Aus dem Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Inaugural-Dissertation
zur Erlangung des Grades eines
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an der
Freien Universität Berlin

vorgelegt von
Lisa Ciesinski, geb. Rabes
Tierärztin aus Brandenburg/Havel

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1 Introduction

1.1 *Escherichia coli*

Escherichia (*E.*) *coli* is a Gram-negative, facultative anaerobic bacterial species belonging to the family of Enterobacteriaceae. Bacteria of this species are rod-shaped and in most cases motile through peritrichous flagellation. They are distributed worldwide and colonize the intestine of mammals, birds, and reptiles [1, 2]. Through fecal contamination they can also be detected in the environment. The intestinal colonization with *E. coli* starts directly after birth [3] through processes like suckling and nursing. This early intestinal presence of facultative anaerobes like *E. coli* leads to an environment, which supports the colonization with anaerobic phyla [4].

The gut microbiota of adults mainly consists of strict anaerobic bacteria. It is predominantly composed of bacteria belonging to the phyla Firmicutes and Bacteroides followed by Actinobacteria, Proteobacteria, and Verrucomicrobia [5-7]. However, with respect to the microbiota composition on the class, family, genus, and species level of bacteria, a lot of differences can be detected between host species and on the individual level. Here, external factors like diet, probiotics, and administration of antibiotics and internal host properties like age, genetics, and physiological processes lead to variations within one species [8]. Moreover, the composition of the microbiota also varies along the entire intestinal tract [9].

Next to the highly abundant strict anaerobic species, *E. coli* are the predominant aerobic bacteria in the gastrointestinal tract and reside in the large intestine, especially in the caecum and colon [10]. As mentioned above, the host species has a significant influence on the quantity of different bacterial species in the intestine and therefore also on the intestinal *E. coli* abundance. In humans *E. coli* are detected in 90% of the individuals with 10^7 to 10^9 colony forming units (cfu) per gram feces [11, 12]. In contrast to this, an *E. coli* prevalence of 56% in wild mammals, 23% in birds, and around 10% in reptiles is described [1]. In piglets, which were used in one of the animal trials of this study, *E. coli* numbers reaching from 10^7 to 10^9 cfu/g feces are found around weaning [13] and 10^7 cfu/g feces for growing piglets [14, 15].

In the case of *E. coli* it is not only interesting to know the relative abundance in the microbiome, but also which different *E. coli* strains colonize the intestine. *E. coli* is a highly variable species with significant genomic and phenotypic differences between individual strains. They have a diverse genetic background with genome sizes ranging from 4.5 to 6.0 megabases [16, 17]. This diversity can be visualized by data generated through whole genome sequencing [18].

By definition, the complete set of genes encoded by all strains of one bacterial species is its pan-genome. It is divided into the core/maximum common genome (MCG) – containing

genes which are shared by all strains of the same species – and a dispensable genome that contains the genes that are present in some but not all strains [19-21]. Today, the dispensable genome is divided into the accessory genome containing genes which are present in two or more strains and the unique genes which are only present in a single strain [22]. As it is impractical to sequence all strains of one species, this system is also used for a defined set of strains belonging to one species.

Two exemplary studies investigate *E. coli* genome sequences according to the previously described system and assign genes to the core and the dispensable genome. Results of both studies underline the high diversity of *E. coli* as only a low percentage of the pan-genome was identified as the core genome, resulting in a large pool of genes belonging to the dispensable genome. In one study 53 *E. coli* genomes were analyzed. Of the total 13,300 gene families that constitute the pan-genome, only 1,500 conserved gene families belong to the core genome, with the remaining 11,800 gene families being allocated to the dispensable genome [16]. In the second study 2085 *E. coli* genomes were investigated. This time 90,000 gene families assemble the pan-genome with only 3,200 gene families as the core genome, and about 86,800 gene families as the dispensable genome [23]. The high proportion of 88% or 96%, respectively, of the dispensable genome to the pan-genome substantiates the diverse genetic background of *E. coli*.

In addition to the high genetic diversity of *E. coli*, bacteria of this species also show a high variance in their pathogenic potential. *E. coli* can be grouped into non-pathogenic commensals and into different pathogenic *E. coli* (Fig. 1), the latter causing various infections and being a cause of major health problems worldwide. Pathogenic *E. coli* are divided into intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC). InPEC are traditionally classified into the six pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin producing/enterohemorrhagic *E. coli* (STEC/EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) [3]. However, beside these six types, the Shiga toxin producing enteroaggregative *E. coli* (STEAEC) and the adherent invasive *E. coli* (AIEC) are so-called hybrids [24]. For ExPEC the pathotypes uropathogenic *E. coli* (UPEC), newborn meningitic *E. coli* (NMEC), septicemia associated *E. coli* (SePEC), and avian pathogenic *E. coli* (APEC) are known [25, 26].

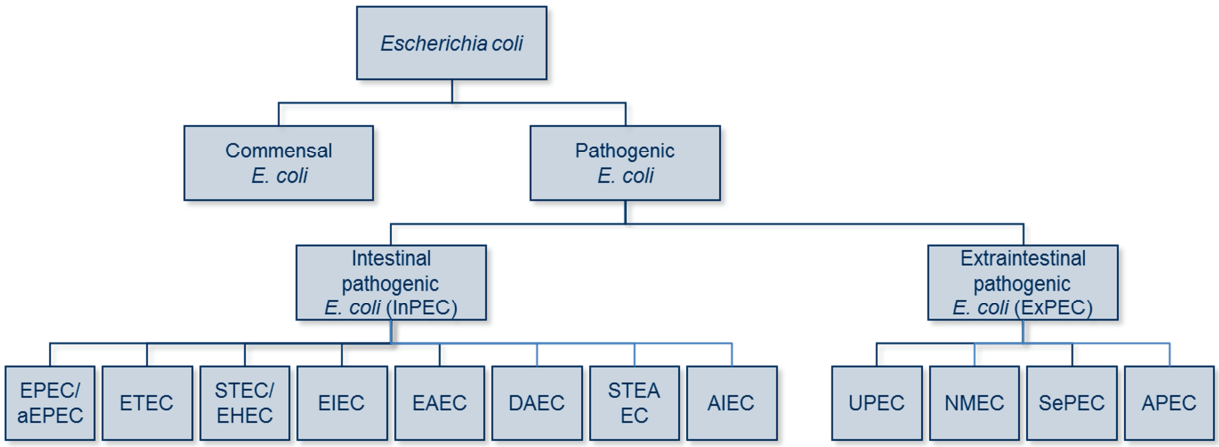

Figure 1: Schematic illustration of the classification of the different *E. coli* groups and pathotypes
EPEC: enteropathogenic *E. coli,* aEPEC: atypical enteropathogenic *E. coli*, ETEC: enterotoxigenic *E. coli*, STEC: Shiga toxin producing *E. coli*, EHEC: enterohemorrhagic *E. coli*, EIEC: enteroinvasive *E. coli*, EAEC: enteroaggregative *E. coli*, DAEC: diffusely adherent *E. coli*, STEAEC: Shiga toxin producing enteroaggregative *E. coli*, AIEC: adherent invasive *E. coli*, UPEC: uropathogenic *E. coli*, NMEC: newborn meningitic *E. coli*, SePEC: septicemia associated *E. coli*, APEC: avian pathogenic *E. coli*

InPEC strains are associated with enteric and diarrheal disease and are important pathogens worldwide. Unfortunately, as the causative agents of enteric infections are often not identified, the real incidence of enteric infections through *E. coli* is difficult to determine. For humans it is known that EPEC, ETEC, and EAEC are major causes of infantile diarrhea in the developing world, which can lead to death if left untreated [24].

EHEC, on the other hand, can cause bloody diarrhea through hemorrhagic colitis, non-bloody diarrhea, and the hemolytic uremic syndrome [3]. These infections are usually foodborne and can lead to large outbreaks or sporadic infections [27]. One exemplary large outbreak occurred from May 1 through July 4 in 2011 in Germany, demonstrating the possible extent of such EHEC outbreaks. During this period infections with STEC of serotype O104:H4 in 3816 cases (845 cases of the hemolytic uremic syndrome and 2971 cases of STEC gastroenteritis) were reported to public health authorities [28].

Besides the four already mentioned pathotypes also EIEC exist. They can cause an invasive inflammatory colitis and dysentery, but usually lead to watery diarrhea [3]. The incidence of EIEC in developed countries is relatively low. However, occasional foodborne outbreaks can occur [27]. For the sixth pathotype – DAEC – the role as a pathogen is still discussed. Though in the existing literature it is suggested that these strains may be important diarrheal pathogens in the developed world [27].

In addition to their human relevance, InPEC strains are also major animal pathogens and play a role in the field of veterinary medicine. These bacteria can cause diarrhea in different animal species for example in pigs, cattle, horses, rabbits, cats, and dogs [29-32]. Moreover, animals can also represent a reservoir for human infections. One example for their reservoir

function is the colonization and shedding of STEC from healthy cattle which can lead to transmission and infection of humans [33]. As this work focusses on pigs, the next section provides detailed information on the different InPEC pathotypes in this species.

Overall, InPEC are important infectious agents in diarrhea of suckling and weaned piglets. Diarrhea of these young animals leads to large economic losses in the pig industry [34, 35] due to increased mortality, morbidity, medication costs, and decreased growth rates [36].

Frequently, the post weaning diarrhea in pigs is caused by ETEC strains [37]. ETEC pathogenicity is based on two types of virulence factors namely colonization factors and enterotoxins. The first one, colonization factors, e.g. fimbrial antigens, allows an adherence to the intestinal mucosa. In cases of ETEC diarrhea in suckling and weaned piglets the fimbriae F18 [38, 39] and F4 [40] are commonly found [36, 41]. F18 positive strains occur almost exclusively in weaned pigs whereas strains positive for F4 can be isolated in suckling and weaned piglets [42].

The second mentioned virulence factor, enterotoxins, is produced either as heat-labile (LT) and/or heat-stable forms (STa and STb) by ETEC. While these toxins do not cause pathological lesions or morphological alterations to the mucosa, they induce hypersecretion of water and the electrolytes Na^+ and Cl^- and reduced intestinal absorption [43]. LT positive strains also often produce STb as both genes commonly exist on the same plasmid [44].

Next to ETEC also EPEC play a role in post weaning diarrhea. However, identification is difficult and veterinary diagnostic laboratories do not focus on the identification of this pathotype [36].

Apart from the diseases with the clinical symptom of diarrhea, edema disease in pigs can be caused by InPEC strains belonging to the STEC group and producing the Shiga toxin 2e. This disease is associated with subcutaneous edema of the eyelids and neurological symptoms including ataxia, convulsions, and paralysis [45]. For strains causing these symptoms also the pathotype designation edema disease *E. coli* (EDEC) is used.

In addition to the described cases of diarrhea and edema disease, there are also cases in which both clinical symptoms exist [46]. The causative agents are *E. coli* strains producing both the Shiga toxin 2e and enterotoxins [41].

Besides InPEC strains, which are associated with enteric and diarrheal disease, the group of ExPEC strains exists. Strains of this group are associated with different extraintestinal diseases in humans and animals.

In humans the most common ExPEC infections are urinary tract infections (UTI) and bacteremia/sepsis [47]. However, also pneumonia, meningitis, surgical site infections, and infections of the abdomen, pelvis, skin, and soft tissue can be caused by ExPEC [47, 48]. UPEC strains play a major role in human UTI and lead to 90% of community-acquired UTIs and pyelonephritis cases [25]. As UTIs are common and have a high prevalence, this

disease leads to high annual economic costs and a decreased workforce productivity [49]. NMEC are the most common cause of neonatal meningitis through gram negative bacteria [50]. Nowadays mortality rates ranging from 12 to 18% are described for NMEC infections [51] and even for survivors severe neurological defects are observed [52]. Sepsis, a life-threatening systemic blood infection can be caused by SePEC. Their relevance as human pathogen is shown, for example in one surveillance study for bloodstream infections which analyses data from 1995 to 2002 in the United States. During this study 24,179 cases of nosocomial bloodstream infections were detected. *E. coli* were the fifth most detected causative agent in 5.6% of the monomicrobial infections with a mortality rate of 22.4% [53].
In veterinary medicine ExPEC can lead to infections of wounds, the respiratory and the reproductive tract, to UTI, septicemia, and surgical site infections in livestock as well as in companion animals [54, 55]. Beside this, there is also the individual pathotype of APEC with strains leading to high economic loses in the poultry industry [55]. APEC strains are responsible for systemic infections in poultry, which start in the respiratory tract of chicken and turkey chicks [56]. This systemic infection causes a clinical picture of polyserositis with pericarditis, perihepatitis, and peritonitis [26].

1.2 Antimicrobial resistance

Both antimicrobial substances and resistance mechanisms against them occur naturally. In DNA from 30,000 years old permafrost sediments genes encoding for resistance against beta-lactams, tetracyclines and glycopeptides have already been found [57]. However, through the immense use and misuse of antimicrobial substances in human and in veterinary medicine over the past 60 years, the selection for antimicrobial resistant bacteria and the spread of resistance genes increased dramatically [58]. Only a few years after the introduction of new antimicrobial substances to the market, resistances against these substances were detected and the proportion of resistance increased in clinical isolates [59]. From the forties to the early sixties when lots of new antimicrobial substances and substance classes were developed and entered the marked, resistance was still manageable as alternative substances were available.
However, only very few new antimicrobial substances have entered the market in the last four decades [60] and resistance against the existing antimicrobial substances increased further. Therefore, the morbidity, mortality, and the treatment costs of bacterial infections have increased due to infections with resistant strains [61, 62]. The dramatic decrease of the approval of new antimicrobial substances is illustrated by an investigation of the United States Food and Drug Administration (FDA) databases. This investigation reveals a reduction in the number of newly approved antibacterial agents during the period from 1983

to 2002. In more detail, by comparing the time period 1983 to 1987 with that from 1998 to 2002 the FDA approvals dropped by 56% [63].

In general this decrease in approvals can be explained by different circumstances including:

(I) the existence of high technical difficulties for the development of new antimicrobial substances [64]

(II) demanding regulatory requirements for the approval of new antimicrobial substances [65]

(III) a high level of competition on the market with approved antimicrobial substances intensified through the restricted use of new antimicrobial substances as last option treatment [66]

(IV) the short-course therapies scheme of antimicrobial substances vs more profitable long-term treatment of chronic diseases [66]

For antimicrobial substances currently approved, the targeted bacterial mechanisms and the different resistance mechanisms by which bacteria respond can be distinguished in different groups (Fig. 2).

Antimicrobial substances target five mechanisms, essential for bacterial physiology or biochemistry. They inhibit DNA or RNA synthesis, protein biosynthesis, cell wall synthesis, folate synthesis or they depolarize the membrane potential [59, 67]. All common antimicrobial targets do not exist or are different in eukaryotic cells.

For the resistance against antimicrobial substances four different mechanisms are described:

(I) inactivation of antimicrobial substances

(II) modification of the antimicrobial targets

(III) efflux of antimicrobial substances

(IV) bypass

One classical example for the inactivation of antimicrobial substances by specific enzymes is the mechanism of action of beta-lactamases. These enzymes cleave the beta-lactam ring hydrolytically, which is characteristic for the entire class of beta-lactam antimicrobials and essential for the antimicrobial effect. The modification of the antimicrobial target is achieved by mutation/s of the target encoding gene (e.g. resistance to fluoroquinolones) or through the modification of the target by enzymes (e.g. resistance to macrolides). In the case of efflux, antimicrobial substances are ejected by protein pumps and consequently an accumulation of the antimicrobial substance in the bacterial cell is prevented. In cases of multidrug efflux pumps different antimicrobial substances can be removed from the cell. Beside these mechanisms also immunity and bypass is described. In case of bypass, a resistant alternative target is produced in parallel to the sensitive native target [67-69].

