4	−4.7 ± 0.4	−5.3 ± 0.4
86	−5.0 ± 0.3	−4.6 ± 0.4	−5.2 ± 0.4	−5.0 ± 0.3	−4.6 ± 0.4	−5.1 ± 0.4
114	−5.0 ± 0.3	−4.6 ± 0.2	−5.2 ± 0.3	−5.1 ± 0.3	−4.6 ± 0.2	−5.2 ± 0.3
p-value	0.689	0.440	0.474	0.609	0.749	0.338
n-Butyrate						
0	−5.0 ± 0.2	−4.5 ± 0.3	−5.1 ± 0.2	−5.0 ± 0.2	−4.5 ± 0.2	−5.1 ± 0.2
38	−5.1 ± 0.4	−4.2 ± 0.7	−5.1 ± 0.4	−5.0 ± 0.4	−4.2 ± 0.4	−4.9 ± 0.4
76	−5.0 ± 0.4	−4.3 ± 0.2	−5.1 ± 0.4	−5.0 ± 0.3	−4.3 ± 0.2	−5.1 ± 0.3
114	−5.1 ± 0.5	−4.5 ± 0.2	−5.2 ± 0.4	−4.9 ± 0.5	−4.5 ± 0.2	−5.1 ± 0.4
152	−4.6 ± 0.5	−4.0 ± 0.4	−4.7 ± 0.5	−4.6 ± 0.5	−4.1 ± 0.3	−4.7 ± 0.5
p-value	0.439	0.116	0.442	0.302	0.102	0.320

[1] Conjugation frequency (CF) calculated as CF/D = transconjugants/donor, CF/R = transconjugants/recipients or CF/T = transconjugants/total bacterial count; SIF = stress impact factor (Supplementary data, Table S3); *p*-values were calculated comparing CF of different levels of exposure to the stressor using the nonparametric Kruskal–Wallis test and Mann–Whitney test. Significant differences ($p \leq 0.05$) between values are indicated by different superscript letters. Raw data are provided in supplementary data, Table S1.

4. Discussion

The aim of this study was to investigate the impact of nutrition related stress factors on conjugation frequencies in an in vitro trial with an ESBL-producing *E. coli* donor strain and a *Salmonella* Typhimurium recipient.

Conjugation frequency (CF) is frequently calculated by dividing the number of transconjugants/mL by the donor count per mL [40–45]. However, CF can also refer to the recipient instead of donor count [12,43]. Both donor and recipient growth are generally considered independent of each other but undergo dynamic growth states in the intestine. Therefore, this study was designed to investigate the impact of both donor and recipient, as well as the total bacterial count on conjugation frequencies. The rationale behind this approach is the view that in vivo bacteria are under constant stress, and, therefore, different stressors affect the physiological response of both donor and recipient. Thus, the results of this study differ depending on the method of calculation as they address different questions. To evaluate the risk of transmission following an infection with ESBL-producing *Enterobacteriaceae*, it is important to know, how many ESBL-producing *E. coli* cells transfer their plasmid to a potential recipient. However, the health-related risks and clinical importance of antibiotic resistant pathogens may be better characterized by their uptake of resistance genes.

The impact of different agents on conjugation events is commonly displayed as a change in CF. However, these agents do not only impact the formation of transconjugants, but also their growth and viability, as well as the growth and viability of recipient and donor. This creates a bias neglected by the common methods to calculate the CF as transconjugants/donor or transconjugants/recipient [14]. If the donor and recipient concentrations remain steady during a certain period of time, one may, therefore, refer instead to conjugation efficiency (Equation 4) [14,27]. This, however, did not apply for the current study and does not resemble the environment in the gastrointestinal tract, where changes in diet, treatment, or infection alter microbial composition [4,44,45]. Therefore, we developed a method to monitor conjugation frequencies for donor, recipient, and transconjugants under the effect of different stressors. By calculating the relative impact on growth and viability and multiplying this factor by their respective cfu/mL, all parts of the in vitro system were corrected for the growth impact of the stressor itself.

From the above it is concluded that the method of calculation has a significant impact on the results, and, thus, different methods should be considered in studies on the impact of stressors on conjugation frequency.

In the gastrointestinal tract (GIT) of poultry, pH levels range mainly between 5 and 8, if gizzard and proventriculus are neglected [46,47]. The crop and caecum are the compartments with the highest bacterial density, where bacterial interaction is most likely to occur [4,5]. Therefore, these pH values were chosen when studying the impact of pH on CF. When investigating pH as a stress factor, no impact on conjugation was observed in this study. Similarly, the growth of both donor and recipient strain was not significantly affected. In contrast, a positive impact on CF was observed in an experiment with an *E. coli* donor and a *Salmonella* Typhimurium recipient where an Inc GpI1 plasmid was transferred at a pH value of 4.3 [25]. Similar results were observed with other different *E. coli* donors and recipients [18]. Unfortunately, conjugation frequency was only based on the recipient count, and no information on the impact of the acid stress on the growth of the strains was stated. In the same study, the impact of low temperatures was investigated, showing a significantly higher impact on the conjugation rate compared to results with different pH values. When a combination of low temperatures and a pH value of 5 was tested for two different mating pairs, the effect was very similar to the impact of the low temperature alone [18]. This suggests that the pH of 5 did not have an impact on the conjugation, corresponding to the findings in this study. The increase of transconjugants at low pH levels was also observed in a study investigating the impact of HCl at 0.032–0.128 M on conjugation [18]. However, no information on conjugation frequencies or donor and recipient growth at these levels was provided. Thus, very low pH values may have an impact on conjugation, but pH levels commonly observed in the major parts of the intestinal tract may not be low enough to have an influence.

Osmolality in the gastrointestinal tract of broilers varies between individual bowel segments. Accordingly, osmolalities of 540 (crop), 312 (gizzard), 571 (duodenum), 650–573 (jejunum), and 514–451 (ileum) mOsm/kg were reported [48], descending the intestinal tract, while osmolarities of 390 (duodenum), 430 (jejunum), and 340 (ileum) mOsm/L were observed [47]. Despite the numeric

differences between these two studies, in the small intestine, the highest values were always observed in the jejunum, followed by the duodenum and ileum. In contrast to the findings from the pH setup, osmolality reduced the conjugation frequency exponentially, when calculating CF(D) and CF(T), while a more linear reduction was observed when calculating CF(R). Similar to experiments with different pH values, the donor growth was not affected by increasing osmolality, while the recipient strain showed reduced growth/viability. Thus, it appears that conjugation is more likely to occur in the gizzard or ileum as far as osmolality is concerned.

Since the ban of antibiotic growth promoters in the European Union (EC (No) 1831/2003), alternatives have especially gained importance [49]. Zinc and copper are commonly used in animal production to increase health, feed efficiency, and body weight [50]. In this study, the impact of zinc and copper on conjugation was the most profound among all tested stressors. Conjugation frequencies were reduced with approximately 2.3 and 0.8 log levels for copper and zinc respectively after correcting for stress impact on growth. Interestingly, in the zinc set up, the highest reduction occurred for medium levels of zinc supplementations contrary to experiments with copper, which showed a decreasing frequency of conjugation with increasing copper concentrations. The decrease of CF at medium zinc concentrations, followed by an increase with increasing zinc concentrations, might indicate that zinc up to a certain concentration can reduce conjugation. However, as bacterial stress increases at higher zinc concentrations, horizontal gene transfer might also be enhanced. This suggests a correlation between concentration of stressor and CF, which must not be linear. One must acknowledge that numerically, the observed decrease of CF at 321 and 642 µM Zn(II) is rather small (<1 log cfu/mL), indicating that interpretations should be discussed critically. Further studies should investigate this observation in detail. Zinc acetate (0.2 mM zinc) reduced the transfer of an ESBL-carrying plasmid from an *Enterobacter* donor to an *E. coli* recipient under detection limit [51]. As results from this study did not show such a severe impact, it must be considered that the conjugative pair used in the present study was more prone to transfer plasmids and did so in a shorter time period. Varying conjugation frequencies were also observed for ESBL-producing *Enterobacteriaceae* in the presence of different metal surfaces with an *E. coli* and a *Klebsiella pneumoniae* donor and a *E. coli* recipient [45]. While CF declined after 2 h on stainless steel, it fell below the detection limit after 2 h incubation on the copper surface. This agrees with our results, where increasing concentrations of copper led to higher reductions of conjugation rates. Similarly, different copper supplementations of $CuSO_4$ and copper nanoparticles reduced conjugation frequencies [52]. From this comprehensive perspective, the copper surface should be considered a substantial high concentration. A link between the usage of zinc and copper feed additives and the occurrence of antibiotic resistance was established repeatedly [53–57]. Consequently, the European Medicines Agency (EMA) and European Food Safety Authority (EFSA) recommends reducing zinc and copper in animal production [58,59]. The positive impact of copper and zinc on the reduction of CF must, therefore, be weighed against the risk of increasing prevalence of antibiotic resistant bacteria by other mechanisms and treatments.

Bacterial metabolites may also have an impact on bacterial physiology in vivo. For instance, a reduction of conjugation frequency was previously reported in the presence of lactic acid producing bacteria, such as *Streptococcus thermophilus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *L*actobacillus bulgar*icus* [19,60–63]. This effect was assumed to be due to their lactate production among other factors. This hypothesis could not be confirmed here, as lactate did not lead to a reduced conjugation frequency. Interestingly, while numeric differences between the control and highest lactate concentrations were negligible, n-butyrate actually showed higher conjugation rates at its highest concentration, while acetate numerically decreased the conjugation frequency. Thus, apart from pH reduction, bacterial metabolites may also play different roles for the transfer of mobile genetic elements. This may also be the case for propionic acid, which showed a negative concentration dependent effect on conjugation. Similarly, a reduction of CF(D) was observed in an experiment with *Salmonella enterica* serovar Typhimurium donor and recipient strains derived from mice in the presence of propionate [64]. As pH as a factor can be ruled out, and the growth corrected conjugation frequency also declined

drastically, it can be concluded that propionate acts differently on conjugation than acetate or n-butyrate. Finally, observed effects for propionate were only significant at concentrations that exceeded the usually observed threshold in the hindgut of poultry. However, propionate is used in quite high doses for its antibacterial and antifungal properties in animal nutrition, and, therefore, further in vivo studies should investigate the effect of propionic acid as a feed additive to counteract the transfer of ESBL-carrying plasmids in *Enterobacteriaceae*.

The determined conjugation frequency depends on two factors—The bacterial concentration and the transconjugants' growth. The bacterial concentration shapes the chance for donor and recipients to meet close enough to perform a plasmid transfer. On the other hand, transconjugant growth directly affects the number of detected transconjugants and thus the calculation and result of CF. Hence, it would be tempting to conclude that changes in CF can be explained solely mathematically due to variations of donor, recipient, and/or transconjugant concentrations in the presence of stressors. To investigate this further, an experiment with sublethal amounts of antibiotics was designed, to reduce the growth of (a) donor, (b) recipient, or (c) both donor and recipient. Assuming that conjugation comes at no fitness cost or gain, the transconjugants should grow similar to the recipients, as they are basically identical apart from their additional plasmid harboring resistance against CTX. If the explanation for the changes in CF were solely mathematical, the following situation would arise for the mentioned scenarios:

a. Lower numbers of donor cells would lead to a higher ratio of transconjugants per donor cell count.
b. Lower numbers of recipient cells would lead to lower numbers of transconjugants and a decreased ratio of transconjugants per donor cell count (except for CTX supplementation, as the transconjugants grow better than the recipients).
c. As recipient and donor are affected equally, CF will not differ significantly from control.