Although the resistance mechanisms can be grouped according to the mentioned scheme, a high diversity for the genes encoding for resistance is recorded. The actual list of “The

Comprehensive Antibiotic Resistance Database" comprises of 2172 reference protein sequences which are tagged specifically for antibiotic resistance [70, 71].

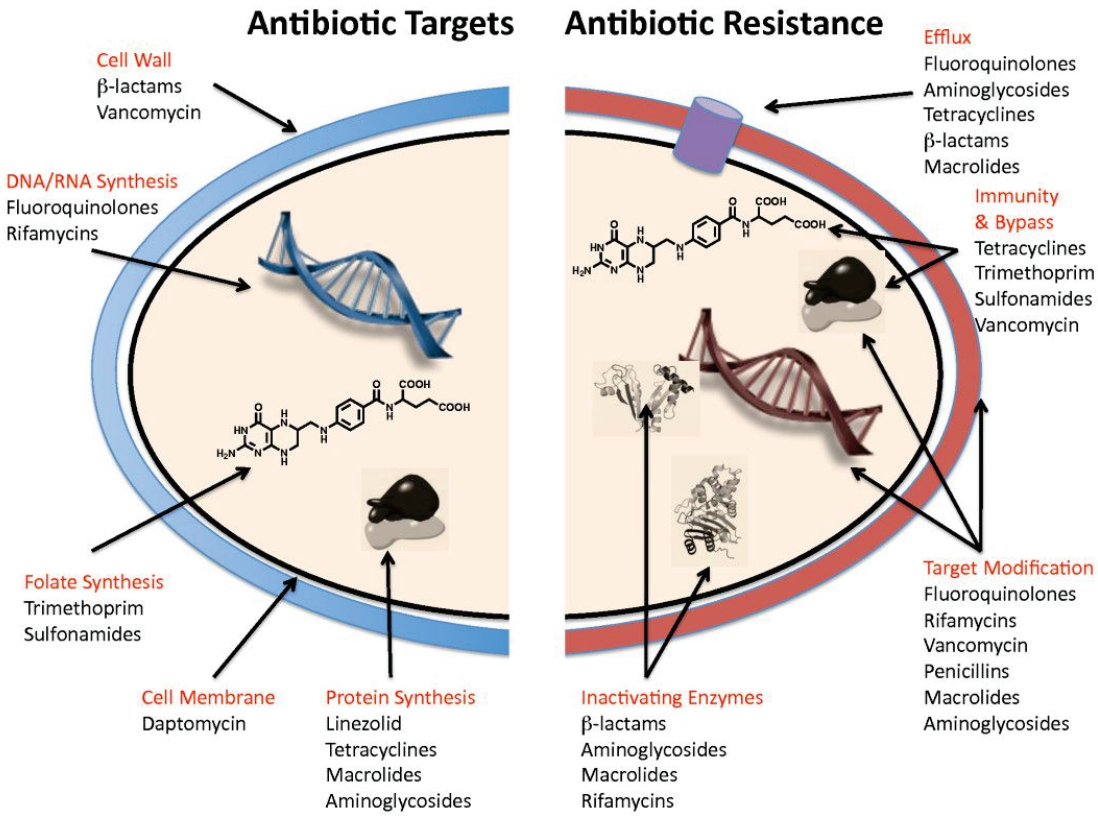

Figure 2: Antibiotic targets and mechanisms of resistance
Wright, BMC Biology 2010, 8:123, original publisher: BioMed Central http://www.biomedcentral.com/1741-7007/8/123 [67]

As mentioned above, the abundance of antimicrobial resistant bacteria and the spread of resistance genes have been increasing worldwide. For humans several articles describe and deal with this problem. In 2014, the World Health Organization published its first global report on antimicrobial resistance, which reveals a serious and worldwide threat to the public health [72].

This statement is underlined by different national resistance reports like "Antibiotic Resistance Threats in the United States, 2013" by the Center for Disease Control and Prevention (CDC) [73], which emphasize this threat. More than two million cases of illnesses with antibiotic resistant bacterial or fungal infections per year resulting in 23,000 deaths were estimated for the United States. With regard to *E. coli*, the species in focus of this work, the CDC mentions extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae as a serious problem. These bacteria are able to hydrolyze various types of beta-lactam antibiotics like third-generation cephalosporins and monobactams leading to resistance to these antibiotics. They are worldwide distributed, often multi-resistant and therefore exhibit only limited treatment options [74]. However, classical ESBL-producing bacteria are not able

to hydrolyze cephamycins and carbapenems. 9,000 cases of infections with ESBL-producing *E. coli* per year, leading to 600 deaths were estimated for the United States [73].

For Europe, data notified to the European Antimicrobial Resistance Surveillance Network (EARS-Net) were published in the report “Antimicrobial resistance surveillance in Europe, 2014” of the European Centre for Disease Prevention and Control (ECDC) [75]. In accordance with the articles mentioned above, this report also assesses antimicrobial resistance as a serious threat to the public health in Europe. Especially the situation for Gram-negative bacteria is alarming as high and often increasing resistance percentages were detected for many parts of Europe. For *E. coli*, more than half of the notified isolates in 2014 were at least resistant against one group of antimicrobial substances. Besides this, a significant increase of the resistance against third-generation cephalosporins and of the resistance against fluoroquinolones, aminoglycosides, and third-generation cephalosporins was observed between 2011 and 2014 [75].

In veterinary medicine the occurrence of antimicrobial resistant bacteria displays an important problem for two reasons: On the one hand, there is the ethical standard for veterinarians to treat diseased animals and to prevent their suffering. In case of infections with resistant bacteria, the limited treatment options complicate the situation. On the other hand, animals colonized with resistant bacteria can transmit these to humans. For livestock animals antimicrobial resistant bacteria can be transmitted via food consumption and direct or indirect contact to humans [76]. Companion animals can transmit resistant bacteria via their often close contact to their owner [77]. Beside this, in case of illness and therapy difficulties emotional burden for the owners is often high.

The already mentioned increase of antimicrobial resistance can also be detected in the area of veterinary medicine [77-79]. As *E. coli* is the main species of this study, the resistance rates of the next section will display only data for *E. coli*.

For livestock, different studies display high resistance rates for several antimicrobial substances. For the European Union, “The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014” presented data for bacteria isolated from healthy animals, carcasses, and meat from the poultry industry. For broilers, resistance against ciprofloxacin (66% of isolates were resistant), nalidixic acid (63%), ampicillin (59%), sulfamethoxazole (53%), tetracycline (50%), and trimethoprim (41%) were very often detected. For fattening turkeys comparable data were gained. However, this time the highest resistance was detected against tetracycline (71%) [80].

Information for cattle and pigs are delivered by the European Antimicrobial Susceptibility Surveillance in Animals (EASSA), which was coordinated by the European Animal Health Study Centre (CEESA). In healthy cattle, the resistance rates were in general lower than for

pigs and chickens. The highest resistance rate was detected for tetracycline, where 8.2% of bovine isolates were resistant. Resistance rates for piglets, however, were remarkably higher with 66% of isolates being resistant against tetracycline, 42% against sulfamethoxazole-trimethoprim, 29% against ampicillin, and 16% against chloramphenicol [81]. For industrial pig production also high resistance levels against aminopenicillins were detected for the Netherlands (36% of isolates were resistant), Denmark (27%), and Switzerland (25%) [82].

Comparable results can be found in Germany. With focus on pigs, the resistance monitoring GermVet revealed resistance rates of 59% against sulfamethoxazole, 54% against tetracycline, and 38% against ampicillin for isolates of urogenital tract infections and the mastitis metritis agalactia syndrome [83]. For *E. coli* isolated from piglets with enteritis even higher resistance rates were detected [84].

Finally, also alarming data for ESBL-producing *E. coli* exists for livestock. In feces collected in German fattening pig farms with a positive ESBL/AmpC beta-lactamases status, 37% of samples were positive for ESBL/AmpC-producers after a pre-enrichment [85].

Resistance data for *E. coli* isolates of companion animals complete the picture of the global problem of antimicrobial resistant bacteria. For European cats and dogs with urinary tract infections resistance against ampicillin was detected for 47%, against doxycycline for 27%, against amoxicillin/clavulanic acid for 25%, and against sulfamethoxazole-trimethoprim for 19% of isolates [86]. In a Danish dog study with isolates from feces, intestine, and the urogenital tract, also high levels of resistance were detected [87].

In Germany, dog and cat isolates from urinary/genital tract infections show high resistance rates for ampicillin (24% of isolates were resistant), sulfamethoxazole (18%), and tetracycline (16%). For animals with gastrointestinal infections for tetracycline (18%), sulfamethoxazole (15%), and ampicillin (14%) resistance rates over 10% were detected [83].

Beside the mentioned data, 10% ESBL-producing *E. coli* positive fecal samples from dogs underline also the problem of antimicrobial resistant bacteria in companion animals [88].

Although differences between humans, animals, sample sites, countries, and studies exist, the overall picture is the same; namely, that antimicrobial resistant bacteria are widely distributed and are a fixed component during bacterial sampling. Whatever numbers are published, it has to be considered how representative they actually are. Thus, the main message is to state that resistance is a common feature, and to reduce this trend, intervention strategies have to be implemented.

1.3 Conjugation as a main contributor to the spread of antimicrobial resistance genes

As described above, the global problem of antimicrobial resistant bacteria is well known and therefore, a lot of investigations and research is performed. On the strain level, antimicrobial resistance is caused by genetic mutation or the acquisition of resistance genes by horizontal gene transfer (HGT). For the general spread of antimicrobial resistance genes HGT was known to be the major vector [62]. However, with the emergence of pandemic clones, the clonal spread of multi-drug resistant bacteria lineages gains more attention [89]. Examples for these pandemic clones can be found in ESBL-producing bacteria of sequence types 131 [89] and 648 [90], in methicillin-resistant *Staphylococcus aureus* (MRSA) of clonal complexes 22 [91] and 398 [92], and in vancomycin resistant enterococci of complex 17 for *Enterococcus faecium* [93]. However, this work focusses on the spread of antimicrobial resistance genes via HGT and the next sections will therefore reveal information on this aspect.

The genetic exchange via HGT is in contrast to lateral gene transfer independent of asexual cell division. During HGT, DNA can be exchanged between bacteria of one species and also cross species barriers by the following mechanisms:

(I) transformation (uptake of DNA from the environment)

(II) transduction (DNA transfer via viral particles)

(III) conjugation [94]

As this work investigates bacterial conjugation of plasmids, the next section will further describe this mechanism, particularly the transfer of conjugative plasmids.

During conjugation, plasmids (Fig. 3), transposons, integrons, and integrative and conjugative elements are transferred by direct cell-cell contact [95-97]. Plasmids are circular or linear DNA segments. They are autonomously replicating and can be found in bacterial cells in parallel to the chromosome [98]. Conjugative plasmids encode for their own conjugation machinery to transfer themselves to a recipient cell [98]. In cases of non-conjugative plasmids, mobilization and transfer via conjugation can take place through self-transmissible helper plasmids [99] or conjugative transposons [100].

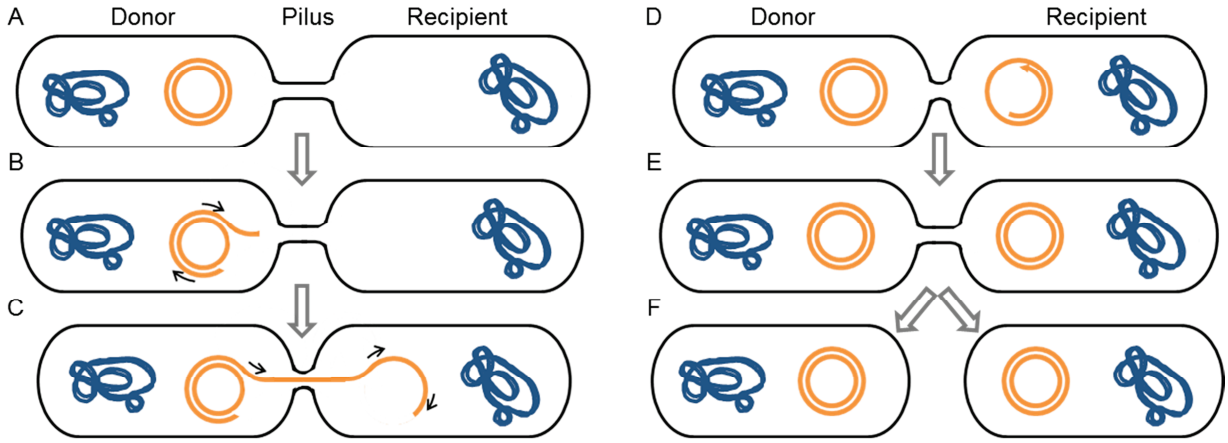

Figure 3: Simplified schematic diagram of the conjugation of a conjugative plasmid Chromosomal DNA is presented in blue and DNA of the conjugative plasmid in orange. A) pilus mediated cell-cell contact, B) beginning of replication, C) plasmid transfer to recipient cell, D) plasmid replication in recipient cell, E) completion of replication, and F) separation of the mating pair. Adapted from Bushman F., Lateral DNA Transfer [101]

Conjugation is mediated by a type IV secretion system (T4SS). These systems are exceptionally versatile and can be found in Gram-negative as well as Gram-positive bacteria [102]. According to their function three distinct groups of T4SS can be differentiated:

(I) T4SS for bacterial conjugation

(II) T4SS which mediate the uptake and release of DNA with the extracellular milieu

(III) T4SS which translocate DNA or protein substrates to eukaryotic cells and are involved in the virulence of the strain [102]

The architecture of all T4SS consists of four domains:

(I) pilus

(II) core channel complex

(III) inner membrane platform

(IV) three hexameric ATPases [60]

The pilus is composed of helical assembled pilin molecules and adhesion molecules at the distal end and the core channel complex has two subcomplexes, the outer membrane cap and the periplasmiatic domain. The three hexameric ATPases are located at the base, attached to the inner membrane and supply the energy for the system [60].

One of the best studied T4SS is the VirB-VirD system of *Agrobacterium tumefaciens* which is similar to many T4SS found in Gram-negative bacteria [103]. It is composed of 12 proteins named VirB1-VirB11 and VirD4 and the genes encoding these proteins can be grouped in genes involved in mating pair formation and DNA transfer replication [104].

The transport of the nucleoprotein by a T4SS is characterized by different steps:

(I) Relaxase [105] and auxiliary transfer proteins bind to the origin of transfer (*oriT*) region of the conjugative plasmid to form the relaxosome [106]. The relaxase, a phosphodiesterase cleaves the *nic* site within the *oriT* resulting in a single stranded DNA.

(II) Supported by the auxiliary proteins the coupling protein [107] binds the relaxosome and binds it to the T4SS transferosome. Beside this, the retraction of the pilus leads to a membrane fusion process.

(III) DNA covalently bound to the relaxase is transferred via the secretion system. It is hypothesized that the relaxase is unfolded during this transfer [60].

(IV) The nicking reaction is reversed in the recipient, leading to a circular plasmid molecule free of relaxase.

(V) Replication of the plasmid occurs in the donor and recipient cell to stabilize the plasmid [60, 108].

Conjugation of plasmids are described as the major vector for the horizontal gene transfer of antimicrobial resistance genes [62] and have therefore a high impact on the spread of resistance genes. This is due to the fact that plasmids often harbor resistance genes and that their genetic structure is also predisposed to contain different resistance genes [62, 109]. Interestingly, also the transfer of initially chromosomal encoded resistance genes on plasmids was described [110].

The clinical relevance of resistance plasmids dissemination is underlined by the fact that epidemiological investigations of disease outbreaks need to determine both the clonal background of strains and the relatedness of plasmids of the respective strains [111-113]. During a hospital outbreak with *Klebsiella (K.) pneumoniae* carbapenemase (KPC)-producing carbapenem resistant Enterobacteriaceae, eleven unique strains were isolated, of which nine harbored the same plasmid carrying the KPC gene. The nine strains harboring this plasmid belonged to four different species of three different genera. This again proofs the ability of plasmids to disseminate between different species and genera [111]. In addition, over the last decade conjugation has become a potential target for the development of novel ecology and evolution drugs to fight antimicrobial resistance (for review see [114]). ESBL-producing bacteria, with their mostly plasmid encoded ESBL-phenotype, are also one example for the relevance of resistance plasmid distribution. Although the spread of successful clonal lineages is a major driving part for their worldwide distribution, the spread of epidemic plasmids is relevant as well [115, 116].

1.4 The use of gnotobiotic mice and rats in conjugation experiments

As direct cell to cell contact is required for bacterial conjugation, the intestine with its high cell density represents a favorable place for the exchange and spread of resistance genes [61]. Therefore, *in vivo* investigations with gnotobiotic animals were frequently used for qualitative and quantitative examination of the exchange of conjugative elements. The term gnotobiotic comes from the Greek words “gnotos”, meaning “well known” and “biota”, meaning “life” [117]. It is used for germfree animals or animals with a defined and known microbiota. The use of gnotobiotic animals for experiments allows a microbiological standardization of the animal stock, the evaluation of interactions between microbiota and host [118], and investigations in a simpler environment than in that of the complex microbiome [119]. To implement a new germfree mouse line, unborn mice have to be removed sterile from the womb by hysterectomy or hysterotomy and raised and kept in a sterile surrounding [119]. Therefore, isolators with a double door transfer chamber with sterile feed, water, bedding, and equipment are used. Once a line with proofed germfree animals is established, germfree mothers can give natural birth. As the management of these animals is laborious, alternative protocols exist based on the administration of different antimicrobial substances to deplete the cultivable microbiota. These antimicrobial substances treated animals can be used as germfree animals or animals with an adjusted microbiota [120, 121].

During the past years, different elements of conjugation were investigated in gnotobiotic mice or rats including:

(I) genetic exchange between Gram-positive and Gram-negative bacteria [122]

(II) genetic exchange between isolates of animal and human origin [123, 124]

(III) transfer of resistance genes from food borne strains to members of the gastrointestinal microbiota [125, 126]

(IV) influence of nutritional components [127] and of the therapeutic treatment with antimicrobial substances [128] on the genetic exchange

(V) differences in the genetic exchange between several intestinal sections [129]

The influence of high dietary zinc feeding on bacterial conjugation, which was investigated during this study, has not been examined in gnotobiotic mice previously.

1.5 High dietary zinc feeding as alternative to antimicrobial growth promoters

One of the concepts to counteract the problem of globally distributed resistance genes and resistant bacteria is the prudent use of antimicrobial substances. Therefore, the European Union banned in-feed antibiotics in 2006 (REGULATION (EC) No 1831/2003) [130]. This regulation has enforced the search for possible alternatives in livestock breeding to maintain gut health and growth performance. In this study we focus on pig production as important part of the livestock industry. As already mentioned, post weaning diarrhea in pigs leads to

important economic loses. To prevent these frequently occurring disease, several in-feed alternatives like probiotics (e.g. *Enterococcus faecium*, *Bacillus cereus* variant Toyoi or *Lactobacillus sobrius* [131-133]), prebiotics (e.g. fructo- and galacto-oligosaccharides or stabilized rice bran [134, 135]), enzymes, herbal products and other bioactive compounds (for review see [136, 137]) where propagated by science and industry. Besides the mentioned alternatives also cationic trace elements such as zinc or copper [138, 139] are described. This work further investigates the strategy of high dietary zinc oxide feeding as alternative to antimicrobial growth promoters.

The trace element zinc is a cofactor for more than 300 enzymes [140, 141] and an essential ingredient of food and feed. For example, zinc is necessary for DNA synthesis, cell growth, cell division, the immune system, cognitive functions and blood clotting (for review see [142]). Beside its relevance for humans and animals, zinc is also essential for bacteria. Approximately 5% of the total bacterial proteins are putative zinc-binding proteins [143]. However, too high internal zinc concentrations are toxic [144]. To regulate the internal zinc concentration, *E. coli* have different zinc uptake and export systems. For uptake across the cytoplasmic membrane, the high-affinity ABC-type zinc uptake system ZnuABC [145] and the low-affinity uptake system ZupT [146] are described. In the ZnuABC system, *ZnuA* encodes for the periplasmic component binding zinc, *znuB* for the transmembrane component, and *znuC* for the ATPase subunit providing energy [147]. The export of zinc is primarily conducted by the P-type ATPase ZntA [148] and the two subsidiary cation diffusion facilitator transporters ZitB [149] and YiiP [150].

As described above, zinc is an essential ingredient of feed. In cases of growing piglets, current dietary recommendations range between 80 to 100 mg zinc/kg diet. However, the strategy of feeding zinc oxide in concentrations from 2000 – 3000 mg zinc/kg diet to prevent diarrhea in young pigs is widely used [151-153]. Within the EU, zinc oxide is applied as feed-additive, veterinary medical product and in combination with colistine [154]. As feed-additive the maximum allowance is 150 mg zinc/kg diet (COMMISSION REGULATION (EC) No 1334/2003) [155]. The use of zinc oxide as veterinary medical product is authorized for some but not all member states of the EU and different medical products are approved. Combination products with colistine and zinc oxide are again only allowed for some countries within the EU. For example in Germany the pharmaceutical Enteroxid “Ogris” (aniMedica GmbH, Senden-Bösensell, Germany) is approved for the therapy and metaphylaxis of bacterial infections of the digestive tract of young pigs. This pharmaceutical contains 25 mg colistin sulfate in addition to 480 mg zinc oxide in 1 g of powder.

The main mechanism by which zinc increases the growth performance and reduces post weaning diarrhea is still not known and several studies identified different mechanisms. In an infection study with an ETEC strain (K88), piglets fed high dietary zinc had a reduced rectal

temperature, a reduced fecal consistency score (standing for a more physiological texture), reduced fecal shedding of the ETEC strain and an increased average daily gain [156]. Beside this, it is described that high dietary zinc feeding reduces intestinal permeability and increases expression of tight junction proteins [157]. Zinc also reduces the intestinal mucosal susceptibility to substances, which activate the chloride secretion [158]. Furthermore high dietary zinc feeding increases the expression of the insulin like growth factor I and its receptor in the small intestine mucosa [159], it increases gene expression of antimicrobial peptide PR-39 [160], and it influences the variety, diversity and composition of the microbiota [161, 162].

1.6 Association of zinc feeding with antimicrobial resistance

To reach the goal of a prudent use of antimicrobial substances the ban of antimicrobial growth promoters was a step in the right direction. However, the resulting massive use of zinc oxide for weaning piglets might have consequences which are poorly investigated until now.

The effect of co-selection of antimicrobial and metal resistance is well known [163]. In the case of zinc, this knowledge is gaining increasing recognition [164-166]. For livestock, a connection of the zinc concentration in liquid pig manure with the phenotypic antimicrobial resistance in *E. coli* [167] and an increased prevalence and persistence of MRSA through feeding therapeutic doses zinc oxide [168] is described.

Furthermore, previous zinc pig feeding trials performed in the Collaboration Research Center 852 by our group found an increase of multi-resistant *E. coli* under the influence of high dietary zinc feeding [169]. During this trial, 36 piglets were fed either a diet containing high dietary zinc (2425 mg zinc/kg diet) or a control diet (57 mg/kg). Overall, 1.481 *E. coli* were isolated from digesta and initially their phylogenetic relatedness was investigated via pulsed-field gel electrophoresis. Afterwards, the antimicrobial resistance characteristics were determined for selected isolates, each representing one clone (a phylogenetic related group of the isolates). Interestingly, 18.6% of the *E. coli* clones isolated in the zinc group were multi-resistant whereas not a single multi-resistant clone was detected in the control group. These results were substantiated through real time PCR investigations of the same zinc feeding trial. An increase of the copy numbers for tetracycline and sulfonamide resistance genes was detected for the zinc group in the gut of piglets over time after weaning [170].

However, in that study of Bednorz et al. (2013), the mechanisms causing the increase of multi-resistance under high dietary zinc feeding have not been analyzed. Suggestions for involved mechanisms were gained as isolates originating from piglets of both feeding groups and belonging to the same clone were further characterized via minimum inhibitory concentration (MIC) determination for defined antimicrobial substances, investigation of their

plasmid profiles, and zinc tolerance testing. As some isolates originating from the zinc group showed additional resistance paired with increased zinc tolerance, an involvement of co-selection was proposed. Beside this, some variations in the resistance pattern could be linked with differences in the plasmid profiles of some isolates and, therefore, an enhanced plasmid uptake under the influence of high dietary zinc feeding was suggested [169].
Interestingly, it has also been shown that zinc interferes with the conjugation process. Earlier conjugation experiments with *E. coli* displayed a negative influence of zinc on the fertility of the male donor strain but a positive influence on the ability of F- recipient cells to form mating pairs [171, 172]. As already mentioned, the high bacterial density in the intestine leads to an optimal condition for bacterial conjugation [61], and it is also known that food and feed ingredients can affect the genetic exchange rates by conjugation [127, 173].

1.7 Aim of the study

As mentioned above, during zinc oxide feeding as alternative to the feeding of antimicrobial growth promoters, an increased multi-resistance in porcine *E. coli* was observed. To both proof these results and also gain insight into the underlying mechanisms a complex study design for an animal trial was chosen. Key components were:

(I) investigation of the phenotypic antimicrobial resistance status of the *E. coli* population without a prior clonal selection
(II) parallel quantitative investigation of the resistant and overall numbers of *E. coli*
(III) extension of the sampling scheme to feces, digesta, and mucosa samples
(IV) investigation of the status quo of the *E. coli* population before weaning and the start of the zinc feeding

As the complex microbiota of piglets does not allow a clarification of the mechanism, we also investigated the influence of zinc on bacterial conjugation *in vivo* with gnotobiotic mice as well as *in vitro*. For the *in vitro* investigations a protocol for mating in liquid media was used.

2 Materials and Methods

2.1 Solutions and nutrition media

1x PBS buffer

NaCl	8 g
KCl	0.2 g
KH_2PO_4	0.24 g
Na_2HPO_4 $2H_2O$	1.805 g

pH=7

The indicated chemicals were dissolved in distilled water, the volume was added up to 1000ml and the solution was autoclaved at 121°C for 15 minutes.

CHROMagar Orientation plates

CHROMagar Orientation powder	33 g
Agar	15 g
Peptone and yeast extract	17 g
Chromogenic mix	1 g

The powder was dissolved in distilled water, the volume was added up to 1000ml and the solution was autoclaved at 121°C for 15 minutes. In case an antimicrobial substance was added, the agar was cooled down to 50°C before supplementation and afterwards filled in sterile petri dishes.

LB broth (Luria/Miller)

LB broth powder (Luria/Miller)	25 g
Tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g

The powder was dissolved in distilled water, the volume was added up to 1000ml and the solution was autoclaved at 121°C for 15 minutes.

LB agar (Luria/Miller)

LB agar powder (Luria/Miller)	40 g
Tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g
Agar-Agar	15 g

The powder was dissolved in distilled water, the volume was added up to 1000ml and the solution was autoclaved at 121°C for 15 minutes. In cases an antimicrobial substance was added, the agar was cooled down to 50°C before supplementation and afterwards filled in sterile petri dishes.

Mueller-Hinton-II broth (Cation-Adjusted)

Mueller-Hinton-II broth powder	22 g
Beef extract	3 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g

The powder was dissolved in distilled water, the volume was added up to 1000ml and the solution was autoclaved at 121°C for 15 minutes.

LB broth (Lennox)

LB broth powder (Lennox)	20 g
Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g

The powder was dissolved in distilled water, the volume was added up to 1000ml and the solution was autoclaved at 121°C for 15 minutes.

Columbia agar for anaerobic cultivation

Columbia agar (base) powder	42,5 g
Casein peptone	10 g
Meat peptone	5 g
Heart peptone	3 g
Yeast extract	5 g
Corn starch	1 g
Sodium chloride	5 g
Agar	13.5 g
L-Cysteinium chloride monohydrate	0.3 g
Agar	3 g
Hemin	5 ml
Vitamin K1 10mg/ml	1 ml
Lysed sheep blood	5 ml
Defibrinated sheep blood	50 ml

The Columbia agar powder was dissolved in distilled water. L-cysteinium chloride monohydrate, agar, and hemin were added, the volume was added up to 9944ml and the solution was autoclaved at 121°C for 15 minutes. The agar was cooled down to 50°C, vitamin K1, lysed sheep blood, and defibrinated sheep blood was added and after mixing the agar was filled in sterile petri dishes.

2.2 Zinc pig feeding trial

2.2.1 Animals, Housing and Diets

A zinc pig feeding trial was performed with a total of 32 landrace piglets in the Institute of Animal Nutrition of the Department of Veterinary Medicine at the Freie Universität Berlin. Husbandry conditions were adapted from the recent zinc pig feeding trial performed in this institute [169, 174] and are already published by Zetzsche et al. [175]. In detail, 25±1 days after birth, piglets were weaned and randomly allocated to two different feeding groups balancing for gender, litter, and body weight. Animals had *ad libitum* access to water and one of the two feeding diets, containing either a high level of dietary zinc or a background level of zinc, and were housed in pairs in straw bedding pens in a stable separated from the stable of their mothers.

Both diets met the nutritional requirements of weaned piglets and were based on wheat, barley, corn, and soybean meal (Tab. 1). For adjusting the zinc content of the feed, corn starch was partially replaced by analytical grade zinc oxide (Sigma-Aldrich Chemie, Taufkirchen, Germany) and the final zinc concentration was determined by atomic absorption spectrometry using an AAS vario 6 spectrometer (Analytik Jena, Jena, Germany). A final concentration of 2103 mg zinc/kg diet was measured for the zinc group and 72 mg/kg diet was measured for the control group.

To exclude an effect by antimicrobial active pharmaceuticals, piglets and their mothers received no antimicrobial substances. The animal trial was approved by the local state office of occupational health and technical safety ‘Landesamt für Gesundheit und Soziales, Berlin’ (LaGeSo Reg. Nr. 0296/13).

Table 1: Ingredients and chemical composition of the diets used in the zinc pig feeding trial

Ingredients	g/kg diet	Chemical composition	Control diet	Zinc diet
Wheat	300			
Barley	200	g/kg		
Corn	230	Dry matter	892	891
Soybean meal	200	Crude protein	188	192
Monocalcium phosphate	13	Crude fiber	30	32
Limestone	14	Ether extract	29	30
Vitamine-mineral pre-mix*	15	Starch	424	445
Soy oil	10	Ash	51	52
Lysine-HCl	3.5	Minerals (mg/kg)		
Tryptophan	1	Iron	183	156
Methionine	1	Manganese	96	113
Salt	2.5	Copper	18	19
Zinc oxide / Corn starch	10	Zinc	72	2103

*600,000 IU vitamin, 120,000 IU vitamin D3, 8,000 mg vitamin E, 300 mg vitamin K3, 250 mg vitamin B2, 400 mg vitamin B6, 2,000 µg vitamin B12, 2,500 mg nicotine acid, 100 mg folic acid, 1,000 mg pantothenic acid, 80,000 mg choline chloride, 30 mg cobalt, 45 mg iodine, 35 mg selenium, 6,000 mg manganese, 1,000 mg copper, 5,000 mg iron

Adapted from Zetzsche et al. [175]

2.2.2 Sampling

In this study, feces, digesta, and mucosa samples were investigated at three different time points (Fig. 4). Feces was collected from the ampulla recti while digesta and mucosa samples were taken from the colon ascendens after sacrificing the piglets. To collect mucosa samples a 2-5cm long piece of intestine was used. Digesta was removed by gently massage and afterwards the intestine was opened by longitudinal incision and washed two to three times in 1x phosphate buffered saline (PBS) solution to remove the remaining digesta and the not adherent bacteria. Mucosa samples with adherent bacteria were then gained by scraping the mucosal side of the intestine with a sterile slide.