This would lead to a lower number of transconjugants per donor in the osmolality and CTX experiments. This effect should be more profound for osmolality, since both the recipient count and transconjugant growth are affected negatively. CTX, on the other hand, reduces the growth of the recipient more than transconjugant growth. Thus, one expects decreasing CF(D), CF(R), and CF(T) with increasing concentrations of CTX and higher osmolality. Similarly, SXT supplementation would result in an increase of transconjugants per donor and higher CF(D), while lower CF(R) would be expected at increasing antibiotic concentration. Nitrofurantoin inhibited the growth of donors and recipients in an equal manner. Therefore, no significant differences were predicted. The results from the experiments differed from these assumptions. Thus, the changes in conjugation frequencies cannot solely be explained by changes in bacterial growth, justifying the conclusion that some stress factors may directly influence conjugation.

Antibiotics are frequently used in livestock as therapeutics, meta- and prophylaxis and, outside the European Union, in subtherapeutic levels to enhance performance [5,10,65]. The usage of antibiotics always comes with the risk of developing antibiotic resistant bacteria due to selective pressure, especially when used at subtherapeutic levels [5,10,66]. Simultaneously, the potential threat to survival is posing stress to the microorganisms resulting in changes of metabolism and activity [24,36,37]. This may also impact conjugation [35]. The influence of gentamycin on the transfer of an ESBL-carrying plasmid from an *E. coli* donor to *E. coli* and *Pseudomonas aeruginosa* recipients had similar prerequisites as the CTX experiment, since donor growth was not affected as much as the recipient, which declined with increasing concentrations [67]. Similar results were observed in a further study investigating the impact of three different antibiotics in a conjugation experiment with resistant donors and transconjugants but sensitive recipients [68]. *Pseudomonas aeruginosa* accepted the ESBL-carrying plasmids at a higher rate only at a state where the antibiotic concentration changed from low impact to high impact on recipient growth. The results agree with the results from the CTX experiment (CF(D)). In the case of the *E. coli* recipient, CF(D) decreased with increasing gentamycin [68] supplementation, corresponding with the results from the presented nitrofurantoin experiment. However, nitrofurantoin had a different impact

on donor growth. The gentamycin effect was observed at a stage of severe impact on recipient growth, and this was not considered when CF was calculated. Thus, the low detection of transconjugants may be the reason for these results, rather than an actual change in conjugation. Similar to the nitrofurantoin experiment, it was previously described that an antibiotic substance, affecting donors and recipients equally, may lead to a reduction of conjugation frequencies [68]. The impact of antibiotics on donor, recipient, and transconjugant growth over a period of time shows how incorrect assumptions on the impact of different factors easily arise [20]. Even after recipient counts fell under the detection limit, transconjugants kept rising. Simultaneously, the ratio of transconjugants/donor and transconjugant/recipient changed with time. Both amoxicillin and ampicillin reduced transconjugant counts similar to the cefotaxime experiment with increasing antibiotic concentrations [20]. CF(D) increased in the presence of sulfamethoxazole/trimethoprim. However, when corrected for growth impact, the effect was opposite, showing a significant decrease in conjugation frequency with an increasing concentration of SXT, which was also observed for CF(R)(SIF) and CF(T)(SIF).

5. Conclusions

In conclusion, a negative impact on conjugation frequency was observed for osmolality, zinc, copper, and propionic acid, as well as subtherapeutic levels of antibiotics. No effects were found for pH or the bacterial metabolites lactate, acetate, or n-butyrate. Furthermore, no stressors increased conjugation frequency, and, thus, the hypothesis that stress generally increases bacterial conjugation should be viewed with caution. The results also show that, in studies focusing on stress related effects on gene transfer, the calculation of conjugation frequency should include the impact of a stressor on donor, recipient, and transconjugant. Still, it must be considered that the observed impact on conjugation frequencies might be strain specific. Future studies should, therefore, investigate if these observations can be repeated with different donor and recipient strains. In the present study, the impact on conjugation events was investigated for one stressor at a time. However, the intestinal tract of broilers combines these and further stressors. Thus, further studies should be anticipated to examine conjugation events in complex systems.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/9/8/324/s1, Figure S1: Bacterial concentration at different pH values, Figure S2: Bacterial concentration at different osmolalities, Figure S3: Bacterial concentration at different levels of cefotaxime (CTX), Figure S4: Bacterial concentration at different levels of sulfamethoxazole/trimethoprim (SXT), Figure S5: Bacterial concentration at different levels of nitrofurantoin (F), Figure S6: Bacterial concentration at different levels of copper, Figure S7: Bacterial concentration at different levels of zinc, Figure S8: Bacterial concentration at different concentrations of acetate, Figure S9: Bacterial concentration at different concentrations of D/L-lactate, Figure S10: Bacterial concentration at different concentrations of propionate, Figure S11: Bacterial concentration at different concentrations of n-butyrate, Table S1: Conjugation frequencies: measured values, Table S2: Bacterial growth: measured values, Table S3: Values of stress impact factor.

Author Contributions: E.-M.S. planned and carried out the experiment and wrote the manuscript with input from all authors. M.E. designed the technical details for the osmolality, zinc, copper and pH media. W.V., J.Z., and E.-M.S. conceived the original idea. W.V. supervised the project. J.Z. helped to supervise the project. All authors discussed the results and commented on the manuscript.

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The Impact of Direct-Fed Microbials and Phytogenic Feed Additives on Prevalence and Transfer of Extended-Spectrum Beta-Lactamase Genes in Broiler Chicken

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Abstract: Poultry frequently account for the highest prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* in livestock. To investigate the impact of direct-fed microbials (DFM) and phytobiotic feed additives on prevalence and conjugation of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*, an animal trial was conducted. *Lactobacillus agilis* LA73 and *Lactobacillus salivarius* LS1 and two commercial phytogenic feed additives (consisting of carvacrol, cinnamaldehyde, and eugenol) were used as feed additives either alone or as a combination of DFM and phytogenic feed additive. An ESBL-producing *E. coli* donor and a potentially pathogenic *Salmonella* Typhimurium recipient were inoculated at 5×10^9 cells/mL in cecal contents from 2-week-old broilers. Conjugation frequencies were determined after 4 h aerobic co-incubation at 37 °C and corrected for the impact of the sample matrix on bacterial growth of donor and recipient. Surprisingly, indigenous *Enterobacteriaceae* acted as recipients instead of the anticipated *Salmonella* recipient. The observed increase in conjugation frequency was most obvious in the groups fed the combinations of DFM and phytogenic product, but merely up to 0.6 log units. Further, cecal samples were examined for ESBL-producing *Enterobacteriaceae* on five consecutive days in broilers aged 27–31 days. All samples derived from animals fed the experimental diet showed lower ESBL-prevalence than the control. It is concluded that *Lactobacillus* spp. and essential oils may help to reduce the prevalence of ESBL-harboring plasmids in broilers, while the effect on horizontal gene transfer is less obvious.

Keywords: extended-spectrum ß-lactamases; ESBL; phytobiotics; probiotics; essential oils; Lactobacillus; plasmid transfer; horizontal gene transfer; stress impact; conjugation

1. Introduction

Broiler chickens are the livestock with the highest prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* in many regions of the world. In this context, *E. coli* and *Salmonella* spp. are the bacteria most commonly identified as the host of *bla* (ESBL encoding genes) carrying plasmids [1]. Transmission of ESBL-producing bacteria between animals happens rapidly and undetected, as no specific symptoms accompany the inoculation and establishment of ESBL-producing *Enterobacteriaceae* in broilers. These primarily non-pathogenic bacteria can, however, transfer mobile genetic elements to pathogenic bacteria and thereby cause infections, which are difficult to cure [2–5]. As they also may be transmitted to humans, these multi-resistant bacteria pose a major hazard to public health, causing tremendous costs worldwide [6–9].

As the problem gained global attention and the importance to intervene in the development and spread of antibiotic-resistant bacteria has gained high political priority, different measurements were developed to reduce the prevalence of ESBL-producing bacteria [8,10,11]. One possible approach to reduce the ESBL prevalence may be the use of feed additives, such as direct-fed microbials (DFM) or phytogenic feed additives [12,13]. The negative impact of DFMs, such as various *Lactobacillus* strains, on the prevalence of pathogens has been described frequently [12,13]. To our best knowledge, only three studies demonstrating the impact of DFMs and competitive exclusion cultures on prevalence and transfer of ESBL-producing *Enterobacteriaceae* in broilers have been published [14–16]. These studies comprise commercial products and competitive exclusion cultures, however no exact qualitative and quantitative specification of the containing microorganisms was provided.

Phytogenic products are used in poultry farming due to their beneficial impact on health and production [13,17]. These products can be grouped into four categories: herbs, botanicals, essential oils, and oleoresins [18]. Antibacterial activities against pathogens such as *Salmonella* spp. and *E. coli* among others have been observed in various essential oils [13,19,20]. Moreover, phytogenic feed additives were associated with a reduced plasmid transfer in *E. coli* [21].

Besides their prevalence, the spread of antibiotic resistance plays an important role in the dissemination of ESBL-producing *Enterobacteriaceae*. On the one hand, the transmission between animals and from animals to humans must be considered. Several studies have targeted this topic with one trial specifically covering the transmission of ESBL-producing *Enterobacteriaceae* between broilers receiving DFM [16]. On the other hand, horizontal gene transfer of ESBL-carrying plasmids must also be considered as antibiotic resistance genes are frequently exchanged between bacteria [22,23].

Thus, the approach of this study was to use DFMs with previously characterized components (qualitatively and quantitatively) as well as phytogenic products to reduce the prevalence and conjugation frequency of ESBL-producing *Enterobacteriaceae*. Results were obtained from in vivo (ESBL prevalence) and ex vivo (conjugation) experiments to investigate the natural occurrence and spread of ESBL genes as well as conjugation between artificially added strains.

2. Materials and Methods

2.1. Animals and Husbandry

For the animal trial, newly hatched male Cobb 500 broiler chicks were randomly allocated to nine feeding groups with seven replicates each and reared for five weeks. Three animals were reared together in cages of 50 × 35 × 68 cm (depth × width × height) for two weeks, subsequently, the animal density was reduced to 1–2 animals per cage. Metal walls separating cages reduced the contact between animals. As the cage floor comprised a metal net, excreta were automatically excluded from the animals' environment, reducing the contact of the animals with the excreta and thereby decreasing the risk for bacterial contamination. The initial temperature was 34 °C for 48 h and reduced by 3 °C weekly. After 72 h of constant light, a cycle of 18 h light and 6 h darkness was applied. Water and experimental diet were constantly available ad libitum. The nine experimental diets comprised a control diet (Table 1), two diets supplemented with *Lactobacillus salivarius* LS1 or *Lactobacillus agilis* LA73 (10^{10} cfu per kg feed) [24], two experimental diets were supplemented with the phytogenic products Formulation C or Formulation L (250 mg/kg feed; EW Nutrition, Germany) and four diets supplemented with a combination of one DFM and one phytogenic product (Table 2). Formulation C contained the essential oils carvacrol and cinnamaldehyde, while Formulation L additionally contained eugenol. These feed additives were chosen due to their ability to reduce the viability of the ESBL-producing *E. coli* strain ESBL10716 in a previous in vitro experiment [25]. The animal trial was approved by the Regional Office for Health and Social Affairs Berlin (LaGeSo Reg. A 0437/17).