The first sampling time point was to investigate the status quo of the feces *E. coli* population. Therefore, feces of all 32 piglets were collected two to zero days before weaning at the age of 24±2 days. The second sampling time point was at the age of 38±2 days (two weeks after weaning). This time, feces were collected from all 32 piglets and afterwards, 16 animals were sacrificed (eight per feeding group), and 16 times digesta and mucosa were collected. The third and last sampling time point was at the age of 52±2 days (four weeks after weaning). Feces were collected from the remaining 16 animals prior to euthanasia and collection of digesta and mucosa samples.

For euthanasia, animals were sedated [20 mg/kg bodyweight (BW) of ketamine hydrochloride (Ursotamin®, Serumwerk Bernburg AG, Germany) and 2 mg/kg BW of azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany)] and euthanized with intracardial injection of 10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide, and embutramide (T61®, Intervet, Unterschleißheim, Germany). After sacrificing, the entire intestinal tract was removed from the abdominal cavity after a midline abdominal incision and samples from colon ascendens were gathered.

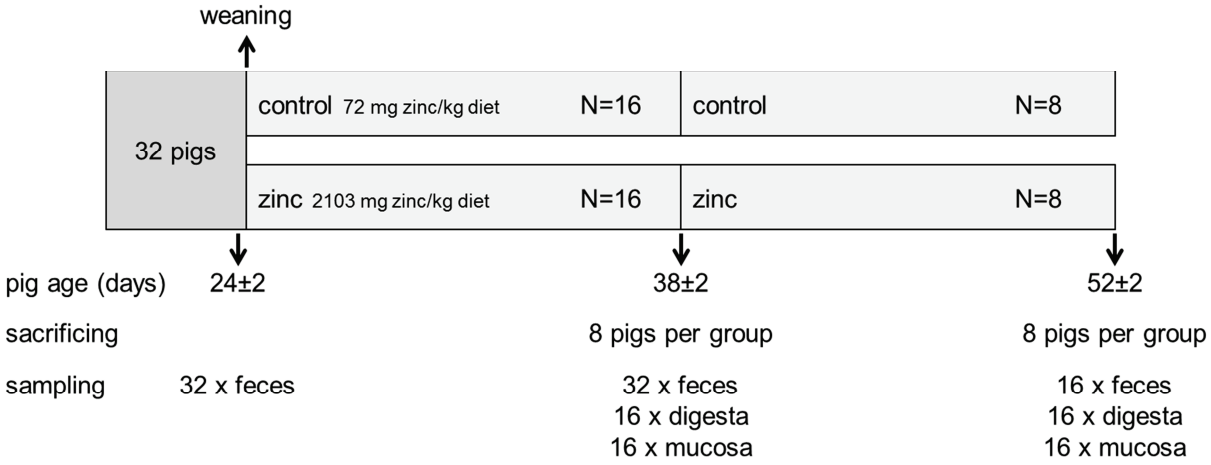

Figure 4: Schematic diagram of the zinc pig feeding trial
32 piglets were weaned 25±1 days after birth, randomly allocated to two feeding groups, and sacrificed at the age of 38±2 days (8 animals per group) or 52±2 days (8 animals per group). N values present the number of piglets per experimental stage and feeding group. Arrows indicate the sampling time points related to the pig age with the number of sacrificed animals (sacrificing) and the amount and habitat of taken samples (sampling).

2.2.3 Isolation of *E. coli*

For isolation of *E. coli* with a highly diverse genetic background considering antimicrobial resistance, a protocol adapted from Bednorz et al. was used [169]. Samples were suspended in 1x PBS and serially diluted. To suspend mucosa scrapings, samples were homogenized with a dounce homogenizer. Three different serial dilutions were plated on sheep blood agar plates (Oxoid, Wesel, Germany), CHROMagar Orientation plates (CHROMagar, Paris, France), and on CHROMagar Orientation plates supplemented with one of nine antimicrobial substances. Concentrations of antimicrobial substances were derived from the breakpoint concentrations of the Clinical and Laboratory Standards Institute [176, 177] or were adapted from Guenther et al. [178] (ampicillin 32 µg/ml, streptomycin 64 µg/ml, nalidixic acid 32 µg/ml, sulfamethoxazole-trimethoprim 76 µg/ml / 4 µg/ml, tetracycline 16 µg/ml, cefotaxime 4 µg/ml, gentamicin 16 µg/ml, enrofloxacin 2 µg/ml, or chloramphenicol 32 µg/ml, further information to the used antimicrobial substances are given in Tab. 2).
Agar plates were incubated under aerobic conditions at 37°C for 18-24 hours. Up to 22 colonies per specimen with each colony representing a single isolate were picked according

to a specific scheme, which allowed a maximum diversity of antimicrobial resistant isolates and isolates, chosen from the non-selective agar plates. In detail, if possible, ten colonies were randomly chosen from different antimicrobial substances containing agar plates with an equal distribution for the different substances added to the agar. If colonies could be detected on five or more different types of selective agar plates, a maximum of two randomly chosen colonies per agar type were chosen for further investigation, resulting in a maximum of twelve colonies isolated from selective agar plates. Remaining colonies were randomly picked from sheep blood agar plates and CHROMagar Orientation plates. For sheep blood agar plates, colonies which were suspected to be *E. coli* and for CHROMagar Orientation plates colonies with typical dark pink to reddish morphology were chosen.

Each isolate was two times sub-cultured and incubated overnight on CHROMagar Orientation and sheep blood agar plates. Afterwards, a single colony of a pure culture was grown under aerobic conditions at 37°C and 200 rounds per minute (rpm) for three hours in lysogeny broth (LB) media (Luria/Miller) (Carl Roth, Karlsruhe, Germany) and stored in 20% glycerol (Carl Roth, Karlsruhe, Germany) stocks at -80°C for further investigation. Overall, 1.610 feces, 550 digesta, and 505 mucosa isolates were selected.

Table 2: General information on the antimicrobial substances [179] used for the isolation and the phenotypic resistance screening of *E. coli* isolates

Antimicrobial substance	Antimicrobial class	Main acting mechanism	Resistance conferred via
Ampicillin (Amp[1])	Beta-Lactams	inhibition of the synthesis of bacterial cell walls	enzymatic hydrolysis of the β-lactam ring
Streptomycin (Strep[1])	Aminoglycosides	target the 30S ribosomal subunit, leading to mistranslation of proteins	aminoglycoside modifying enzymes, increased efflux, target modification by methylation
Nalidixic acid	Quinolones	inhibition of DNA synthesis by topoisomerases II (DNA gyrase) and IV	target modification by mutations, increased efflux, target protection by a protein
Sulfamethoxazole/ Trimethoprim (Sul/Tri[1])	Sulfonamides/ Diaminopyrimidine	inhibition of folic acid metabolism	target modification or altered gene expression by mutations, replacement of sensitive enzymes by resistant ones
Tetracycline (Tet[1])	Tetracyclines	blocking of aminoacyl tRNA access to the ribosom by binding the 30S ribosomal subunit	increased efflux, ribosomal modification, (tetracycline inactivating enzyme)
Cefotaxime	Beta-Lactams	see ampicillin	see ampicillin
Gentamicin	Aminoglycosides	see streptomycin	see streptomycin
Enrofloxacin	Quinolones	see nalidixic acid	see nalidixic acid
Chloramphenicol	Amphenicols	inhibition of the elongation step of translation by binding the 50S ribosomal subunit	target modification by methylation, chloramphenicol acetyltransferase, increased efflux
Kanamycin	Aminoglycosides	see streptomycin	see streptomycin

[1] **Abbreviations for the resistance against the respective antimicrobial substance used in the analysis of the resistance pattern (see 3.1.5)**

2.2.4 Quantification of the *E. coli* population

For each of the ten different CHROMagar Orientation plates (one without supplementation and nine with single antimicrobial substances added) plated per specimen, colonies with typical dark pink to reddish morphology were counted. To diminish technical variations of the results, colonies were counted for two different serial dilutions plated. The cfu/g sample were calculated for both counted agar plates, and because of the assumed normal distribution of

both values, the mean of both values was used for further statistical analysis. Due to the initial dilution of samples in 1x PBS the minimum detection limit was 300 cfu/g feces or digesta and 100 cfu/g mucosa.

To compare the cfu/g sample values between the two feeding groups, we stratified the quantitative data according to the three different habitats, the three different time points, and the ten different types of agar plates. Additionally the cfu values were compared between different time points. For feces the cfu/g at the age of 24±2 days were compared with the cfu/g at the age of 52±2 days for the 16 animals which were sacrificed at the age of 52±2 days. Cfu/g digesta and cfu/g mucosa were compared between the sampling time points 38±2 days of age and 52±2 days of age for the sacrificed animals.

2.2.5 Quantification of isolates belonging to the family of Enterobacteriaceae

According to the above mentioned protocol for quantification of the *E. coli* numbers, the quantitative data for some other Enterobacteriaceae for example of the genus *Klebsiella, Enterobacter, Citrobacter, Serratia,* and *Pantoea* were investigated. For colonies with a typical metallic blue (±reddish halo) colony appearance, again, the cfu/g was determined for each specimen of digesta and mucosa samples and all ten different CHROMagar Orientation plates. For feces, the amount of *E. coli* was so high in relation to the metallic blue colonies, that it was not possible to count these blue colonies. Therefore, the cfu/g sample values were determined for the digesta and mucosa samples, for the three different time points, and the ten different types of agar plates and compared between the two feeding groups.

2.2.6 Phenotypic resistance screening of the *E. coli* isolates

For the phenotypic resistance screening of 1.610 feces, 550 digesta, and 505 mucosa isolates, a protocol for the phenotypic resistance screening of large numbers of isolates was adopted [178]. Isolates of the -80°C stock were subcultured and incubated on sheep blood agar plates under aerobic conditions at 37°C overnight. Afterwards, material of one single colony was subcultured on LB (Luria/Miller) agar plates supplemented with one of six antimicrobial substances (ampicillin, streptomycin, sulfamethoxazole-trimethoprim, tetracycline, enrofloxacin (concentrations as mentioned above), or kanamycin 64 µg/ml, for further information about the antimicrobial substances used see Tab. 2). As aminoglycosides strongly differ in their chemical structure and there exists no cross-resistance and no lead substance, two different aminoglycosides were used for the screening. After overnight aerobic incubation at 37°C growth of the subcultured colonies was evaluated visually. Isolates which were resistant against antimicrobial substances of at least three different antimicrobial classes were subsequently defined as multi-resistant isolates [180].

2.2.7 Validation of the resistance screening by MIC determination

For multi-resistant isolates the resistance screening method was validated by minimum inhibitory concentration (MIC) determination for ampicillin, sulfamethoxazole-trimethoprim, tetracycline, and enrofloxacin. Therefore, for each habitat a representative random sample of multi-resistant isolates was created based on a representative freedom survey using the website http://epitools.ausvet.com.au/. Sample sizes were calculated with the parameters prevalence 5% and population sensitivity 95% and consequently 56 feces, 50 digesta, and 50 mucosa isolates were investigated.

For MIC determination, the broth microdilution method was performed with Micronaut-S livestock susceptibility plates (Merlin, Bornheim-Hersel, Germany) according to the supplier's protocol. In short, bacteria were suspended in 0.9% sodium chloride pH=6.5 (Carl Roth, Karlsruhe, Germany) to a 0.5 McFarland turbidity standard, transferred in Mueller-Hinton-II broth (Becton, Dickinson and Company, Heidelberg, Germany), inoculated on the test plate, and incubated for 18-24 hours at 37°C. Evaluation was performed visually.

2.2.8 Multi-locus sequence typing of *E. coli* isolates

Those isolates collected at the age of 52±2 days were representatively whole genome sequenced by choosing a random sample. As already described, calculation was based on a representative freedom survey using the website http://epitools.ausvet.com.au/. This time the isolates were stratified according to the three different habitats (feces, digesta, and mucosa), the two different feeding groups (control and zinc group), and their multi-resistance status (not multi-resistant and multi-resistant) for sample size calculation with the parameters prevalence 15% and population sensitivity 95% (Tab. 3).

Table 3: Representative random sample for MLST

Feeding group	Multi-resistance status	Feces		Digesta		Mucosa	
		Initial isolates	Random sample size	Initial isolates	Random sample size	Initial isolates	Random sample size
Control group	Multi-resistant	21	12	9	7	7	6
Control group	Not multi-resistant	129	18	116	18	113	19
Zinc group	Multi-resistant	45	16	42	15	37	15
Zinc group	Not multi-resistant	110	18	97	18	91	18

For DNA isolation of cultures which were incubated overnight at 37°C in LB (Luria/Miller), the MasterPure™ DNA Purification Kit (Epicentre, an Illumina Company, Madison, WI, USA) was

used according to the manufacturer's guidelines for cell samples. Sequencing was performed on an Illumina MiSeq with v3 chemistry (MiSeq Reagent Kit v3, Illumina Inc., San Diego, CA, USA) resulting in 300 bp paired-end reads and an average coverage of 100x. After quality trimming reads were de novo assembled into scaffolds using CLC Genomics Workbench v.9.0 (CLC bio, a Qiagen Company, Aarhus, Denmark).

Multi-locus sequence type (MLST) determination was performed on the whole genome sequences according to the scheme provided by the MLST website (University of Warwick, http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) [181]. Sequences were analyzed by the software package Ridom SeqSphere+ v. 3.1 (Ridom website, http://www.ridom.de/seqsphere/). Sequence types (STs) were computed automatically and for visualization minimum spanning trees were constructed with BioNumerics version 6.6 (Applied Maths, a bioMerieux Company, Sint-Martens-Latem, Belgium).

2.2.9 Statistics

Quantitative *E. coli* data were tested for normal distribution with the Kolmogorov-Smirnov test [182, 183]. As normal distribution could be rejected, for the comparison of the quantitative *E. coli* data (cfu/ g sample) between the two feeding groups and between the two time points 38±2 and 52±2 days of age performed for digesta and mucosa samples, the Mann-Whitney U test [184] was used. Quantitative *E. coli* data for cfu/g feces were compared between the time points 24±2 and 52±2 days of age by a paired-samples sign test [185]. Outliers in the boxplot diagrams are indicated as points and lay 1.5 to 3 lengths of boxes outside of the box. The proportions of resistant and multi-resistant *E. coli* between both feeding groups and over time were compared based on results of the Pearson chi-square-test [186]. For statistical analysis of the MLST data beside the Pearson chi-square-test, the Fisher's exact test [187] was used, as the sample size for some cells of the contingency tables were lower than five. The Pearson chi-square-test [186] was used for the comparison of the proportion of isolates belonging either to ST10 plus ST34 or to the remaining 18 STs. The Fisher's exact test [187] was used for the comparison of the proportion of multi-resistant isolates of ST10 or ST34 between both feeding groups. IBM SPSS Statistics version 20 was used for all statistical analysis and the randomized sampling of the isolates for MIC determination and MLST.

2.3 Conjugation experiments

2.3.1 Bacterial strains

For conjugation experiments two different recipient strains were used. Selection criteria for both strains were a chromosomally encoded resistance which was not encoded on the conjugation plasmid. Without this resistance it would only be possible to detect donor and transconjugant on the agar plate supplemented with the corresponding antimicrobial substance and recipient, donor, and transconjugant on the plates without antimicrobial substances added. In this case, counting of the cfu values of the three different strains is not really possible, especially if one strain grows with noticeable higher cfu values. However, with recipients encoding for an additional resistance it was possible to detect the recipient and the transconjugant on separate agar plates (for further information see 2.3.4 Quantification of the strains). The first recipient used was the sodium azide resistant strain *E. coli* J53. This strain is a derivate of the laboratory *E. coli* K-12 strain and has been widely used as a recipient for conjugation experiments [188]. The sodium azide resistance was acquired by spontaneous mutation [189] and was explained by a single nucleotide substitution in the *secA* gene [190]. As second recipient the enrofloxacin resistant strain *E. coli* IMT30552 was used. This strain was isolated from the colon ascendens digesta of a 34 day-old piglet fed the high zinc diet (2425 mg zinc/kg feed) in our recent zinc pig feeding trial [169]. This strain was previously tested for its phenotypic resistance against eight different antimicrobial substances via agar disk diffusion testing. It was resistant against sulfamethoxazole-trimethoprim, tetracycline, and enrofloxacin and belonged to the sequence type (ST) 641.