Table 1. Composition of the basal diet.

Ingredients	g/kg
Maize	320.3
Wheat	247.8
Soybean meal 49% CP	323.3
Soybean oil	59.5
Mineral–Vitamin Premix [1)]	12.0
Limestone	14.6
Monocalcium phosphate	18.4
Salt	1.0
DL-Methionine	1.8
L-Lysine	1.3
Skim milk powder	0.3
Nutrient Composition	
Crude Protein (%)	22.00
Crude Fat (%)	8.19
Crude Fiber (%)	2.42
Methionine (%)	0.51
Lysine (%)	1.28
Threonine (%)	0.84
Calcium (%)	0.96
Phosphorus (%)	0.80
Calculated Apparent Metabolizable Energy	
AME_N (MJ/kg) [2)]	12.6

[1)] Contents per kg diet: 4800 IU vit. A; 480 IU vit. D3; 96 mg vit. E (α-tocopherole acetate); 3.6 mg vit. K3; 3 mg vit. B1 ; 3 mg vit. B2; 30 mg nicotinic acid; 4.8 mg vit. B6; 24 µg vit. B12; 300 µg biotin; 12 mg calcium pantothenic acid; 1.2 mg folic acid; 960 mg choline chloride; 60 mg Zn (zinc oxide); 24 mg Fe (iron carbonate); 72 mg Mn (manganese oxide); 14.4 mg Cu (copper sulfate-pentahydrate); 0.54 mg I (calcium Iodate; 0.36 mg Co (cobalt-(II)-sulfate-heptahydrate); 0.42 mg Se (sodium selenite); 1.56 g Na (sodium chloride); 0.66 g Mg (magnesium oxide). [2)] Nitrogen-corrected apparent metabolizable energy estimated from chemical composition of feed ingredients (based on the EU Regulation - Directive 86/174/EEC): 0.1551 × % crude protein + 0.3431 × % ether extract + 0.1669 × % starch + 0.1301 × % total sugar.

Table 2. Feed additives applied to feed groups.

Feed Group	Diet Supplementation
Control	None
LS	DFM 1:10^{10} cfu *Lactobacillus salivarius* LS1/kg diet
LA	DFM 2: 10^{10} cfu *Lactobacillus agilis* LA73/kg diet
Formulation C	Phytogenic product 1: 0.25 g Formulation C/kg diet
Formulation L	Phytogenic product 2: 0.25 g Formulation L/kg diet
LS + C	0.25 g Formulation C + 10^{10} cfu *Lactobacillus salivarius* LS1/kg diet
LS + L	0.25 g Formulation L + 10^{10} cfu *Lactobacillus salivarius* LS1/kg diet
LA + C	0.25 g Formulation C + 10^{10} cfu *Lactobacillus agilis* LA73/kg diet
LA + L	0.25 g Formulation L + 10^{10} cfu *Lactobacillus agilis* LA73/kg diet

2.2. Strains and Cultivation Conditions

The experimental design comprised the ESBL-producing donor strain *Escherichia coli* ESBL10682, derived from broiler excreta within the RESET program [26]. This strain belonged to the phylogenic group B1 and produced the enzyme CTX-M-1. Furthermore, the strain *Salmonella* Typhimurium L1219-R32 served as the recipient. Susceptibility of donor and recipient against various antibiotics was investigated by disc diffusion test (Table S1 Saliu et al., manuscript submitted). This conjugative pair was known to transfer plasmids in vitro at a conjugation frequency (CF) of 10^{-4}–10^{-5} when incubated in Mueller Hinton 2 Broth (Sigma-Aldrich, Chemie GmbH, Germany) for 4 h [27]. All samples were cultivated aerobically at 37 °C for 4 h.

2.3. Collection of Samples

Pooled excreta samples were collected weekly from each experimental group. During days 13–17, these samples were derived from the cages used for the conjugation experiment only. The samples from the last collection originated from fewer cages as several animals had been sacrificed according to the trial design. At all remaining time points, all cages were sampled. After dilution with equal volumes of sterile glycerol (50% Glycerol, Carl Roth GmbH + Co. KG, Germany, 50% Phosphate Buffered Saline (PBS), Sigma-Aldrich Chemie GmbH, Germany), the samples were stored at −80 °C until further analyzed.

Two animals of each feeding group were sacrificed per day by cervical dislocation subsequent to anesthesia from day 13 to 17. The ceca were removed, immediately transferred to the laboratory facilities, and the content was collected. This procedure was repeated with one animal per feeding group and day on days 27–31.

2.4. Conjugation Experiments

The donor and recipient strains were cultivated in Mueller Hinton 2 Broth supplemented with 8 µg/mL cefotaxime (CTX; Alfa Aesar, Thermo Fisher GmbH, Germany) or 300 µg/mL sulfamethoxazole/trimethoprim (SXT, Sigma-Aldrich, Chemie GmbH, Germany) respectively for 19 h. After washing the cells twice in PBS, cell concentrations were obtained photometrically and adjusted to 5×10^9 cells/mL. Cecal samples were diluted 10-fold in a citric acid–Na-citrate buffer system (pH 6.2; Sigma-Aldrich, Chemie GmbH, Germany). Each 50 µL donor and recipient suspension were added to 900 µL diluted cecal samples in triplicates. The suspensions were thereafter incubated aerobically for 4 h and plated on selective MacConkey agar (Carl Roth GmbH + Co. KG, Germany) containing 300 µg SXT and 8 µg CTX per mL agar, to obtain transconjugants, 300 µg SXT/mL agar for recipient identification or 8 µg CTX/mL agar for the calculation of donor concentrations. CF was calculated as transconjugants/donor (CF/D) and transconjugants/recipients (CF/R). The negative control comprised cecal contents without donor or recipient and was plated on MacConkey agar containing 300 µg SXT and 8 µg CTX per mL agar after 4 h incubation.

2.5. Calculation of a Stress Impact Factor

As the sample matrix influences the growth of donor, recipient, and transconjugants, a bias arises when only evaluating CF based on donor or recipient cfu. Thus, the impact of stressors within the sample must be considered. A stress impact factor (SIF), correcting the results for this bias, aims to normalize the differences by incorporating control incubations [27]:

$$\mathrm{SIF} = \frac{mean\ cfu_{\mathrm{ctr}}/mL}{mean\ cfu_{\mathrm{stress}}/mL} \quad (1)$$

Here, $\mathrm{cfu_{ctr}}$ represents the cfu of the control and $\mathrm{cfu_{stress}}$ stands for the bacterial concentration (cfu) at a specific level of supplementation with a stress factor. No differences in growth are observed when the SIF equals 1, while a SIF smaller than 1 occurs when feed additives result in higher bacterial concentrations. On the other hand, a SIF larger than 1 outlines enhanced bacterial growth in the presence of the stressor compared to no supplementation. This factor can subsequently be used to calculate corrected bacterial concentrations ($\mathrm{cfu_{corr}}$), which most likely would be observed in the absence of the stressor by multiplication of the SIF and $\mathrm{cfu_{stress}}$:

$$\mathrm{cfu_{corr}} = \mathrm{cfu_{stress}} \times \mathrm{SIF} \quad (2)$$

SIF corrected CF were thereafter calculated as CF/D (SIF) = $\mathrm{cfu_{corr}}$(transconjugants)/ $\mathrm{cfu_{corr}}$(donor) and CF/R (SIF) = $\mathrm{cfu_{corr}}$(transconjugants)/ $\mathrm{cfu_{corr}}$(recipient). In the latter, the SIF for transconjugants equals the SIF for recipients. Hence, CF/R=CF/R (SIF) and CF/R (SIF) can be neglected.

2.6. Prevalence of ESBL-Producing Enterobacteriaceae

The excreta samples were thawed at room temperature and each 1 g sample was diluted in 4.5 mL Buffered Peptone Water (BPW, Carl Roth GmbH + Co. KG, Germany) as described previously [28]. The dilutions were plated on multiple MacConkey agar plates containing 2 µg CTX/mL agar as described previously [29–31] and incubated aerobically at 37 °C for 48 h.

A similar procedure was applied for the cecal content samples collected on day 27–31. The cecal content was diluted in the double amount of PBS and directly spread on MacConkey agar containing 2 µg CTX/mL. The plates were evaluated after 48 h aerobic incubation at 37 °C.

No pre-enrichment was conducted, as quantification of ESBL-producing *Enterobacteriaceae* was intended.

2.7. Statistics

The software IBM SPSS (Version 22, USA) was used for the statistical analysis of the results. The results are presented as mean values and standard deviation. To determine significance and subgroups, the non-parametric Kruskal–Wallis test and Mann–Whitney test were applied for CF and bacterial growth while the chi-squared test was applied for the prevalence of ESBL-producing *Enterobacteriaceae* in the cecal content. Differences at $p < 0.05$ were considered statistically significant and p values between 0.05 and 0.1 were accepted as trends. Pearson correlations were applied to identify correlations between short-chain fatty acid concentrations and CF and were considered significant at the 0.01 level (2-tailed).

3. Results

3.1. Donor and Recipient Growth

The different feed additives and their combinations did not show a significant impact on *Salmonella* Typhimurium L1219-R32 ($p = 0.183$) or *E. coli* ESBL10682 ($p = 0.317$) growth (Table 3). The initial concentration of approximately 8.7 log cfu/mL cecal content of each donor and recipient strain declined after 4 h of incubation to 7.5–7.9 log cfu/g cecal content for *E. coli* ESBL10682 and 7.9–8.4 log cfu *Salmonella* Typhimurium/g cecal content while the indigenous, SXT-resistant *Enterobacteriaceae* showed concentrations of 6.4–7.1 log cfu/g cecal content. On average, the *Salmonella* Typhimurium L1219-R32 showed slightly higher growth than the *E. coli* strain. The lowest growth was observed for SXT resistant *Enterobacteriaceae*, which also varied significantly ($p = 0.002$) between feeding groups (Table 3). Here, LS + L, LA + C, LA and Formulation C showed significantly lower numbers of SXT resistant *Enterobacteriaceae* of up to 0.72 log units than Formulation L or LA + L. However, these were not significantly lower than the observed amount of SXT resistant *Enterobacteriaceae* in samples derived from animals fed the control diet.

3.2. Conjugation Experiments

The donor/recipient pair was known from a previous in vitro study to show CF of 10^{-5}–10^{-4} transconjugants/donor or transconjugants/recipient after 4 h of co-incubation [27]. In the aforementioned in vitro set up, a 10-fold lower initial concentration of donor and recipient was chosen compared to the setup of the present experiment. Still, in the present study, indigenous *Enterobacteriaceae* stepped in as plasmid acceptors instead of the intended *Salmonella* Typhimurium recipient at CF of 10^{-5}–10^{-4} transconjugants/donor and 10^{-3} transconjugants/recipient (Table 4). Differences in CFD (SIF) were observed between different trial groups. The groups receiving feed supplemented with Formulation C, LS +C, LS + L or LA + L showed significantly higher CF/D (SIF) than the control group (Table 4). The applied feed additives did not affect CF/R. The negative control did not grow colonies on plates containing the combination of CTX and SXT or only CTX but on the plates containing SXT.