As donor strain a sodium azide and enrofloxacin sensitive *K. pneumoniae* strain was used as the different colony morphologies on CHROMagar Orientation plates allowed us to distinguish between the *K. pneumoniae* donor and the *E. coli* recipient and transconjugant. These differentiating characteristics allowed us to determine the donor cfu values without prior subtraction of the transconjugant cfu values (for further information see 2.3.4. Quantification of the strains). Therefore, the ESBL-producing, CTX-M-15 positive strain *K. pneumoniae* VB983816.1 was used as donor. For this strain, self-transferability of the ESBL plasmid was already confirmed with the recipient J53 [191]. Further strain information are listed in table 4.

Table 4: General information about the strains used for conjugation experiments

Strain designation	Species	Host species	Country of origin	Source-lab	Disease	Antimicrobial substance used for cfu determination
J53	*E.coli*	Lab strain	United States of America	RKI Wernigerode		Sodium azide
IMT30552	*E.coli*	Swine	Germany	FU, Institute for Microbiology and Epizootics	None	Enrofloxacin
VB983816.1	*K. pneumoniae*	Horse	Finland	VetMed Labor Ludwigsburg	Wound infection	Cefotaxime, Gentamicin

2.3.2 Animals, Housing and Diet

A zinc mouse feeding trial was performed with 28 germfree male C3H/HeOuJ mice in the Max Rubner Laboratory of the German Institute of Human Nutrition in Potsdam-Rehbrücke. Mice were housed in cages within a sterile Trexler type isolator, originated from the in-house breeding, and were weaned three to four weeks after birth. Afterwards, they were housed in cages, either alone or in pairs, and with approximately six weeks of age their sterilized standard diet (Altromin 1314) (Altromin, Lage, Germany) was changed against the experimental diet. Prior to bacterial association, germfree status was confirmed regularly by gram staining and aerobic and anaerobic incubation of feces samples as previously described [192].

All mice had *ad libitum* access to autoclaved water and to sterilized and pelleted zinc diets. Diets refer to our zinc pig feeding trial (Tab. 1) and a previous zinc pig feeding trial in which an increasing effect of zinc on bacterial conjugation was suggested [169]. As described above, cornstarch of the diet was partially replaced by analytical grade zinc oxide. For the zinc group a final concentration of 1905 mg zinc/kg diet and for the control group a final concentration of 100 mg/kg diet were determined by atomic absorption spectrometry. To confirm differences between both feeding groups, zinc concentrations in the caecum digesta were additionally measured for all mice. An average of 95.3 mg/kg for the zinc group and 11.7 mg/kg for the control group were measured by atomic absorption spectrometry. A negative effect on the health status by feeding this pig adapted diet was not observed. The experimental trial was approved by the Ministry of Rural Development, Environment and Agriculture of the Federal State of Brandenburg (LUVG Brandenburg Reg. Nr. 2347-04-2014).

2.3.3 Experimental setup for *in vivo* mating

The experimental setup was adapted from a different *in vivo* mating protocol [122]. Mice were randomly allocated to four different groups (seven mice per group). Group A and C were fed with the zinc diet, group B and D with the control diet and adaptation to the feed started two weeks prior to the experiment (day -14, experimental setup see Fig. 5). On day zero, 10^8 bacteria of the recipient were applied to each mouse by gastric gavage. Group A and B were associated with the sodium azide resistant recipient *E. coli* J53. Mice of group C and D were associated with the enrofloxacin resistant recipient *E. coli* IMT30552. On day seven, 10^8 bacteria of the ESBL-producing donor *K. pneumoniae* VB983816.1 were applied likewise to all groups. Therefore, group A and B were associated with strain combination I (J53 x VB983816.1) and group C and D with strain combination II (IMT30552 x VB983816.1). Feces were collected daily from day one to 21. On day 20+1 all mice were sacrificed by cervical dislocation and digesta were isolated from the small intestine and caecum. During the ongoing experiment, the association status was controlled by aerobic and anaerobic incubation of the feces samples on sheep blood agar plates. (Oxoid, Wesel, Germany; anaerobic plates see 2.1. Solutions and nutrition media).

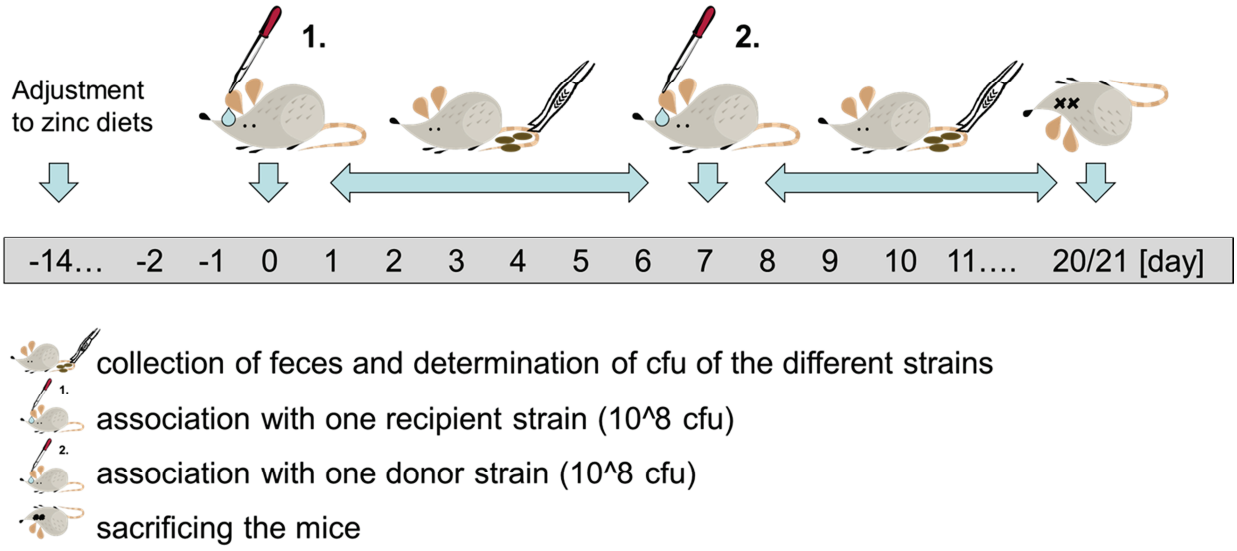

Figure 5: Schematic diagram of the zinc mouse feeding trial
Arrows indicate the performed actions in relation to the timeline. 14 days prior to the first bacterial association and at the age of approximately six weeks, mice were fed with the experimental zinc diets. On day zero and seven mice were associated with the bacterial strains. Feces were collected daily from day one to 21. On day 20+1 all mice were sacrificed and caecum digesta and small intestine digesta were collected in addition to feces.

2.3.4 Quantification of the strains

Feces and digesta samples were dissolved and serially diluted in 1x PBS solution and plated on CHROMagar Orientation plates (CHROMagar, Paris, France), supplemented with different antimicrobial substances (Fig. 6). For each strain, three different serial dilutions

were plated. The cfu/g sample for recipient, donor and transconjugant were determined after 20±2 hours incubation at 37°C under aerobic conditions or after 44±2 hours for sodium azide containing plates respectively.

For group A and B (strain combination I – J53 x VB983816.1), the recipient was detected on sodium azide (100µg/ml), the donor on cefotaxime (4µg/ml) and the transconjugant on sodium azide-cefotaxime (100µg/ml / 4µg/ml) plates. For group C and D (strain combination II – IMT30552 x VB983816.1), the recipient was detected on enrofloxacin (1µg/ml), the donor on cefotaxime (4µg/ml) and the transconjugant on enrofloxacin-cefotaxime-gentamicin (1g/ml / 4µg/ml / 16µg/ml) plates.

Two different plated serial dilutions were counted to diminish technical variations of the results. The cfu/g sample values were calculated for each agar plate. Because of the assumed normal distribution of both values, the mean of both serial dilutions per sample was used for further statistical analysis. Due to the initial solution of samples in 1x PBS, the minimum detection limit was 100 cfu/g sample.

Transconjugants grew also on recipient and donor plates. Different colony characteristics on CHROMagar Orientation plates allowed us to distinguish between donor and transconjugant on the donor plates (*K. pneumoniae* donor with metallic blue colonies and *E. coli* transconjugant with dark pink to reddish colonies). Therefore, the cfu values of the donor could be counted directly. However, for cfu values of the recipient, cfu values of the transconjugant had to be subtracted from the cfu values counted for recipient plates.

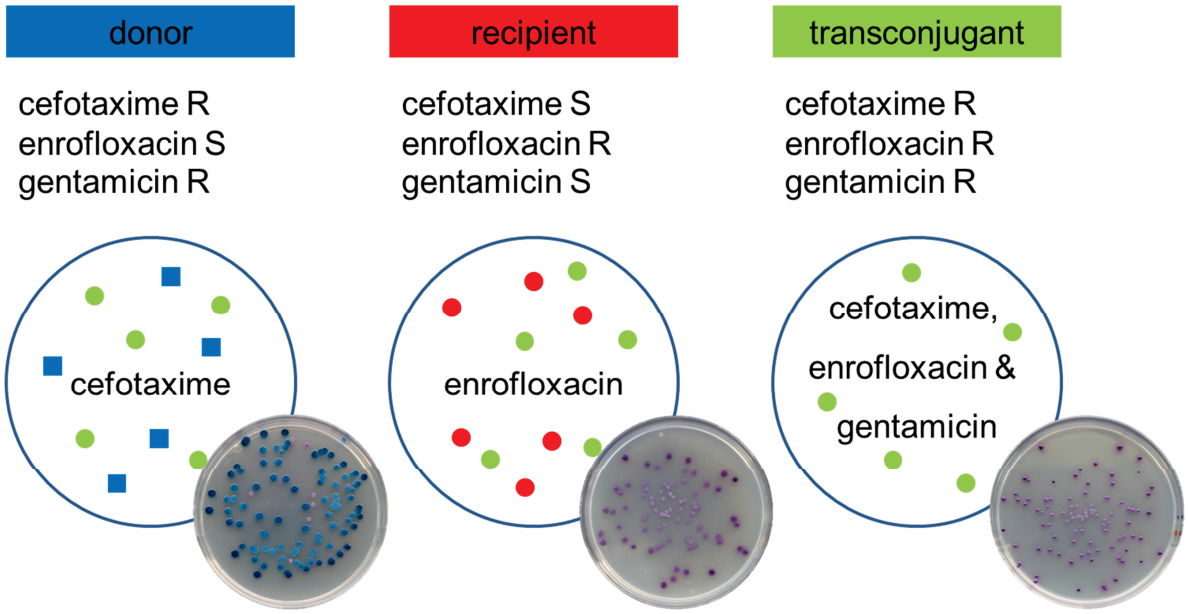

Figure 6: Plating model used for the determination of the cfu values for strain combination II (IMT30552 x VB983816.1)
CHROMagar orientation plates were supplemented with different antimicrobial substances (indicated in the plates) to allow the differentiation of strains. Antimicrobial properties of the strains used for the screening, schematic, and real plates are indicated. R: resistant against the antimicrobial substance; S: sensitive against the antimicrobial substance; blue rectangles: blue colonies of the *K. pneumoniae* donor; red circles: dark pink to reddish colonies of the *E. coli* recipient; green circles: dark pink to reddish colonies of the *E. coli* transconjugant.

2.3.5 *In vitro* mating

For *in vitro* mating, the same two strain combinations as already utilized *in vivo* were used for mating in liquid media. For the entire *in vitro* mating procedure, LB (Lennox) (Carl Roth, Karlsruhe, Germany) was used instead of LB (Luria/Miller). LB (Lennox) contained only 5 g/l sodium chloride instead of 10 g/l and was therefore more suitable to dissolve zinc chloride (Sigma-Aldrich Chemie, Taufkirchen, Germany).

In five independent replicates, recipient and donor were grown separately overnight in LB media, diluted with fresh LB to an optical density at 600nm (OD_{600}) of 0.05, and incubated at 37°C and 200rpm to an OD_{600} of 0.6. Initial cfu/ml for recipient and donor were determined as described above and for mating 1.5ml of recipient and 1.5 ml of donor were added to 7ml LB media containing one of four different zinc concentrations. Zinc was added as zinc chloride in form of a 164.5 mg zinc/l medium containing LB stock (concentration was determined by AAS) resulting in the final concentrations: A 1.1 mg/l; B 7.7 mg/l; C 76.8 mg/l and D 115.1 mg zinc/l medium. Conjugation media were incubated at 37°C statically and after 1.5 and 24 hours, cfu values for recipient, donor and transconjugant were determined. The same plating design as described above was used, beside that, for the transconjugant of strain combination I (J53 x VB983816.1), sodium azide-cefotaxime-gentamicin (100µg/ml / 4µg/ml /

16µg/ml) plates were used. The minimum detection limit for *in vitro* mating was 10 cfu/ml medium.

2.3.6 Verification of transconjugants

For verification of transconjugants, colonies of transconjugant plates were randomly chosen, subcultured on CHROMagar Orientation plates supplemented with cefotaxime (4µg/ml), incubated at 37°C overnight and growth was evaluated visually. Typical dark pink to reddish colonies indicated *E. coli* colonies and growth on cefotaxime plates indicated a plasmid transfer.

2.3.7 Statistics

For statistical analysis of the feces samples from the zinc mouse feeding trial, two different approaches were chosen for the evaluation of zinc influence the bacterial conjugation. First, the cfu values for the different strains were compared between the two feeding groups. Therefore, the logarithm to the basis ten (lg) of cfu values was used. In case the cfu equals zero, the cfu was artificially changed to cfu equals one so that the lg (cfu/g feces) value equals zero. To integrate cfu data over the range of sampling time points, the area under the curve (AUC) value for each mouse and strain was calculated. For the AUC approach, only data from day eight to 20 were used. As each strain represents a different population, differences between both feeding groups were investigated for each strain separately. Normal distribution of AUC values were evaluated based on results of the Kolmogorov-Smirnov test [182, 183] and could not be assumed for all AUC values of all three strains of both strain combinations. Therefore, AUC values for each strain were compared between the two feeding groups based on results of the Mann-Whitney U test [184].
In the second approach, the conjugation rates were compared between the two feeding groups. Therefore, the conjugation rate cfu transconjugant/cfu recipient (recipient conjugation rate) and the conjugation rate cfu transconjugant/cfu donor (donor conjugation rate) were calculated. To integrate values of all different time points, again, the AUC value per conjugation rate was calculated for each mouse. The AUC values were again compared between the two feeding groups with the Mann-Whitney U test [184].
With the analysis of the data generated on the day of sacrificing, the influence of the integrative AUC approach was filtered out. Again, both already described approaches were used and therefore the lg transformed cfu values for each strain and the calculated conjugation rates were compared between both feeding groups with the Mann-Whitney U test [184] for all three samples (feces, caecum digesta, and small intestine digesta). As already described, in case the cfu equals zero the cfu was artificially changed to cfu equals one so that the lg (cfu/g sample) value equals zero. Beside this specification, it was defined

that in cases that a recipient cfu values equals zero or that the calculated conjugation rate was greater than one, the corresponding conjugation rate was defined as one.

For the *in vitro* conjugation experiments, only the approach with conjugation rates was chosen. Both conjugation rates were calculated and normal distribution could be rejected with the Kolmogorov-Smirnov test [182, 183]. Therefore, the Kruskal-Wallis test [193] was used to analyze if there existed a central tendency that the different zinc concentrations influenced the conjugation rates. In significant cases, Dunn-Bonferroni [194] post hoc method was used to identify significant differences between defined zinc groups by pairwise comparisons.

AUC values were calculated with NCSS Statistical Software version 9 whereas IBM SPSS Statistics version 20 was used for all statistical analysis. Outliers in boxplot diagrams are indicated by points and asterisks. Values which are indicated as points lay 1.5 to 3 lengths of boxes outside of the box and values indicated as asterisks lay more than 3 lengths of boxes outside the box.

3 Results

3.1 Zinc pig feeding trial

3.1.1 Quantitative analysis of the *E. coli* population

For the three different time points investigated (24±2, 38±2, 52±2 days of age) and the three different habitats (feces, digesta, and mucosa), the absolute numbers of *E. coli* were determined. The cfu/g sample values varied for feces from approximately 10^4 to 10^9, for digesta from 10^4 to 10^8, and for mucosa from 10^3 to 10^7 (Fig. 7). For the absolute numbers of *E. coli*, grown on non-selective CHROMagar Orientation plates for all time points and habitats, no significant difference between the control and the zinc group was detectable. However, a substantial decrease of the *E. coli* population was detected after weaning in all three habitats (feces paired-samples sign test; p=0.001, digesta Mann-Whitney U test; p=0.001, mucosa Mann-Whitney U test; p=0.012).

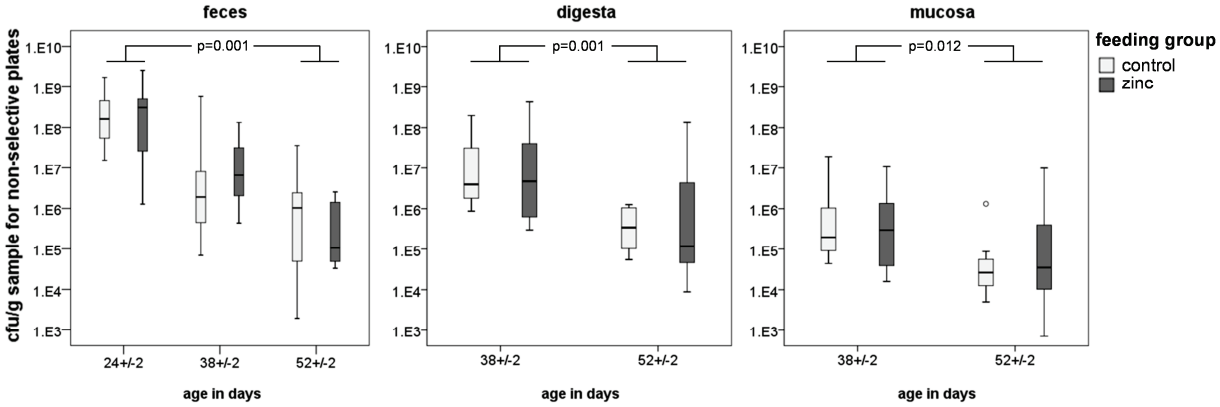

Figure 7: Absolute numbers of the porcine *E. coli* population of the zinc pig feeding trial
Data are displayed in association to intestinal habitat (feces, digesta, mucosa) and age of piglets (24±2, 38±2, 52±2 days of age). Colonies were counted on CHROMagar Orientation plates and the cfu/g values per specimen were calculated. Significant differences could only be detected between different time points and are indicated by p values. Outliers are displayed separately.

For selective agar plates supplemented with one of nine antimicrobial substances, *E. coli* colonies could be detected on ampicillin, streptomycin, nalidixic acid, sulfamethoxazole-trimethoprim, tetracycline, and enrofloxacin containing plates (Tab. 5). However, not a single *E. coli* colony could be detected for all samples on cefotaxime, gentamicin, and chloramphenicol containing plates. Overall, for all habitats and samples, we observed most frequent growth on ampicillin plates, followed by tetracycline and sulfamethoxazole-trimethoprim containing plates (n=144 samples, 85% positive for ampicillin resistant isolates, 72% for tetracycline, 66% for sulfamethoxazole-trimethoprim, 37% for nalidixic acid, 26% for enrofloxacin, and 25% for streptomycin). Regarding differences between the feeding groups across the three different time points, the three different habitats and the nine different

cultivation media, no significant differences between numbers of *E. coli* (cfu/g sample) were detectable between the control and the zinc feeding group.

Table 5: Quantitative data of the *E. coli* population grown on selective agar plates of the zinc pig feeding trial

	Antimicrobial substance of the agar plate	Median lg (cfu/g sample) (percentage of samples with growing colonies[1])					
		24±2 control[2]	24±2 zinc[2]	38±2 control[2]	38±2 zinc[2]	52±2 control[2]	52±2 zinc[2]
Feces	Ampicillin	5.4 (100%)	5.6 (100%)	4.5 (94%)	5.2 (88%)	3.0 (63%)	1.2 (50%)
	Streptomycin	0 (25%)	0 (44%)	0 (13%)	0 (31%)	0 (0%)	0 (0%)
	Nalidixic acid	4.1 (69%)	4.6 (100%)	0 (13%)	0 (44%)	0 (13%)	0 (13%)
	Sulfamethoxazole-Trimethoprim	6.0 (100%)	6.3 (100%)	3.0 (56%)	3.6 (81%)	0 (25%)	0 (38%)
	Tetracycline	4.4 (100%)	4.5 (100%)	3.5 (88%)	3.7 (69%)	2.9 (63%)	1.2 (50%)
	Enrofloxacin	3.6 (56%)	4.5 (81%)	0 (0%)	0 (19%)	0 (0%)	0 (0%)
Digesta	Ampicillin			5.5 (100%)	6.0 (100%)	3.5 (75%)	2.8 (63%)
	Streptomycin			3.5 (63%)	3.4 (63%)	0 (0%)	0 (0%)
	Nalidixic acid			0 (38%)	1.7 (50%)	0 (13%)	0 (0%)
	Sulfamethoxazole-Trimethoprim			4.0 (75%)	5.3 (75%)	0 (38%)	1.2 (50%)
	Tetracycline			3.5 (100%)	4.2 (75%)	0 (25%)	0 (38%)
	Enrofloxacin			0 (25%)	3.5 (63%)	0 (0%)	0 (0%)
Mucosa	Ampicillin			4.2 (88%)	4.6 (100%)	2.3 (88%)	1.2 (50%)
	Streptomycin			1.4 (50%)	1.6 (50%)	0 (0%)	0 (0%)
	Nalidixic acid			0 (38%)	1.0 (50%)	0 (0%)	0 (0%)
	Sulfamethoxazole-Trimethoprim			3.4 (75%)	3.8 (88%)	0 (25%)	0 (25%)
	Tetracycline			2.3 (75%)	3.6 (75%)	0 (38%)	0 (38%)
	Enrofloxacin			0 (25%)	1.0 (50%)	0 (0%)	0 (0%)

The median of the lg (cfu/g sample) values and the percentage of samples in which colonies were detectable on the respective agar plate are displayed. Data are stratified according to intestinal habitat (feces, digesta, mucosa), age of piglets (24±2, 38±2, 52±2 days of age), feeding group (control, zinc) and the different cultivation media.

[1] for feces at 24±2 and 38±2 days of age 16 samples per feeding group existed, for all other samples eight samples per feeding group existed /[2] age of piglets in days and feeding group

3.1.2 Quantitative analysis of isolates belonging to the family of Enterobacteriaceae

Besides the dark pink to reddish *E. coli* colonies, also the typical metallic blue (±reddish halo) colonies representing colonies of the family Enterobacteriaceae as for example of the genus *Klebsiella, Enterobacter, Citrobacter, Serratia,* and *Pantoea* were counted for cfu determination (Fig. 8). For feces plated on non-selective agar plates, the percentage of these metallic blue colonies in relation to the *E. coli* colonies was too low to count. In serial dilutions, which were adequate to count blue colonies, plates were overgrown by *E. coli* colonies and therefore the colony sizes were so small that we were not able to distinguish between the bigger desired metallic blue colonies and the small turquoise blue colonies which represent isolates of the genus *Enterococcus*. Therefore, only the data for digesta and mucosa were investigated whereby, also two digesta and three mucosa samples were excluded from the dataset because of the mentioned fact.

Like for the quantitative *E. coli* data for digesta and mucosa, no significant difference could be detected between the feeding groups, but a decrease of the cfu/g values over time was identified (Mann-Whitney U test; digesta p=0.001; mucosa p=0.004).

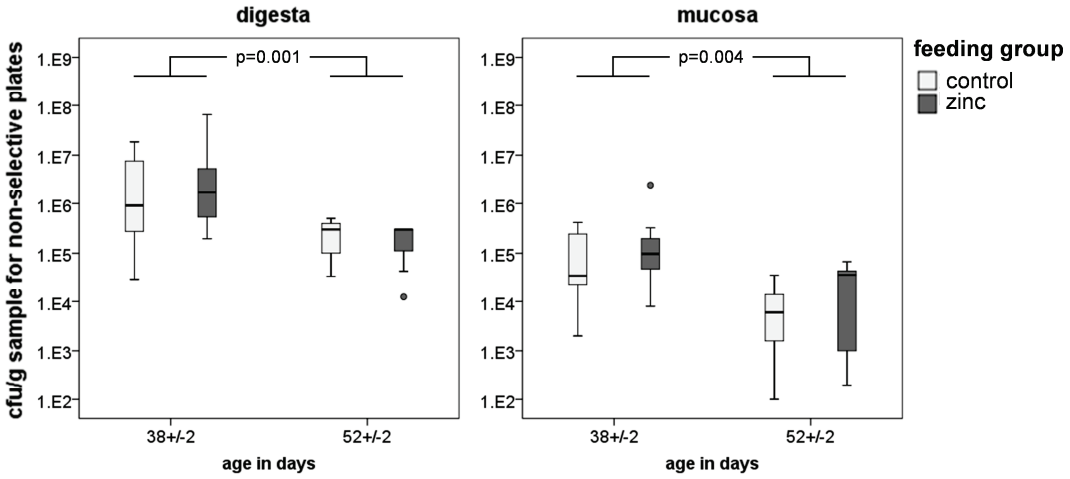

Figure 8: Quantitative data of colonies of the family Enterobacteriaceae as for example of the genus *Klebsiella, Enterobacter, Citrobacter, Serratia,* and *Pantoea* of the zinc pig feeding trial
Data are shown for digesta and mucosa at 38±2 and 52±2 days of age. Colonies were counted on CHROMagar Orientation plates and the cfu/g values per specimen were calculated. Significant differences could only be detected between the different time points and are indicated by p values. Outliers are displayed separately.

3.1.3 Isolation of *E. coli* and screening for antimicrobial resistance

Up to 22 *E. coli* colonies were isolated for each sample from different agar plates and stored for phenotypic resistance screening. In total, 1.610 feces, 550 digesta, and 505 mucosa isolates, which were distributed equally between the two feeding groups, were screened for their phenotypic resistance against six antimicrobial substances from five different antimicrobial classes.

Regarding the first analysis, isolates were grouped in two categories, (I) isolates sensitive against all tested antimicrobial substances and (II) isolates resistant against at least one antimicrobial substance tested. For these categories significant differences between the two feeding groups could be detected for isolates from feces and digesta. For feces, 66.8% of all isolates from the zinc group were resistant and only 60.4% of the control group. With regard to the three different time points tested, only at the age of 38±2 days a significant difference was detectable between zinc and control group. In the zinc group 69% resistant isolates were detected whereas 57.6% in the control group (chi-square-test; $p=0.004$).

Regarding digesta, 57.1% of all isolates of the zinc group and 44.1% of the control group were resistant against at least one antimicrobial substance (chi-square-test; $p=0.002$). With respect to the different time points, a significant difference was detectable at the age of 38±2 days with 78% resistant isolates in the zinc group and 63.5% in the control group (chi-square-test; $p=0.007$) and at the age of 52±2 days with 36% resistant isolates in the zinc group and 21.6% in the control group (chi-square-test; $p=0.01$). For mucosa, no significant differences could be detected between the two feeding groups and 53.4% of isolates from the zinc group and 47.6% of the control group were resistant. Overall, the proportion of isolates which were resistant against at least one antimicrobial substance decreased for all different habitats over time (feces, digesta, and mucosa: chi-square-test, $p<0.001$).

In the second analysis, the influence of high dietary zinc feeding on the proportion of multi-resistant isolates was investigated. In contrast to the analyses regarding isolates resistant against at least one antimicrobial substance, these analyses revealed a strong increasing effect of high dietary zinc feeding on the proportion of multi-resistant isolates in all three tested habitats at the age of 52±2 days (Fig. 9). For feces isolates, the proportion of multi-resistant isolates did not differ between the two feeding groups directly before weaning (24±2 days of age) and at the age of 38±2 days (chi-square-test; 24±2 days of age $p=0.579$; 38±2 days of age $p=0.254$). However, at the age of 52±2 days a highly significant difference with 29% multi-resistant isolates in the zinc group and only 14% multi-resistant isolates in the control group was detectable (chi-square-test; $p=0.001$).

For digesta isolates, comparable results were observed at the age of 52±2 days where 30.2% multi-resistant isolates were detected in the zinc group and only 7.2% in the control group (chi-square-test; $p<0.001$). Interestingly, at the age of 38±2 days also a significant difference between the feeding groups could be detected but this time the proportion of multi-resistant isolates was higher in the control group (36.2% multi-resistant isolates in the zinc group and 49.7% in the control group, chi-square-test; $p=0.021$).

Finally, also for mucosa isolates similar results could be detected. At the age of 38±2 days no significant differences between the feeding groups were observed (chi-square-test; $p=0.695$).

However, at the age of 52±2 days, a highly significant difference occurred (28.9% multi-resistant isolates in the zinc group and 5.8% in the control group, chi-square-test; $p<0.001$).
In general, in the control group the proportion of multi-resistant isolates diminished in all habitats after 38±2 days of age, whereas in the zinc group, multi-resistant isolates maintained at a higher proportion.