Table 3. Impact of direct-fed microbials (DFM) and phytobiotic feed additives on bacterial growth after 4 h incubation of inoculated cecal samples with the donor (*E. coli* ESBL10682) and recipient (*Salmonella* Typhimurium L1219-R32) strains [$\log_{10}$(cfu/mL)].

Trial Group	*E. coli* ESBL10682	*Salmonella* Typhimurium L1219-R32	Indigenous, SXT Resistant *Enterobacteriaceae* [2]
Control	6.86 ± 0.48	7.10 ± 0.37	5.93 ± 0.61 ^{abe}
LS	6.81 ± 0.27	7.04 ± 0.39	5.92 ± 0.52 ^{be}
LA	6.90 ± 0.45	6.92 ± 0.47	5.49 ± 0.42 ^{abcd}
Formulation C	6.94 ± 0.31	7.06 ± 0.42	5.50 ± 0.52 ^{abcd}
Formulation L	6.91 ± 0.28	7.36 ± 0.24	6.12 ± 0.80 ^{e}
LS + C	6.76 ± 0.54	7.21 ± 0.31	5.85 ± 0.49 ^{abde}
LS + L	6.56 ± 0.71	7.14 ± 0.32	5.40 ± 0.64 ^{abcd}
LA + C	6.49 ± 0.73	7.10 ± 0.37	5.45 ± 0.68 ^{abcd}
LA + L	6.59 ± 0.53	7.14 ± 0.24	6.02 ± 0.51 ^{e}
p [1]	0.317	0.183	0.002

[1] Significant differences were determined using the Kruskal–Wallis test, [2] different letters indicate significant differences ($p < 0.05$, Mann–Whitney test) in the abundance of indigenous, SXT resistant *Enterobacteriaceae* after 4 h incubation between trial groups.

Table 4. Impact of DFM and phytobiotic feed additives on CF after 4 h incubation of donor (*E. coli* ESBL10682) and the recipient (*Salmonella* Typhimurium L1219-R32) in cecal contents [$\log_{10}$(CF [1])].

Trial Group	CF/D [3]	CF/R	CF/D (SIF) [3]
Control	−4.43 ± 0.45 ^{ab}	−3.46 ± 1.01	−4.43 ± 0.45 ^{a}
LS	−4.21 ± 0.57 ^{b}	−3.32 ± 0.74	−4.29 ± 0.57 ^{ab}
LA	−5.07 ± 1.46 ^{a}	−3.65 ± 1.12	−4.57 ± 1.46 ^{a}
Formulation C	−4.60 ± 0.40 ^{a}	−3.16 ± 0.68	−4.20 ± 0.40 ^{b}
Formulation L	−4.27 ± 0.52 ^{ab}	−3.48 ± 0.96	−4.66 ± 0.52 ^{a}
LS + C	−4.10 ± 0.52 ^{b}	−3.19 ± 0.56	−3.98 ± 0.52 ^{b}
LS + L	−3.98 ± 0.96 ^{b}	−2.82 ± 0.71	−3.82 ± 0.96 ^{b}
LA + C	−3.95 ± 0.55 ^{b}	−2.90 ± 0.68	−3.98 ± 0.55 ^{b}
LA + L	−4.10 ± 0.94 ^{b}	−3.53 ± 0.86	−4.33 ± 0.94 ^{ab}
p [2]	0.010	0.172	0.031

[1] CF = conjugation frequency; [2] significant differences were determined using the Kruskal–Wallis test, [3] different letters indicate significant differences ($p < 0.05$, Mann–Whitney test) in conjugation frequency between trial groups within a column; CF/D = transconjugants/donor; CF/R = transconjugants/recipients; SIF = stress impact factor.

3.3. Prevalence of ESBL-Producing Enterobacteriaceae

ESBL-producing *Enterobacteriaceae* (ESBL-PE) were detected in the excreta of newly hatched chicks and throughout the entire rearing period (Table S2). To investigate the day-to-day differences of cecal ESBL-PE, the fifth week of the feeding trial was monitored closely. Significant differences ($p = 0.001$) were observed between feed groups both in regards to the number of days ESBL-PE were above the detection limit, as well as to the amount of detected ESBL-PE/g cecal content (Table 5). The groups LA and LS + L were negative at all sampling times. Quantitatively, groups LS + C, LA + L, LS, LA + C, Formulation C and Formulation L followed. When evaluating the qualitative results, LS + C and LA + L showed a lower prevalence than LS, LA + C, Formulation C and Formulation L. The ESBL-producing *Enterobacteriaceae* were not characterized further, but the microbial composition of the cecal content was described elsewhere [32].

Table 5. Impact of probiotic and phytobiotic feed additives on the prevalence of ESBL-producing *Enterobacteriaceae* in cecal contents of broilers [cfu/g].

Trial Group \ Day	27	28	29	30	31	Prevalence per Trial Group (%)
Control	13	nd	7	8340	nd	60 [a]
LS	20	nd	nd	nd	40	40 [b]
LA	nd	nd	nd	nd	nd	0 [b]
Formulation C	13	nd	nd	nd	80	40 [b]
Formulation L	7	nd	nd	539	nd	40 [b]
LS + C	nd	nd	20	nd	nd	20 [b]
LS + L	nd	nd	nd	nd	nd	0 [b]
LA + C	nd	63	nd	25	nd	40 [b]
LA + L	nd	nd	nd	7	nd	20 [b]
Prevalence (%): positive samples per day	44	11	22	44	22	
p [1]						0.001

nd: not detected. [1] Significant differences were determined using the chi-squared test, different letters indicate significant differences ($p < 0.05$) in the overall prevalence of ESBL-producing *Enterobacteriaceae* between trial groups.

4. Discussion

The threat that ESBL-producing *Enterobacteriaceae* from poultry pose to public health must be taken into serious consideration when discussing antibiotic resistance in farm animals. Different approaches to reduce the prevalence and spread of these bacteria in poultry by the use of feed additives showed promising results [14–16,21]. However, to our best knowledge, their impact on both prevalence and horizontal gene transfer has not been studied in poultry. A previous study by our working group focused on the identification of lactic acid bacteria and phytogenic products with inhibitory potentials against the ESBL-producing *E. coli* ESBL10716 [25]. Subsequently, the current trial followed to investigate their ability to reduce in vivo prevalence and ex vivo transfer of extended-spectrum beta-lactam resistance.

To investigate the impact of these feed additives on conjugation frequency, donor and recipient strains were added to cecal contents obtained from 2-week-old broilers. The initial concentrations of the donor and recipient strains were chosen at very high levels compared to the common abundance of bacterial strains in the gastrointestinal tract of broilers [33–35] to investigate the theoretical possibility of gene transfer, as high concentrations increase the chance of detecting changes. Only a part of the initial donors and recipients added to the cecal contents were able to establish in the matrix, but *E. coli* and *Salmonella* Typhimurium concentrations still exceeded natural levels of single strains. Here, the detection of higher concentrations for *Salmonella* Typhimurium L1219-R32 than *E. coli* ESBL10682 was unexpected, as previous in vitro experiments with this mating pair constantly led to reverse results [27]. Apparently, the chosen habitat (cecum) was more favorable for the *Salmonella* strain.

As both donor and recipient were able to survive the conditions in cecal contents, one would expect to find transconjugants after 4 h of co-incubation. Surprisingly, the complex system of an intestinal milieu revealed a tremendous impact on conjugation, as no *Salmonella* transconjugants could be recovered. The chosen *Salmonella*/*E. coli* mating pair has been established as an excellent system for in vitro experiments [27]. Furthermore, the growth of the *Salmonella* strain was also not affected by the cecal matrix. Therefore, other factors of the cecal matrix may have been present. During previous in vitro experiments, the simple addition of propionate for instance significantly reduced conjugation frequencies for the *Salmonella*/*E. coli* mating pair [27]. Cecal propionate concentrations were not significantly different between feed groups (Table S3) [32] and no correlation between propionate ($p = 0.662$), acetate ($p = 0.96$) or total short-chain fatty acids ($p = 0.905$) and CF/D (SIF) were detected. Still, this and other factors of the cecal matrix (bacterial competition, quorum sensing molecules, intermediary metabolites) may have triggered a physiological state in either donor and/or recipient strain that inhibits the formation of the conjugation machinery [36–38].

Instead, within the period of 4 h, a time frame resembling the passage time of cecal content in 14-day-old broilers [39], *E. coli* donor successfully transferred the $bla_{CTX\text{-}M\text{-}1}$ carrying plasmid to indigenous *Enterobaceriaceae*. This also leads to the conclusion that conjugation was not inhibited for the donor and thus, conjugation may have only been inhibited for the *Salmonella* recipient. As only the SXT resistant transconjugants were detected, one must expect that further, SXT sensitive enterobacteria additionally served as recipients and affected the CF observed in this experiment. As the negative control did not show growth on plates identifying donor or transconjugants, it was concluded that the chosen donor strain was accountable for the observed transconjugants. Further studies should investigate longer incubation times, simulating the establishment of donor and recipient in the gastrointestinal tract. Additionally, a wider screening of recipients should be included.

Comparing conjugation frequencies is a common method applied when evaluating horizontal gene transfer [40]. To calculate the CF, the amount of transconjugants is either divided by the number of recipients or donors. Results from the present study display major differences between these approaches and stress the importance of considering both methods when evaluating plasmid transfer. Also, the impact of the environment on bacterial growth is frequently neglected. Thereby, a bias arises [40–42], which was circumvented in the current study by applying the stress impact factor (SIF). In the present study, the results of SIF correction were rather similar to the CF/D and CF/R values besides group LA, Formulation C and Formulation L, where differences of 0.5, 0.4, and 0.3 log cfu/mL were observed, respectively. In these groups, the SIF for transconjugant and recipient growth were among the most pronounced at 2.8, 2.3, and 0.4 respectively. This highlights the importance of considering changes in bacterial growth when interpreting results from conjugation trials. Observed CF/R in this ex vivo setup exceeded results from previous in vitro experiments, while the CF/D was rather similar to earlier findings [27]. This might be explained by the amount of different possible indigenous recipients compared to one *Salmonella* recipient strain.

The lowest detected CF/D (SIF) among indigenous enterobacteria was observed when fed the experimental diets supplemented with *L. agilis* or Formulation L. Similarly, it was previously described that *Lactobacillus plantarum* strains can reduce CF/R in vitro, independent of their ability to produce bacteriocins [43]. Similar results were observed in an in vitro trial with *Klebsiella pneumoniae* (SHV-5) and *Salmonella enterica* serovar Typhimurium (CTX-M-15) donors and an *Escherichia coli* K-12 recipient in the presence of different *Bifidobacterium* spp., reducing transconjugant counts with up to 2.6 logs [44]. These results were confirmed in in vivo trials with gnotobiotic mice, where a strain and incubation time-dependent reduction of transconjugants of up to 3.3 logs was reported. However, no information is provided on the impact of DFM on horizontal gene transfer of *bla* in a complex matrix comprising a diverse microbial community. Compared to the mentioned studies, the reduction of CF observed in the current study in the presence of *L. agilis* was less pronounced and statistically not significantly different from the control. The results from the current study also revealed species-specific differences as, opposite to *L. agilis*, *L. salivarius* induced a slight increase of CF/D, CF/R, and CF/D (SIF) compared to the control.

The phytobiotic product Formulation C seemed to reduce CF/D, but correction for stress impact on bacterial growth reversed this into an increase. On the contrary, the observed increase in CF/D by the group receiving Formulation L was reversed into a decrease when corrected by SIF. Similarly, components of *Thymus vulgaris* essential oils reduced the transfer of the *bla*-carrying pKM101 plasmid between *E. coli* strains. The highest reduction was observed as an effect of linalool supplementation, followed by S- and R-carvone, eugenol, and borneol [21]. This implies that different phytogenic compounds affect CF differently.

Interestingly, the combination of DFM and phytobiotic feed additives had an enhancing impact on conjugation frequencies. The only exception was observed for the combination of *L. agilis* and Formulation L, where a minor numeric reduction was observed. In summary, a non-significant reductive impact on conjugation (CF/D (SIF)) was observed for *L. agilis* and Formulation L, while a significantly higher CF was detected in Formulation C, LS + C, LS + L, and LA + C than in the control.

This suggests that the chosen DFM and phytobiotics are unlikely to reduce conjugation frequencies of the investigated ESBL-carrying plasmid in broilers.

In poultry, ESBL-PE prevalence of up to 100% were previously reported [1]. This corresponds with findings from excreta samples at week 4, where all samples were tested positive (Table S2). The early detection of ESBL-PE in newly hatched broiler chicks corresponds with results from the literature, identifying chicks and eggs as a potential risk factor for transmission between farms [45,46]. In cecal contents, two feed groups were negative for ESBL-PE at all sampling times. Comparing quantitative and qualitative results from the other feed groups, differences appear. The groups characterized as equal in the qualitative approach (20% prevalence) showed obvious quantitative differences. Qualitative evaluation of ESBL-PE prevalence, often after pre-enrichment, is commonly used to report antimicrobial resistance in poultry [1]. The results of this study suggest that qualitative evaluations may distort the picture.

It was previously shown in vivo, that DFM can reduce the prevalence of ESBL-producing *E. coli* in the ceca of broiler chicks [14,15], corresponding with the results from this in vivo trial. Another study investigated the transmission of ESBL-producing *E. coli* (CTX-M-1) between animals, where animals perceiving the DFMs were less susceptible to ESBL-producing *E. coli* and excreted lower numbers of these bacteria [16]. The effect observed in these studies were based on competitive exclusion. In contrast to the present study, the aforementioned trials performed an ESBL-PE challenge. Additionally, commercial products comprising a diverse bacterial community, which was not characterized quantitatively, was used compared to the single-strain approach of the present study.

Besides the impact of the DFM, phytogenic feed additives may have an effect on the prevalence of ESBL-PE [47,48]. A reduction of ESBL-PE prevalence was observed both qualitatively and quantitatively in the presence of the phytogenic products in cecal contents. As quantitative differences were observed between the products, the most severe effect was observed when combined with a DFM strain.

The results of this study displayed differences in the efficiency of different lactobacilli strains and phytobiotic products regarding the ability to reduce ESBL-PE prevalence and their plasmid transfer. In addition, no consistent correlation between the ability to reduce the prevalence and the CF was observed. This suggests that feed additives reducing the ESBL-PE prevalence should be combined with supplements targeting plasmid transfer to achieve the highest possible reduction of ESBL-producing bacteria. As this may sound logical in theory, the interaction of combinations may lead to different results [32]. Thus, it is crucial to determine the combined effect of such products, as they may be additive but also can neutralize or even reverse the effect of the single components. In this study, only one combination (LA + L) did not increase CF. With regards to the ESBL-PE prevalence, 2 (LS + C, LS) of 4 combinations were superior to the quantitative results induced by the DFM supplementation and all 4 combinations performed better than the single supplementation of phytobiotics. Further studies, where the broilers are challenged with defined amounts of ESBL-producing *E. coli*, should be conducted in the future to compare the results with these results from natural colonization with ESBL-PE.

5. Conclusions

Out of the tested feed additives, the effect of DFM on ESBL-PE prevalence was superior to the phytobiotic products. *L. agilis* showed the most promising ability to reduce both the prevalence of ESBL-producing *Enterobacteriaceae* as well as the ESBL-carrying plasmid transfer between *Enterobacteriaceae*. Combinations of phytogenic additives and DFM did not enhance the effect of the single components on CF. The impact of DFM and phytobiotic feed additives on conjugation was less obvious than the impact on ESBL-PE prevalence.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/3/322/s1, Table S1: Susceptibility of donor and recipient strain, Table S2: Prevalence of ESBL-producing *Enterobacteriaceae* in the feces of broilers, Table S3: Raw data short-chain fatty acids, Table S4: Raw data.

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6. General discussion and conclusion

6.1. Conclusions from literature

6.1.1. Antibiotic resistance

The relevant literature discloses ancient existence of antibiotic resistant bacteria, with the ability to produce lactamases since approximately 2.4 billion years (Hall and Barlow, 2004). These drug resistant bacteria were promoted by the introduction of antibiotic substances to medicine and animal farming, among others, by humans in the 20th century (Barlow and Hall, 2002; Gaze et al., 2013; Gniadkowski, 2008; Perry and Wright, 2014). As a countermeasure, the use of antibiotics was restricted, and antibiotic growth promotors were prohibited in the European Union (Regulation (EC) No 1831/2003). Unfortunately, this did not stop the spread of antibiotic resistant bacteria. On the contrary, a future post-antibiotic era with tremendous consequences for global health and economics is a realistic scenario (Tang et al., 2017). It can be concluded that measures to reduce the prevalence of multi drug resistant bacteria are vital for a secure future for mankind and civilization. This also includes controlling the spread of resistance genes to other bacteria, especially of pathogenic nature, and the transmission of antibiotic resistant bacteria between animals and between animals and humans.

6.1.2. ESBL-producing *Enterobacteriaceae*

One of the most dangerous multi drug resistant bacteria in this context is the group of ESBL-producing *Enterobacteriaceae* (WHO, 2017). This group is well represented in poultry and poultry products (Saliu et al., 2017). CTX-M-1, one of the ESBL types with the highest prevalence in animals and humans, might even originate from poultry (Cloeckaert et al., 2010). ESBL encoding genes are frequently located on plasmids (Apata, 2009). This enables the genes to additionally spread horizontally by conjugation and increases the chance for severe health related consequences (Gaze et al., 2013). Also, the transfer from ESBL-producing *Enterobacteriaceae* from poultry to bacteria of human origin is possible (Sarowska et al., 2009; Smet et al., 2011). The common assumption that the spread by conjugation comes with fitness costs for the bacteria, followed by the discharge of the plasmid by the bacteria, is currently being questioned (chapter 2.2.). Stress, defined as a potential threat to the survival of the bacterial cells induced by unfavorable conditions (Aertsen and Michiels, 2004; Boor, 2006) and displayed as a change in bacterial growth in the presence of the stressor, is believed to enhance conjugation (Mc Mahon et al., 2007).

6.1.3. Measurements to reduce the prevalence and spread of ESBL-producing *Enterobacteriaceae*

Similar to the above explained, reduction of ß-lactam antibiotics reduced the prevalence of ESBL-producing *Enterobacteriaceae,* but the effect is not sufficient to eradicate these bacteria (Borjesson et al., 2013; Mo et al., 2014). Thus, further measurements to combat ESBL-producing *Enterobacteriaceae* are crucial. While direct-fed microbials showed promising effects by reducing the prevalence (Ceccarelli et al., 2017; Methner et al., 2019; Nuotio et al., 2013), a negative impact of their metabolites on horizontal gene transfer was observed (Moubareck et al., 2007). Similarly, a negative impact on conjugation was also observed in the presence of different phytobiotics (Skalicka-Wozniak et al., 2018), trace minerals (Klumper et al., 2017; Warnes et al., 2012), and bacterial metabolites including short chain fatty acids (Garcia-Quintanilla et al., 2008; Maisonneuve et al., 2000; Maisonneuve et al., 2001, 2002; Sabia et al., 2009; Tallmeister et al., 1977).

6.2. The *in vitro* conjugation experiments

6.2.1. Designing the experimental set up

Based on the literature review, some hints were provided on the successful reduction of the prevalence and transfer of ESBL encoding genes in poultry, but no information was provided on the interaction between measurements targeting prevalence and transfer. Also, information about their impact on conjugation came either from *in vitro* (Skalicka-Wozniak et al., 2018) experiments or retrieved from another species (mice) (Garcia-Quintanilla et al., 2008), while

data on prevalence were collected from *in vivo* trials in broilers (Ceccarelli et al., 2017; Methner et al., 2019; Nuotio et al., 2013). Thus, established methods to investigate the prevalence of ESBL-producing bacteria and the impact of different factors *in vivo* were available, while a model for *ex vivo* and *in vivo* conjugation studies had to be designed. *In vitro* experiments involving 11 recipient/donor pairs revealed major differences between strains and incubation time (chapter 3). Based on the gut transit time in young broilers, pathogenicity of the recipient and ESBL-type of the donor, a suitable mating pair and incubation conditions were identified. Still, the strain specific differences were of such obvious character that it cannot be contested that conclusions based on this mating pair will be strain specific. As for the strain specific differences, the conjugation frequency and incubation time until detection of first conjugation event were investigated. Transconjugants were observed the earliest after 4 h, 8 h or 22 h incubation in 4/11, 1/11, and 6/11 mating pairs, respectively at a conjugation frequency of 10^{-9} – 10^{-5} transconjugants/donor. No general conclusions regarding donor and/or recipient strain combinations could be observed.

As this study was designed to investigate the impact of different stressors on the conjugation and prevalence of ESBL-producing *Enterobacteriaceae* in poultry, the impact of different recipient/donor strain concentrations and ratios was not investigated. However, these are factors that may impact the results (Table 2.1) and should be addressed in future studies.

6.2.2. The impact of different stressors on conjugation

In the present study, the most compelling result was the inconsistency of the stressors' impact on conjugation. Feed additives are frequently added to animals' diets to modify the gastrointestinal milieu and bacterial composition (Yadav and Jha, 2019). While shifts in bacterial family, genera and species are well documented (Rehman et al., 2007), the impact on the transfer of resistance genes is less obvious. However, a modification in bacterial concentrations comes with stress for the bacterial community. Studying the relevant literature, it can be hypothesized that conjugation frequency may increase

1. when bacteria are exposed to stress
2. due to co-selection in resistance against minerals like zinc and copper.