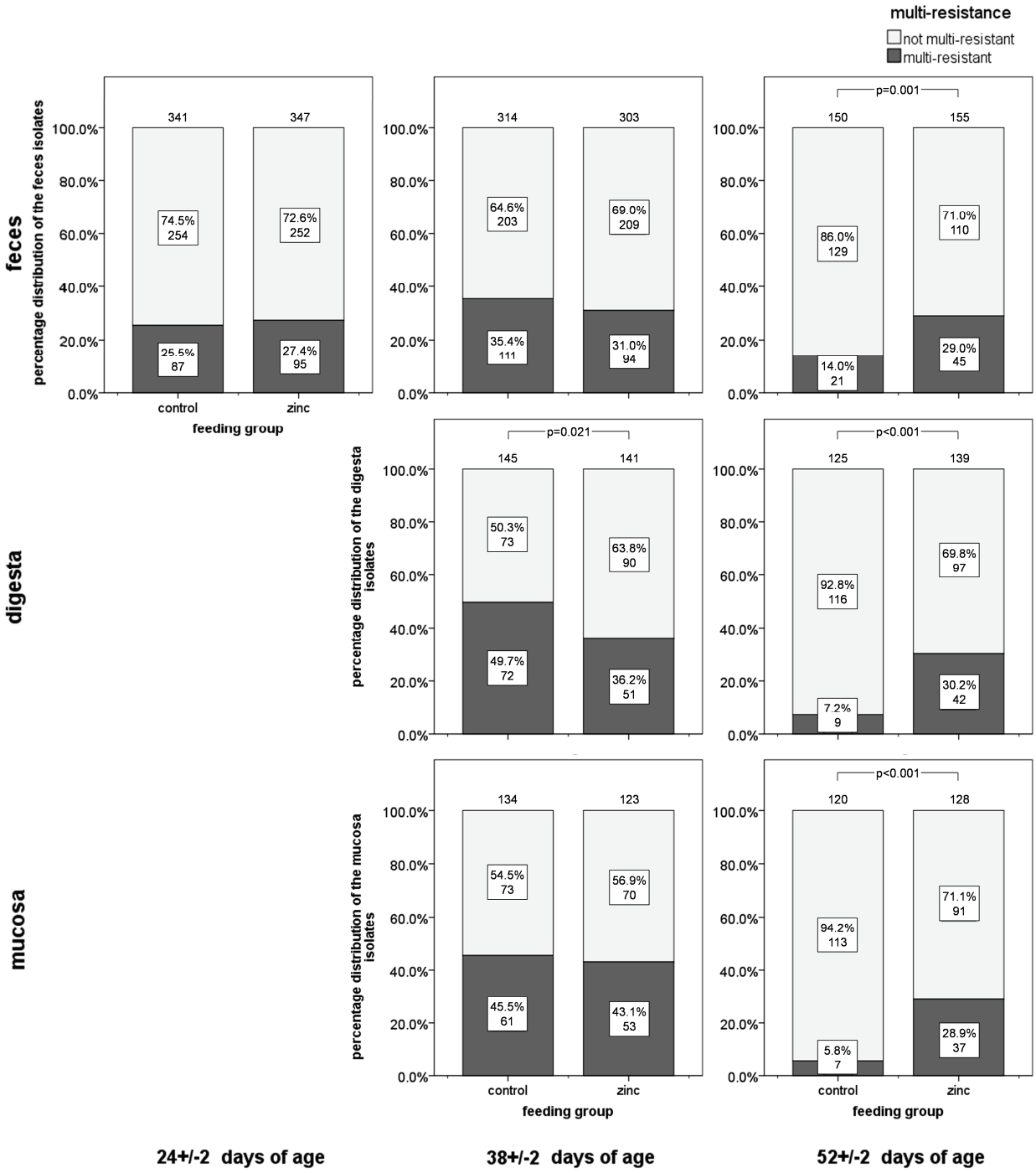

Figure 9: Comparison of the proportion of multi-resistant porcine *E. coli* isolates between the two feeding groups

Piglets of the zinc feeding trial were weaned at 25±1 days of age and data are displayed for feces, digesta, and mucosa isolates collected at the three different sampling time points (24±2, 38±2, 52±2 days of age). Six different antimicrobial substances were used for phenotypic resistance determination and isolates which were resistant against at least three different antimicrobial classes were defined as multi-resistant. Numbers indicate the number of investigated isolates and significant differences are indicated by p values.

3.1.4 Validation of the screening by MIC determination

To validate the phenotypic antimicrobial resistance screening, a standard method was used. MIC determination of ampicillin, sulfamethoxazole-trimethoprim, tetracycline, and enrofloxacin was performed for stratified random samples of the multi-resistant isolates of the three different habitats. Sample size calculation was performed by the website http://epitools.ausvet.com.au/ with the parameters prevalence 5% and population sensitivity 95%. Overall, 56 out of 453 feces, 50/174 digesta, and 50/158 mucosa multi-resistant isolates were investigated with the broth microdilution method. In general, results of the MIC determination confirmed the results of the phenotypic screening as, with the exception of two single mucosa isolates, the results matched the results of the screening. For one mucosa isolate, the MIC value differed for sulfamethoxazole-trimethoprim as it was resistant against 76 µg/ml / 4 µg/ml in the screening assay and showed a MIC value of ≤4.75 µg/ml / 0.25 µg/ml. The other isolate was in the screening assay resistant against tetracycline 16 µg/ml but had only a MIC value of tetracycline ≤1 µg/ml.

3.1.5 Resistance pattern

To get a closer look into and stratify between different multi-resistant populations, all multi-resistant isolates of the antimicrobial resistance screening (453 feces, 174 digesta, and 158 mucosa isolates) were grouped according to their resistance patterns. Analyses of the patterns were stratified according to the different habitats and sampling time points. In general, three main resistance patterns differing in their proportions in each habitat and over time were observed (Fig. 10). Pattern I stands for ampicillin, streptomycin, sulfamethoxazole-trimethoprim, and tetracycline (AmpStrepSul/TriTet), pattern II for streptomycin, sulfamethoxazole-trimethoprim, and tetracycline (StrepSul/TriTet), and pattern III for ampicillin, streptomycin, and tetracycline (AmpStrepTet) resistance.

For the habitat feces at the age of 24±2 days, the three main different resistance patterns did not differ in their proportion between the zinc and the control group. At the age of 38±2 days, the resistance pattern StrepSul/TriTet slightly increased in the zinc group compared to the control group, but the resistance patterns were again nearly identical. However, at the age of 52±2 days, remarkable differences in the resistance pattern were detectable between the two feeding groups. Whereas the resistance pattern of the control group resembled the pattern of the control and zinc group at the age of 38±2 days, in the zinc group, the resistance patterns AmpStrepSul/TriTet and StrepSul/TriTet reached a higher proportion and pattern AmpStrepTet decreased. Especially pattern StrepSul/TriTet differed strongly between both feeding groups (31.1% in the zinc group and 4.8% in the control group).

For the multi-resistant digesta isolates, a partly comparable change in the distribution pattern was detected. Like for the feces isolates at the age of 38±2 days, the resistant patterns of

both feeding groups looked nearly identical, but at the age of 52±2 days, a clear difference between both groups could be detected. The proportion of pattern StrepSul/TriTet was again increased in the zinc group compared to the control group (31% in the zinc group and 11.1% in the control group). However, different to the feces patterns, this time pattern AmpStrepSul/TriTet was less abundant in the zinc group (7.1% of the isolates vs. 44.4% of the isolates in the control group).

For the multi-resistant mucosa isolates on both time points the distribution of the different resistance patterns looked very similar for both feeding groups. AmpStrepTet was the dominant pattern. Resistance pattern AmpStrepSul/TriTet was the second dominant pattern at the age of 38±2 days whereas at the age of 52±2 days only resistance pattern StrepSul/TriTet coexisted beside the dominant pattern AmpStrepTet.

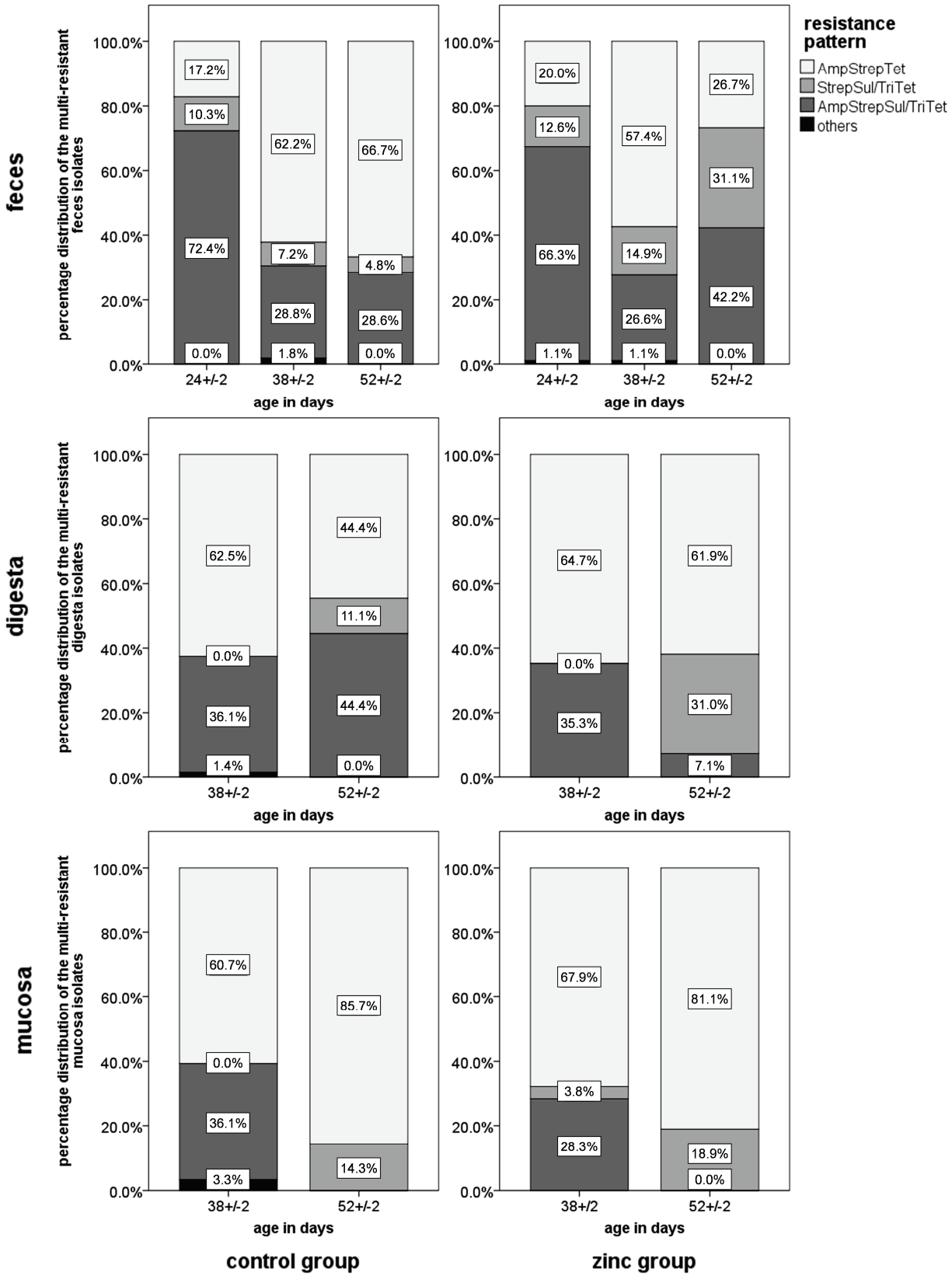

Figure 10: Distribution of the phenotypic resistance patterns of the 453 feces, 174 digesta and 158 mucosa multi-resistant *E. coli* isolates of the zinc pig feeding trial
Isolates which were resistant against at least three different antimicrobial classes were defined as multi-resistant. The distribution of the resistance pattern was analyzed separate for the three different habitats (feces, digesta, mucosa) and three sampling time points (24±2, 38±2, 52±2 days of age). Details on differences are given in the text.

3.1.6 MLST

As at the age of 52±2 days the effect on high dietary zinc feeding on multi-resistance was detectable, representative random samples for this time point with overall 180 isolates were analyzed by whole genome sequencing and MLST. Sample sizes were calculated with the parameters prevalence 15% and population sensitivity 95% and stratified according to the three different habitats (feces, digesta, and mucosa), the two different feeding groups (control and zinc group), and the multi-resistance status (not multi-resistant and multi-resistant) (Tab. 3).

The 180 isolates were assigned to a total of 20 different STs, but most isolates belonged to ST10 and ST34. Isolates of the different STs were found in two or three habitats indicating no association of certain STs with one habitat (Fig. 11 A). Interestingly, when looking at the multi-resistance status of the isolates (Fig. 11 B), with the exception of three isolates of ST1308 and ST1607, all 68 remaining multi-resistant isolates belonged to ST10 and ST34 only.

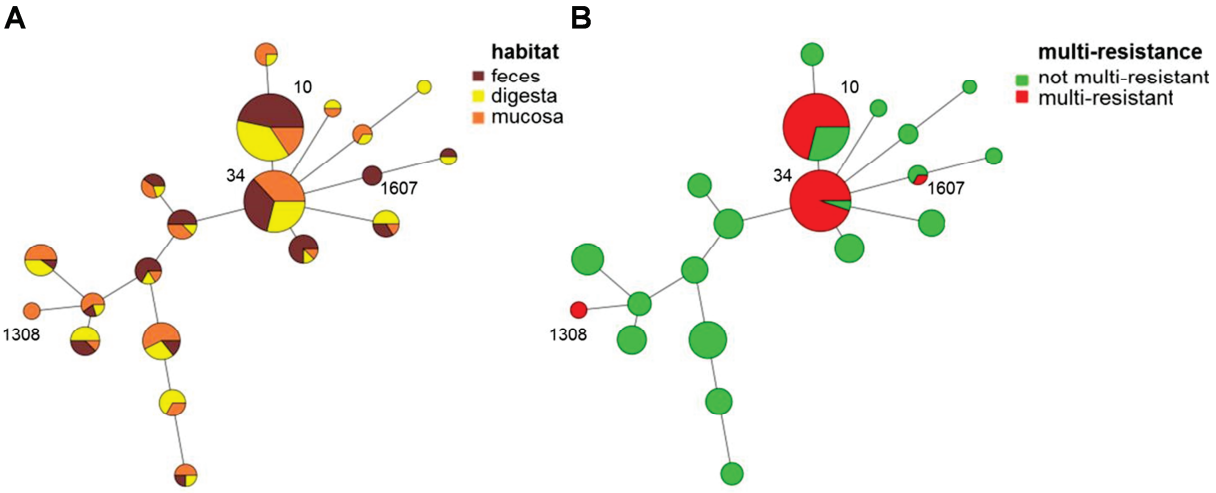

Figure 11: Minimum spanning trees for the 20 STs reflecting the 180 porcine isolates including random samples
For isolates of the zinc pig feeding trial collected at the age of 52±2 days random samples stratified according to the three different habitats (feces, digesta, and mucosa), the two different feeding groups (control and zinc group), and their multi-resistance status (not multi-resistant and multi-resistant) were whole genome sequenced and the ST was determined. Distribution of the isolates according to the different habitats (A) and the multi-resistance status (B). Circles represent different STs, circle sizes the amount of assigned isolates, and numbers indicate the ST designation.

Therefore, for ST10 and ST34, also the distribution of isolates according to multi-resistance status and feeding group was investigated for the three different habitats (Fig. 12). In ST10 of all three habitats, multi-resistant as well as not multi-resistant isolates could be detected. For ST34, this was only the case for feces isolates as all digesta and mucosa isolates assigned to this ST were multi-resistant.