Thus, the impact of nutrition related factors on both prevalence and conjugation was investigated simultaneously. With the mating pair and incubation time being defined, the impact of different stressors at sublethal concentrations on conjugation could be investigated. Here, the impact of bacterial concentrations on conjugation frequency revealed decisive, demonstrating a bias frequently neglected in conjugation experiments (Lopatkin et al., 2016a). Thus, results reporting increases or decreases of conjugation frequency without correction for the impact on bacterial growth must be evaluated with caution. In this thesis, a mathematical correction was conducted by introducing the stress impact factor (chapter 4). Comparing the raw data to the results corrected for the stress impact factor (SIF), the existence of this bias was confirmed in both experiments involving different stressors (chapter 4 and 5). Significant differences between the calculated CF/D and the corrected values were detected at log 0.5 (900 mosm), 0.5-1.2 (CTX), 1.0-2.0 (SXT), 0.4 (2 µg F/mL), 0.4-0.5 (zinc), 0.5-2.2 (copper), 0.5 (*L. agilis*) and 0.4 (Formulation C and Formulation L). Not only was there a difference between CF and CF(SIF), but also a reversion of the relation to the control (increase ↔ decrease) was detected in the cases of Formulation L (decrease → increase), Formulation C (increase → decrease), copper (increase → decrease) and SXT (increase → decrease). In some cases, the significant effect on CF/D observed for certain stressors vanished or revealed as non-significant when a correction for the stress impact on bacterial growth was conducted. This was true for the application of acetate. In contrast, additions of zinc and cefotaxime to the growth media did not result in significant differences between control and increasing concentrations for CF/D, while CF/D (SIF) showed significant differences between samples of different concentrations. Thus, our findings are in line with the conclusion of Lopatkin et al. (2016b), stating that the calculation of CF/D or CF/R may result in misleading assumptions regarding the impact of stressors on conjugation, if changes in bacterial growth are neglected.

While the influence of nutrition related stress factors on conjugation events was studied *in vitro* (chapter 4), the impact of direct-fed microbials, phytogenic compounds and their combinations was investigated *ex vivo* (chapter 5). The different methodical approaches provided the possibility to gain basic knowledge *in vitro* on the effects of pH, osmolality and different short chain fatty acids, zinc, copper and antibiotic concentrations under defined conditions. This aided the understanding of general aspects of the impact of stress on conjugation events and to question the general assumption that stress enhances conjugation frequencies. Additionally, this is the method typically reported in the literature when studying the impact of specific factors on conjugation frequency (Table 2.1) and provides more data for comparison of the results. On the other hand, direct-fed microbials, phytogenic products and their combinations were processed in the intestinal tract before reaching the cecal content in the *ex vivo* experiment. The impact of these substances on ESBL-producing *E. coli* ESBL10716 was known from previous studies (Ren et al., 2019a; Ren et al., 2019b) while closer to *in vivo* conditions, parameters were harder to control and results less clear. Despite relative high concentrations, viable cells and good growth, the recipient, who performed outstandingly *in vitro*, did not form any transconjugants. Instead, indigenous *Enterobacteriaceae* acted as recipients at equal to higher conjugation frequencies. Unfortunately, these recipients could not be identified individually. Future studies on mechanisms behind the donors' choice of mating partners may help to answer this question.

When evaluating the impact of feed additives or competitive exclusion cultures on their inhibitory effect against a certain strain in an animal trial, it is customary to challenge the animals with the targeted strain (Ceccarelli et al., 2017; Methner et al., 2019; Nuotio et al., 2013). Here, a different approach was chosen, as the indigenous prevalence and natural dissemination was investigated instead. The ESBL-PE prevalence in the excreta of animals agreed with the literature on the fact that broiler chicks obtain ESBL-PE at an early stage in life (Nilsson et al., 2014). From this, a hazard of transmission through (international) trading with animals and eggs arises (Borjesson et al., 2015; Mo et al., 2016; Mo et al., 2014). In the existing literature, the prevalence of ESBL-PE is frequently reported in the excreta and cecal content of broilers (chapter 2.1.4.). The common method to report ESBL-PE prevalence is qualitative, displaying the result as the percentage of samples tested positive, commonly after pre-enrichment (MARAN, 2018; Swedres-Svarm, 2015).The explanatory power of this method is limited, as samples with significant quantitative differences are judged as equal. Therefore, a quantitative approach, possibly paired with a qualitative pre-enrichment screening, may be better fitted to describe the prevalence.

In the present study, experiments were performed *in vitro*, *ex vivo* and *in vivo*. The advantage of this concept is not only the stepwise approach from simple and controlled conditions to complex systems, but also that it provides the full picture for one mating pair. This evolved in the awareness, that results from *in vitro*, *ex vivo* and *in vivo* experiments may differ significantly and that conclusions from one setup cannot automatically be transferred to another system. Still, every approach serves its purpose and, if applied correctly and in line with the research question, may contribute to solve complex problems.

6.2.2.1. Physical alterations in the gastrointestinal tract

The physical variables investigated in the present study were pH and osmolality. While pH did not impact on CF, varying osmolality correlated with alterations in the conjugation frequency. Compared to the presented results, the literature reports low pH values to result in an increase of transconjugants and conjugation frequency (Mc Mahon et al., 2007; Schäfer et al., 1994). The decrease of CF/D (SIF) and CF/R at increasing osmolality is not easily compared to results in the literature, as osmolality is not provided but the concentration of chemicals such as NaCl, which does not allow a direct comparison. Still, an addition of 4 % NaCl led to a significant increase of CF/R with *E. coli* donors and *E. coli* and *Salmonella* Typhimurium recipients (Mc Mahon et al., 2007). As lower osmolality was observed in gizzard and ileum compared to other parts of the intestinal tract (Mongin et al., 1976; Mitchell and Lemme, 2008), one would expect higher conjugation frequencies in these segments. Also, the low pH in the gizzard might favor

plasmid transfer (Mc Mahon et al., 2007; Schäfer et al., 1994). One must not forget the impact of bacterial concentrations on conjugation frequency (Händel et al., 2015). Thus, a major spread of antibiotic resistance via conjugation in the gizzard is not likely to occur. This is a fortunate coincidence from the perspective of conjugation control. The ileum on the other hand might be an interesting part to include in future studies, due to the low osmolality of its content.

6.2.2.2. Antibiotic substances

The negative impact of antibiotics on bacterial growth and survival is well proven. Also, the use of antibiotics at sublethal concentrations is known to enhance the prevalence of bacteria resistant against the specific substances as well as other substances (Barlow and Hall, 2002; Gniadkowski, 2008; Perry and Wright, 2014). Consequently, these substances exert stress on susceptible bacteria and thus, an increased conjugation frequency was expected. The impact of antibiotics on the transfer of *bla*-carrying plasmids may be the stressor most frequently investigated in the literature, covering various antibiotic compounds and different mating pairs. Often, one antibiotic substance is tested against one or several mating pairs and compared to other stressors. The fact, that different antibiotics not only affect bacterial growth but also conjugation differently, was demonstrated in chapter 4 and corresponds with findings in the literature (al-Masaudi et al., 1991; Händel et al., 2015; Lopatkin et al., 2016a). In the present thesis, three different antibiotics where chosen due to the resistance profile of donor and recipient. It was concluded that despite posing stress on some bacterial cells while providing a competitive edge to other strains, the conjugation is not increased but rather reduced in the presence of antibiotics.

6.2.2.3. Feed additives

Understanding the complexity of the avian microbiota and the impact feed and feed additives have on it is crucial to battle infections and diseases caused by enteral pathogens. Despite numerous publications, we are still far from a complete and detailed understanding of the bacterial composition in the broilers' intestines (Choi et al., 2014). Still, the information available today seemingly proves that feed additives can impact on bacterial composition, animal health and performance as well as antibiotic resistance (Clavijo and Florez, 2018; EMA and EFSA, 2017; Yadav and Jha, 2019). DFM and essential oils were chosen as potential feed associated inhibitors to the establishment of ESBL-PE in the gastrointestinal tract and conjugative transfer of *bla*-carrying plasmids. The selection procedure of the probiotic strains was described elsewhere (Ren et al., 2019a; Ren et al., 2019c). A massive strain screening method evolved in a handful of potential probiotic lactobacilli strains. This stands in contrast to the literature, were the number of screened strains is significantly lower (Idoui, 2014; Robyn et al., 2012; Salah et al., 2012). The phytobiotic product was provided by EW Nutrition and two of five products with the best inhibitory effect against the tested ESBL-producing *E. coli* (*E. coli* ESBL10716) were chosen for the feeding trial. These pre-experiments enabled higher quality and thereby the likelihood to detect an impact of the DFM and phytobiotics products tested.

6.2.2.3.1. Copper and zinc

Like antibiotics, zinc and copper are used (as feed additives) to reduce the prevalence of certain bacteria (Reed et al., 2018; Yausheva et al., 2018) and linked to increasing numbers of drug resistant bacteria (Aarestrup et al., 2010; Vahjen et al., 2015; Yazdankhah et al., 2014). This was also shown for ESBL-producing *Enterobacteriaceae* (Bednorz et al., 2013; Holzel et al., 2012; Touati et al., 2010). An increase of 18.6 % multi resistant *E. coli* was observed in a trial group of piglets fed high amounts (2500 ppm) of zinc compared to the control group (50 ppm zinc) (Bednorz et al., 2013). Similarly, beta-lactam resistance was linked to zinc and copper concentrations in pigs' manure (Holzel et al., 2012). In contrast to this, all 16 tested ESBL-producing *E. coli* strains isolated from a hospital showed co-resistance to copper but were susceptible to zinc (Touati et al., 2010). In the present study, the recipient strain reduced in growth at higher zinc concentrations while the donor strain was susceptible to copper. This suggests that the donor was resistant to zinc while the recipient was resistant to copper. A similar precondition as in the antimicrobial experiment evolved and again conjugation frequencies were reduced when an increase was predicted (Bednorz et al., 2013; Ou, 1973;

Zhang et al., 2018). The strains were not analyzed molecularly for their resistance genes. The observed decrease was explained in another study where zinc and copper reduced the expression of genes involved in plasmid transfer (Buberg et al., 2020). A significant reduction in conjugation frequency occurred at concentrations of 87 mg copper/L and 21 mg zinc/L. These concentrations are within (zinc) or close to (copper) concentrations observed in the cecum of chickens fed 15 mg copper/kg feed and 100 mg zinc/kg feed (Buberg et al., 2020).

6.2.2.3.2. Short chain fatty acids

In contrast to the minerals, most short chain fatty acids did not impact on conjugation frequency. Here a reduction was anticipated, especially for lactate, which was suggested to be the impetus for reductions in conjugation frequency observed in the presence of direct-fed microbials (Tallmeister et al., 1977; Maisonneuve et al., 2000; Maisonneuve et al., 2001; 2002; Sabia et al., 2009). Only propionate had an impact on conjugation frequencies. This corresponds to findings in mice (Garcia-Quintanilla et al., 2008), but the mechanisms behind the reduction are still not investigated. Propionic acid is an approved feed additive according to the European Union Register of Feed Additives, based on Regulation (EC) No 1831/2003. It may be used in poultry feed as feed preservative, silage additive or flavoring compound. When consumed orally, short chain fatty acids are commonly absorbed and metabolized in the small intestine and do not reach the cecum where they could impact on conjugation (Hume, 2011). Consequently, an increase of propionate must be achieved by other means than feed supplementation if the cecum is targeted. One possible mechanism would be to increase the propionate producing fraction of the bacterial community. However, propionate concentrations required (>109 mmol/L) to reduce plasmid transfer exceed the observed concentrations naturally produced in the ceca (up to 35 mmol/L) significantly (Rehman et al., 2007). Still, propionate might be used to reduce plasmid transfer in the upper gastrointestinal tract.

6.2.2.3.3. Direct-fed microbials and phytobiotics

Compared to the other stressors, the impact of phytogenic products and direct-fed microbials on conjugation frequencies was tested *ex vivo*. In this case, the stressors were fed to the animals instead of adding defined concentrations to the media. However, strain concentrations were defined, as they were added to the cecal content. The hypothesis that direct-fed microbials and phytogenic products reduce conjugation frequencies (Maisonneuve et al., 2000; Maisonneuve et al., 2001, 2002; Sabia et al., 2009; Skalicka-Wozniak et al., 2018; Tallmeister et al., 1977) and act in a synergistic matter could not be confirmed. However, differences between the *in vitro* and *ex vivo* experiments became apparent, as not only the ratio between donor and recipient were overturned, but also indigenous bacteria replaced the anticipated recipient, which did not accept plasmids.

The negative impact of essential oils on plasmid transfer previously reported (Skalicka-Wozniak et al., 2018) was not constant in the present study. Still, the results stress the importance of investigating the impact of combinations of feed additives and not to jump to conclusions based on results from single compounds. While single compounds did not show significant changes in conjugation frequencies, 3 out of 4 combinations had an impact. However, the observed differences were rather low, classifying them as biologically non-significant. Further experiments with increased DFM, donor or recipient strain concentrations, would not represent natural conditions. Thus, the impact of the chosen DFM on plasmid transfer from the chosen donor *in vivo* is rather uncertain. In the case of the phytobiotic feed additives, the product was fed encapsulated at low concentrations. Thus, the equal incorporation by all animals of one trial group cannot be guaranteed. Additionally, the essential oils were probably metabolized before reaching the ceca. The detected effect therefore depends only indirectly on the phytobiotic feed additives and was probably caused by its impact on the microbiota in the upper or middle part of the gastrointestinal tract and its consequences on the cecal microbial community.

6.2.3. Nutrition related factors influencing conjugation frequencies

In summary, osmolality is the physical parameter to consider as a possible influence on conjugation and antibiotic substances appear to have a negative impact on conjugation. This could be used in favor of drug resistance control. Nevertheless, the overall increase of drug resistant bacteria at subtherapeutic levels of antibiotics precludes this as an option (Barlow and Hall, 2002; Gniadkowski, 2008; Perry and Wright, 2014). Still, the fact that treating an infection caused by a specific bacterium does not necessarily trigger the spread of its resistance genes via conjugation is a valuable knowledge gain. While copper revealed the highest impact on conjugation, propionate and zinc were further factors worth considering. Here, the risk of increasing the prevalence of antibiotic resistant bacteria in the presence of zinc and copper must be considered, while it may be hard to reach the required concentrations of propionate *in vivo*. Additionally, the results from these factors must still be confirmed in e*x vivo* and *in vivo* experiments. *L. agilis* showed a reductive impact on both prevalence and conjugation, but at a lower extent than the other feed additives. *L. agilis* should further be compared to results from e*x vivo* and *in vivo* conjugation experiments with propionate, zinc and copper as feed additives to make a correct assessment regarding the differences of the impact of these feed additives. Additionally, when combined with direct-fed microbials, formulation L showed promising results regarding the ESBL prevalence, even though it did not contribute to the reduction of conjugation frequencies. Nevertheless, it may be a crucial factor to address the whole picture of ESBL-producing *Enterobacteriaceae* in poultry.

6.2.4. Future studies

Further *in vivo* and *ex vivo* experiments should focus on osmolality, copper, zinc, propionate, and propionate producing DFM, *L. agilis* and formulation L as conjugation inhibitors. These studies should be designed as challenge trials, where broilers are challenged with the donor and/or recipient, as well as *ex vivo* experiments where donor and recipient concentrations are defined. The gastrointestinal parts which should have the highest priority in *ex vivo* and *in vivo* studies are the crop, the ileum and the cecum. Challenge trials should distinguish between mucosa attached bacteria and bacteria from the intestinal content. This to differentiate between bacteria who pass through and those who establish in the intestinal tract. *Ex vivo* and *in vitro* experiments may investigate differences with different mating pairs and donor and recipient concentrations, as this was previously shown to affect conjugation (Table 2.1). The impact of these feed additives on the prevalence of ESBL-producing *Enterobacteriaceae* should be investigated simultaneously. Studies should also investigate mechanisms behind enhanced or reduced conjugation frequencies. Interesting factors might be the expression of genes regulating plasmid transfer as well as resistance genes against copper and zinc.

6.3. Conclusion

The results of this study revealed an impact of several nutrition related stress factors on the prevalence and conjugation in ESBL-producing *Enterobacteriaceae*, but does not support the general assumption, that stress enhances conjugation frequency. On the contrary, zinc, copper, propionate, *L. agilis* and sublethal concentrations of antibiotics may even reduce conjugation frequencies. Further research, including a challenge trial with an ESBL-producing *E. coli* donor, is warranted to complement the results with *in vivo* data.

7. Summary

Summary of the PhD-thesis:

The impact of feed additives on prevalence and conjugation of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in poultry

Due to their multi-drug resistance, ESBL-producing *Enterobacteriaceae* pose a major hazard on public health and global economy. A transmission from animals to humans is possible and thus, animals may serve as a reservoir for difficult to treat infections in humans. In livestock, the highest prevalence of ESBL-producing *Enterobacteriaceae* is commonly found in poultry. Measures to reduce the prevalence of ESBL-producing *Enterobacteriaceae* are of great importance. Promising results were observed in broilers challenged with ESBL-producing *E. coli* and fed probiotic products. An important aspect concerning the reduction of ESBL-producing *Enterobacteriaceae* is their ability to spread their resistance genes within and across species. As ESBL encoding genes are commonly located on plasmids, conjugation enables horizontal gene transfer and should be considered when developing reduction measures.

The objective of this study was to investigate the impact of different feed additives and nutrition related stress factors on conjugation of ESBL-producing *Enterobacteriaceae* in broilers.

A suitable mating pair evolved from a screening experiment with five different ESBL-producing *E. coli* donor strains and various potential recipients, commonly detected in the gastrointestinal tract of broilers. The chosen mating pair comprised the donor *E. coli* ESBL10682, producing the extended-spectrum beta-lactamase CTX-M-1, and the potentially pathogenic *Salmonella* Typhimurium L1219-R32 strain. Conjugation events occurred at a relative high frequency of 10^{-5} within a period of four hours, resembling the transit time through the gastrointestinal tract of broilers. The initial donor and recipient concentration was 10^8 cfu/mL and the donor/recipient ratio was 1:1. Other mating pairs showed no or lower conjugation frequencies and a kinetic conjugation experiment revealed differences between different incubation durations (0, 2, 4, 6, 8 and 22 hours).

The mating pair was challenged with different nutrition related stress factors in an *in vitro* experiment. To circumvent the bias bacterial growth exerts on conjugation frequency, a stress impact factor was introduced to correct the results. The expected increase in conjugation frequency failed to appear, even when challenged with subtherapeutic levels of antibiotics. Instead, the feed additives copper, zinc and propionate decreased the conjugation frequency significantly with approximately 3, 0.8, and 2 log units respectively. No significant impact on conjugation events was observed for acetate, n-butyrate or lactate. The antibiotics nitrofurantoin, sulfamethoxazole/trimethoprim and cefotaxime also showed conjugation frequencies declining with up to 1.5, 2 and 0.7 log units respectively. Regarding experimental conditions, pH had no significant impact on the results while increasing osmolality reduced conjugation frequencies with up to 0.7 log units.

These results were complemented by an animal trial paired with an *ex vivo* experiment. Newly hatched male Cobb 500 broiler chicks were randomly allocated to nine different feeding groups with seven replicates each. The experimental feed comprised a control, two diets supplemented with one of two *Lactobacillus* strains (*L. agilis, L. salivarius*), two diets supplemented with different phytobiotic feed additives containing the essential oils carvacrol, cinnamaldehyde and eugenol (Formulation C, Formulation L) and four diets comprising the combination of one *Lactobacillus* strain and one phytobiotic product. The *Lactobacillus* strains and the phytogenic products were chosen due to their ability to inhibit an ESBL-producing *E. coli* strain in a previous *in vitro* experiment. Cecal content was obtained at two weeks of age and the mating pair *E. coli* ESBL10682/*Salmonella* Typhimurium L1219-R32 was added at defined concentrations and incubated for four hours. Most surprisingly, the intended *Salmonella* recipient was not detected to form transconjugants, but instead, indigenous SXT-resistant *Enterobacteriaceae* accepted the plasmid. An increase of 0.5 – 0.6 log units was

observed in the conjugation frequencies of the combination groups *L. salivarius* + Formulation C, *L. salivarius* + Formulation L and *L. agilis* + Formulation C. Also, the group fed Formulation C showed an enhanced (0.2 log units) conjugation frequency. Even though statistically significant, from a microbiological view, these differences are rather small and not conclusive.

At five weeks of age, the prevalence of ESBL-producing *Enterobacteriaceae* was determined in the cecal content. The control group revealed a significantly higher prevalence than the groups supplemented with the *Lactobacillus* strains and/or phytobiotic feed additives. Additionally, it was observed that there were quantitative differences between samples, a fact that is commonly neglected in surveys screening for the prevalence of ESBL-producing *Enterobacteriaceae.*

In conclusion, feed additives were able to reduce the transfer of ESBL-carrying plasmids from an *E. coli* donor to a *Salmonella* Typhimurium recipient. The most promising results were observed for copper and propionate.

8. Zusammenfassung

Zusammenfassung der PhD-Arbeit:

Der Einfluss verschiedener Futtermittelzusatzstoffe auf die Prävalenz und Konjugation von Extended Spektrum beta-Laktamase-bildenden *Enterobacteriaceae* in Broilern

Multiresistente, ESBL-bildende *Enterobacteriaceae* haben ein hohes Potenzial, Antibiotikaresistenzen bei Menschen und Tieren zu verbreiten. Eine Übertragung dieser Keime vom Tier auf den Menschen ist denkbar und somit können Tiere als Reservoir mit Bedrohungspotenzial für den Menschen betrachtet werden. Das Huhn ist die Nutztierart, bei welcher die höchste Verbreitung ESBL-bildender *Enterobacteriaceae* verzeichnet wurde. Es ist somit wichtig, Reduktionsmaßnahmen beim Geflügel zu entwickeln. Erste vielversprechende Ergebnisse liegen zum Einsatz von Probiotika bei Broilern vor. Ein wichtiger Aspekt, welcher beachtet werden muss, um die Verbreitung dieser Keime zu unterbinden, ist deren Vermögen, Resistenzgene innerhalb und zwischen Bakterienspezies zu verbreiten. Da die ESBL-kodierenden Gene hauptsächlich auf Plasmiden vorkommen, kann der Mechanismus der Konjugation maßgeblich zu deren Ausbreitung beitragen und sollte bei der Entwicklung von Reduktionsmaßnahmen beachtet werden.

Ziel dieser Arbeit war es, den Einfluss verschiedener Futterzusatzstoffe und ernährungsbezogener potenzieller Stressoren auf die Konjugation ESBL-bildender Enterobakterien im Mastgeflügel zu untersuchen.

Ein für den Konjugationsversuch geeignetes Bakterienpaar konnte bei einem Screening bestimmt werden, das auf fünf ESBL-bildenden *E. coli* Spenderstämmen und einer Vielzahl an potentieller Empfängerstämmen, welche häufig im Gastrointestinaltrakt von Broilern vorkommen, beruhte. Der ausgewählte Donorstamm *E. coli* ESBL10682 bildet die ß-Laktamase CTX-M-1, während der Rezipient *Salmonella* Typhimurium L1219-R32 potenziell pathogen ist. Eine relativ hohe Konjugationsfrequenz von 10^{-5} wurde für das Bakterienpaar nach einer Co-Inkubationszeit von vier Stunden, welche die Zeit der Chymuspassage im Gastrointestinaltrakt von Hühnern widerspiegelt, verzeichnet. Die Ausgangskonzentration von Donoren und Rezipienten betrug 10^{8} cfu/mL, welche in einem Verhältnis von 1:1 vermischt wurden. Die anderen Bakterienpaare, welche das Konjugationsscreening durchliefen, zeigten eine geringere oder keine Konjugationsfrequenz. Die Konjugationskinetik unterschied sich außerdem zwischen den verschiedenen Co-Inkubationszeiten (0, 2, 4, 6, 8 und 22 Stunden).

Ein *in vitro* Versuch diente der Untersuchung des Einflusses verschiedener Stressfaktoren, welche mit der Ernährung in Verbindung stehen, auf die Konjugation. Ein Faktor, welcher den Einfluss der Stressoren auf das Wachstum der Bakterien beschreibt, wurde ermittelt und die Ergebnisse dementsprechend korrigiert. Der erwartete Anstieg der Konjugationsfrequenz blieb jedoch aus, selbst in der Gegenwart von Antibiotika. Stattdessen wurde eine Reduktion um 3, 0.8 beziehungsweise 2 Logarithmusstufen verzeichnet, wenn Kupfer, Zink oder Propionat dem Nährmedium zugeführt wurden. Dahingegen zeigten sich durch Zugabe von D-/L-Laktat, n-Butyrat und Acetat keine signifikanten Einflüsse. Die Antibiotika Nitrofurantoin, Sulfamethoxazol/Trimethoprim und Cefotaxim führten ebenfalls zu einer Abnahme der Konjugationsfrequenz um 1.5, 2 beziehungsweise 0.7 Logarithmusstufen. Während der pH-Wert keinen signifikanten Einfluss auf die Konjugationsfrequenz ausübte, wurde bei steigender Osmolalität eine Verminderung der Konjugationsfrequenz um bis zu 0.7 Logarithmusstufen beobachtet.

Diese Ansätze wurden durch einen Fütterungsversuch ergänzt, welcher mit einem *ex vivo* Experiment verbunden war. Männliche Eintagsküken der Linie Cobb 500 wurden neun Versuchsgruppen zugeteilt, welche je sieben Replikate aufwiesen. Als Versuchsfutter wurden ein Kontrollfutter sowie acht Futter mit verschiedenen Zusätzen gefüttert. Zwei dieser

Futtermischungen wurde je ein *Lactobacillus* Stamm (*L. agilis, L. salivarius*) zugesetzt. Zwei weitere Futter enthielten je ein Phytobiotikum, welche die essentiellen Öle Carvacrol, Cinnamaldehyde und Eugenol enthielten (Formulation C, Formulation L). Die letzten vier Versuchsfutter enthielten verschiedene Kombinationen aus je einem *Lactobacillus* Stamm und einem Phytobiotikum. Sowohl die *Lactobacillus* Stämme als auch die Phytobiotika waren in einem Vorversuch auf Grund ihrer Wirksamkeit gegen einen ESBL-bildenden *E. coli* Stamm ausgewählt worden. Nach zwei Wochen wurde Caecuminhalt gewonnen und mit dem Bakterienpaar *E. coli* ESBL10682/*Salmonella* Typhimurium L1219-R32 versehen. Die Konjugationsfrequenz wurde nach vier Stunden Co-Inkubation ermittelt. Interessanterweise agierten indigene Sulfamethoxazol/Trimethoprim resistente Enterobakterien anstelle des vorgesehenen *Salmonella* Typhimurium Stammes als Empfängerzellen. In den Kombinationsgruppen *L. salivarius* + Formulation C, *L. salivarius* + Formulation L und *L. agilis* + Formulation C wurde ein Anstieg der Konjugationsfrequenz um 0.5 bis 0.6 Logarithmusstufen verzeichnet. Ein Anstieg (0.2 Logarithmusstufen) wurde ebenfalls in der Gruppe Formulation C beobachtet. Obwohl diese Unterschiede statistisch signifikant sind, sind sie so gering, dass ihnen biologisch vermutlich keine Aussagekraft zugeschrieben werden kann.

Die Verbreitung ESBL-bildender Enterobakterien wurde im Caecuminhalt fünf Wochen alter Broiler untersucht. Die Kontrollgruppe zeigte eine signifikante höhere Prävalenz als die restlichen Fütterungsgruppen auf. Darüber hinaus wurden quantitative Unterschiede zwischen den Proben beobachtet.

Zusammenfassend konnte eine Reduktion des Transfers eines ESBL-tragenden Plasmid zwischen eines *E. coli* Spenderstammes und eines *Salmonella* Typhimurium Empfänger-stammes in der Gegenwart von Futterzusatzstoffen beobachtet werden. Den größten dezimierenden Einfluss nahmen hierbei Kupfer und Propionat.

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10. List of publications

10.1. Published and accepted papers (peer reviewed)

Saliu, E.M.; Vahjen, W.; Zentek, J. *In vitro* conjugation kinetics of ESBL-producing *Escherichia coli* donors and various *Enterobacteriaceae* recipients, BMC Microbiol. **2020**, 133, doi: 10.1186/s12866-020-01787-7

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10.2. Congress contributions

10.2.1. Oral presentations

Winter, J.; **Saliu, E.M**.; Eule, C.; Thieme, K.; Zentek, J.; Gehlen, H. (**2020**): Photosensibilisierug in neun Pferden nach Aufnahme von Heu mit Wilder Pastinake (*Pastinaca sativa*): ein Fallbericht. DVG Kongress Berlin – 15.10.-17.10.2020.

Saliu, E.M.; Wessels, A.G.; Martínez-Vallespín, B.; Männer, k.; Vahjen, W.; Zentek, J.; Grześkowiak, Ł. (**2020**): Cortisol levels in saliva and colostrum in gestating and lactating sows fed different fibre sources. 24th ESVCN Congress München – 17.09.-19.09.2020. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 51. ISBN: 978-9-09-033625-1

Saliu, E.M.; Ren H, Goodarzi Boroojeni F, Zentek J, Vahjen W (**2019**). Direct-fed microbials and phytogenic feed additives reduce prevalence but not transfer of extended-spectrum beta-lactamase genes in broiler chicken, 23rd ESVCN Congress Turin – 18.09.-20.09.2018. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 43. ISBN: 979-12-200-5280-1

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Saliu, E.M.; Männer, K.; Vahjen, W.; Zentek, J. (**2018**): ESBL-producing *Enterobacteriaceae* in Turkeys fed probiotics. 11. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences" Berlin – 21.09.2018. In: 11. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences" Berlin: Mensch & Buch, 21. ISBN: 978-3-86387-929-7

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Saliu, E.M.; Vahjen, W.; Zentek, J. (**2018**): The impact of osmolarity, pH and antibiotics at subtherapeutic concentrations on the transfer of ESBL-carrying plasmids. 22nd ESVCN Congress München – 06.09.-08.09.2018. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 82. ISBN: 978-3-00-058952-2

Zentek, J.; **Saliu, E.M.** (**2018**): Entwicklung antimikrobieller Resistenzen bei Nutztieren - Was kann die Tierernährung leisten? Tierernährung und Veredelungswirtschaft - Themen zur Tierernährung Hodenhagen – 25.01.2018.

Ren, H.; Vahjen, W.; **Saliu, E.M.**; Zentek, J. (**2018**): Establishment of a large-scale screening method for probiotic bacteria. 11. Doktorandensymposium & DRS Präsentationsseminar „Biomedical Sciences" Berlin – 21.09.2018. In: 11. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences" Berlin: Mensch & Buch, 30. ISBN: 978-3-86387-929-7

Ren, H.; Vahjen, W.; **Saliu, E.M.**; Zentek, J. (**2018**): Selection of broiler-derived *Lactobacillus* strain active against ESBL *E. coli* based on novel in vitro-ex vivo combined screening. 22nd ESVCN Congress München – 06.09.-08.09.2018. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 81. ISBN: 978-3-00-058952-2

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Saliu, E.M.; Zentek, J (**2017**): Mögliche Beiträge zur Resistenzdiskussion durch die Tierernährung. Mitgliederversammlung der Fachabteilung Mineralfutter im DVT Hamburg – 17.05.2017.

10.2.2. Poster presentations

Saliu, E.M.; Ebersbach, L.; Grzeskowiak, L.; Zentek, J. (**2020**): Abweichungen zwischen deklarierten und analysierten Mineralstoffkonzentrationen in 19 marktüblichen Mineralfuttermitteln für Pferde in Deutschland. DVG Kongress Berlin – 15.10.-17.10.2020

Saliu, E.-M.; Ren, H.; Boroojeni, F. G.; Zentek, J.; Vahjen, W. (**2020**): Der Einfluss fütterungsrelevanter Faktoren auf die Verbreitung von Antibiotikaresistenzen durch horizontalen Gentransfer. DVG Kongress Berlin – 15.10.-17.10.2020

Saliu, E.M.; Grzeskowiak, L.; Ebersbach, L.; Hettmannsperger, M.; Kindler, F.; Kuhr, A.; Lorenz, A.; Lorson, S.; Mussel, R.; Zentek, J. (**2020**): Calcium, phosphorus, zinc and copper content in commercial supplements for horses. 24th ESVCN Congress (virtual) – 17.09.-

19.09.2020. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 69. ISBN: 978-9-09-033625-1

Saliu, E.M.; Zentek, J. (**2020**): Trace mineral contents in different sections of the mane and tale hair in horses. 74. Tagung der Gesellschaft für Ernährungsphysiologie Göttingen – 03.03.-05.03.2020. In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.) DLG-Verlag; 28, 73. ISBN: 978-3-7690-4113-2

Saliu, E.M.; Winter. J, Eule. C, Thieme K, Gehlen H, Zentek, J. (**2019**). Primary photosensitisation in horses fed hay with high amounts of parsnip (*Pastinaca sativa*), 23rd ESVCN Congress Turin – 18.09.-20.09.2018. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 147. ISBN: 979-12-200-5280-1

Winter J., Eule C., Thieme K., **Saliu E.M.**, Gehlen H. (**2019**). Photodermatitis and ocular changes in nine horses after ingestion of parsnip *(Pastinaca sativa),* 12th ECEIM Congress Valencia – 22.11.-24.11.2019

Saliu, E.M.; Vahjen, W.; Zentek, J. (**2016**): Literature review: Spread of ESBL-producing bacteria throughout the poultry production pyramid. 20th Congress of the European Society of Veterinary and Comparative Nutrition Berlin – 15.09.-17.09.2016. In: 20th Congress of the European Society of Veterinary and Comparative Nutrition: September 15th - 17th, 2016, Berlin, Germany: proceedings – European Society of Veterinary and Comparative Nutrition (Hrsg.) Berlin, 179. ISBN: 978-3-00-054219-0

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13. Conflict of Interest

The author declares no conflict of interest.

14. Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

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Eva-Maria Saliu