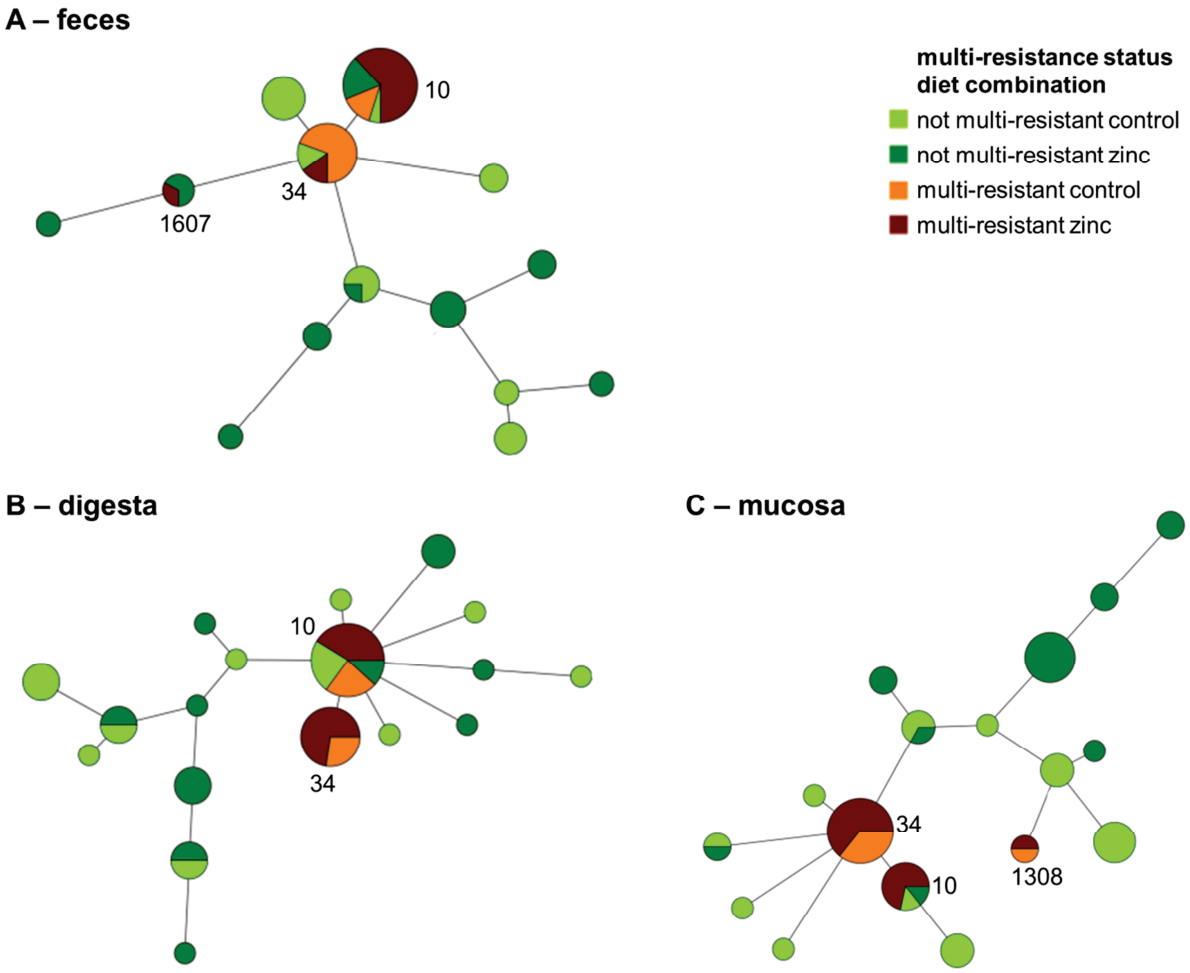

Figure 12: Minimum spanning trees for the STs of the 64 feces, 58 digesta, and 58 mucosa isolates
For isolates of the zinc pig feeding trial collected at the age of 52±2 days random samples stratified according to the three different habitats (feces, digesta, and mucosa), the two different feeding groups (control and zinc group), and their multi-resistance status (not multi-resistant and multi-resistant) were whole genome sequenced and the ST was determined. Distribution of the isolates according to multi-resistance status and feeding group for feces (A), digesta (B), and mucosa (C) isolates. Circles represent different STs, circle sizes the amount of assigned isolates, and numbers indicate the ST designation.

In the first analysis, the proportions of multi-resistant isolates were compared separately for ST10 and ST34 between both feeding groups of all three habitats, to investigate, if under high dietary zinc feeding, a higher proportion of multi-resistant isolates could be detected in one ST. For both STs of the three habitats, no significant differences between the feeding groups were detected.

In the second analysis, the proportion of isolates belonging to the multi-resistance associated STs (ST10 plus ST34) was investigated for each habitat (Fig. 13). Again, no significant differences could be detected between both feeding groups.

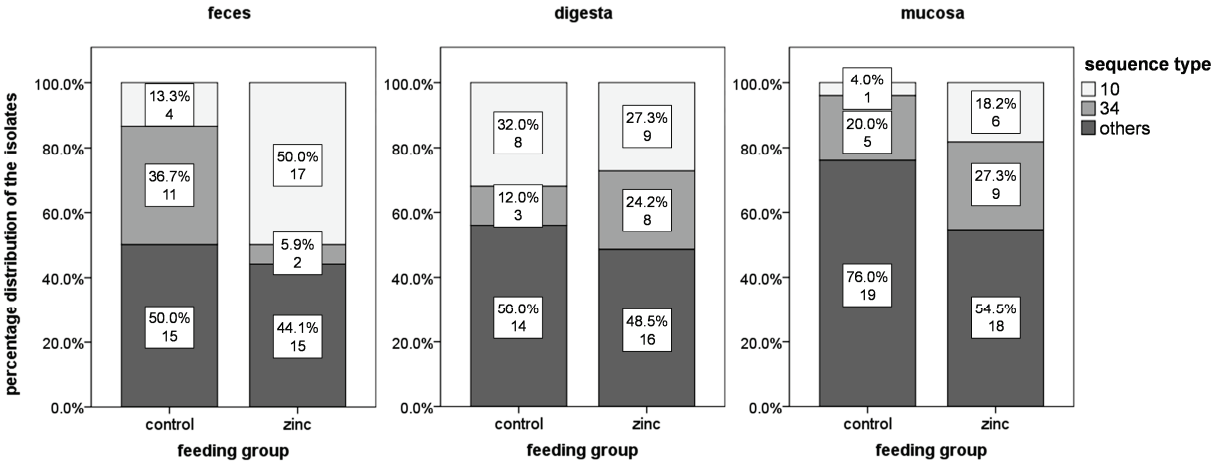

Figure 13: Distribution of the 64 feces, 58 digesta, and 58 mucosa porcine isolates of both feeding groups according to their sequence type.
For isolates of the zinc pig feeding trial collected at the age of 52±2 days random samples stratified according to the three different habitats (feces, digesta, and mucosa), the two different feeding groups (control and zinc group), and their multi-resistance status (not multi-resistant and multi-resistant) were whole genome sequenced and the ST was determined. The proportion of the multi-resistance associated STs (ST10 plus ST34) were not influenced by high dietary zinc feeding.

3.2 Conjugation experiments

3.2.1 *In vivo* colonization and gene transfer frequencies for feces of strain combination I (J53 x VB983816.1)

The recipient, donor and transconjugant successfully colonized the intestine of previously germ-free mice (Fig. 14). The recipient slightly decreased after the association with the donor. The lg (cfu/g feces) values ranged from 6.4 to 9.6 between day eight and day 20. One day after its association, the donor colonized at the highest levels with lg (cfu/g feces) values ranging from 9.3 to 10.3. Transconjugants could already be detected one day after association with the donor and lg (cfu/g feces) values ranged from 4.0 to 7.4. In general, after association with the donor and the appearance of the transconjugants, the three strains formed a stable equilibrium. However, for the recipient and particularly the transconjugant, the cfu values were highly variable.

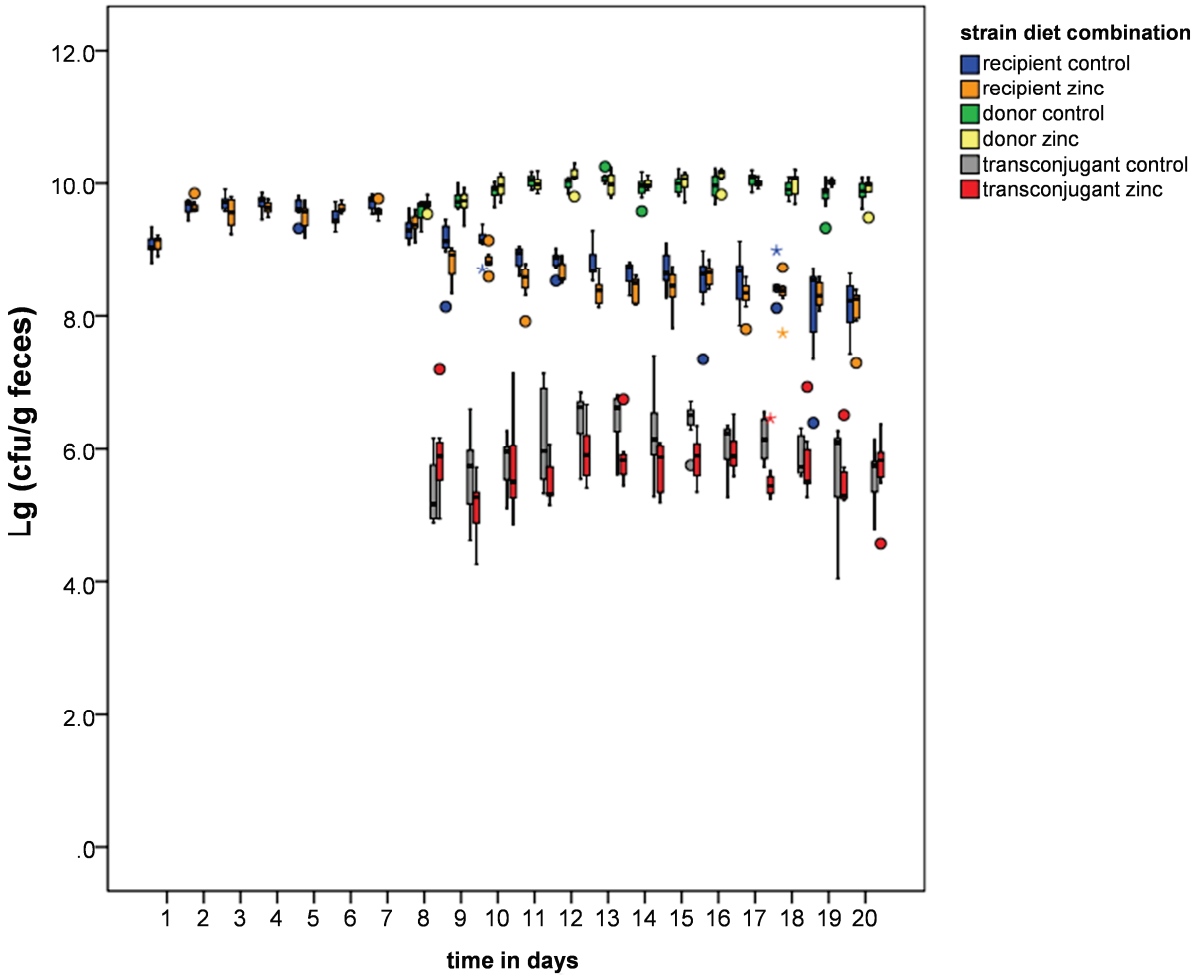

Figure 14: Lg (cfu/g feces) values for recipient, donor, and transconjugant of strain combination I (J53 x VB983816.1) of the zinc mouse feeding trial
On day zero mice were associated with the recipient *E. coli* strain and on day seven with the donor *K. pneumoniae* strain. Data are separated according to the feeding group and are displayed for day one to 20. Colonies were counted on strain differentiating cultivation media and outliers are displayed separately.

To integrate the lg (cfu/g feces) data collected from day eight to 20 and to perform statistical analysis, the AUC values were calculated (Tab. 6). In accordance with the lg (cfu/g feces) values, the AUC values were the highest for the donor, followed by the recipient and the transconjugant. For recipient and donor, no significant effect of high dietary zinc feeding could be detected as for both strains, no significant differences of the AUC values were detectable between both feeding groups. However, AUC values of the transconjugants were significantly higher for the mice of the control group (Mann-Whitney U test; p=0.025).

To evaluate the data in more detail, the recipient conjugation rate (cfu transconjugant/cfu recipient) and the donor conjugation rate (cfu transconjugant/cfu donor) were calculated for each mouse (Fig. 15). As the recipient reached lower cfu values than the donor, the recipient conjugation rate reached higher values than the donor conjugation rate. Recipient conjugation rates ranged from $1.7*10^{-5}$ to $4.7*10^{-2}$ and donor conjugation rates from $4.5*10^{-6}$ to $2.8*10^{-3}$. In general, both conjugation rates were highly variable and multiple outliers could be detected.

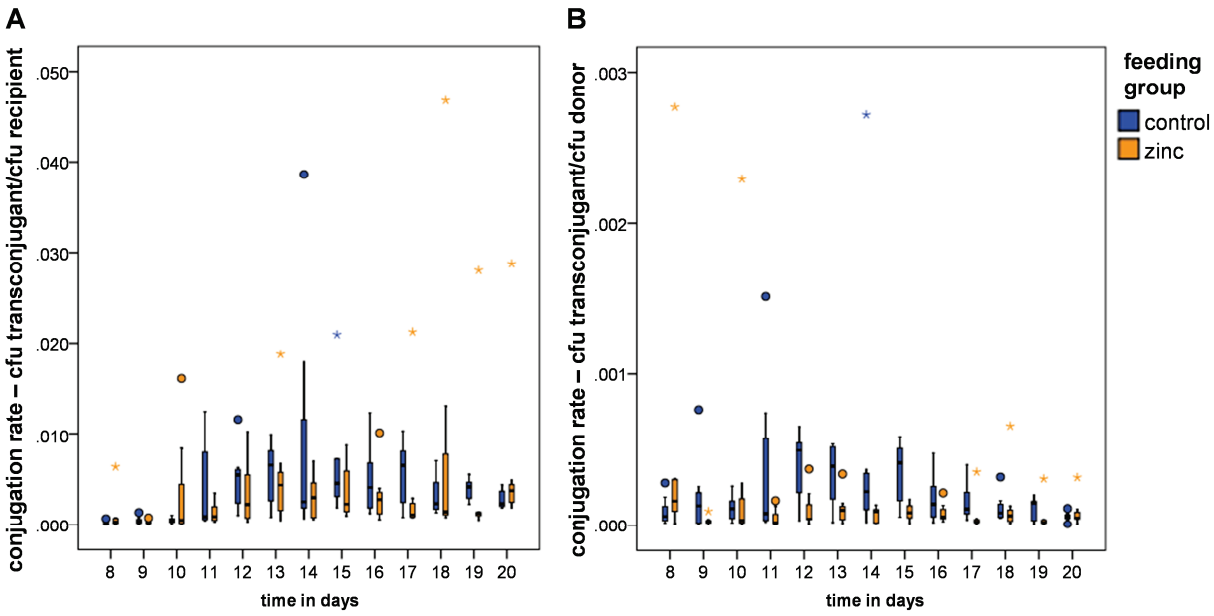

Figure 15: Recipient conjugation rate (cfu transconjugant/cfu recipient – A) and donor conjugation rate (cfu transconjugant/cfu donor – B) for feces of strain combination I (J53 x VB983816.1) of the zinc mouse feeding trial
Data are separated according to the feeding group and are displayed for day eight to 20. Outliers are displayed separately

To integrate the different time points, AUC values of both conjugation rates were calculated for each mouse (Tab. 6). For the AUC of the recipient conjugation rate, no significant difference could be detected between control and zinc group. However, for the AUC of the donor rate, a significant difference was detected with higher conjugation rates in the control group (Mann-Whitney U test; p=0.035).

Table 6: Data of the calculated AUC values for recipient, donor, transconjugant, recipient conjugation rate, and donor conjugation rate for feces of strain combination I (J53 x VB983816.1) of the zinc mouse feeding trial

	Control group		Zinc group	
Strain/conjugation rate	AUC minimum – maximum	AUC median	AUC minimum – maximum	AUC median
Recipient	100.2 – 108.1	104.0	100.6 – 104.1	101.9
Donor	117.4 – 120.4	119.1	118.2 – 121.2	119.6
Transconjugant*	68.0 – 75.4	71.6	65.9 – 73.6	67.1
Recipient conjugation rate	0.017 – 0.103	0.042	0.011 – 0.159	0.024
Donor conjugation rate*	0.001 – 0.005	0.004	0.001 – 0.003	0.001

Minimum, maximum, and median values are displayed for both feeding groups. Significant differences between both feeding groups could be detected for AUC values of the transconjugant and of the donor conjugation rate and are displayed by underlined description and asterisked.
*** significant differences – Mann-Whitney U test; AUC transconjugant p=0.025; AUC donor conjugation rate p=0.035**

3.2.2 *In vivo* colonization and gene transfer frequencies for feces of strain combination II (IMT30552 x VB983816.1)

For strain combination II (Fig. 16), again, all three strains colonized very well. Like for strain combination I, the recipient diminished after donor application and the donor colonized at the highest levels. Lg (cfu/g feces) values ranged from 7.3 to 9.6 (recipient – day eight to day 20) and from 8.9 to 10.1 (donor). Besides for one animal of the zinc group, for all animals, transconjugants could be detected already one day after donor application. In contrast to strain combination I, this time the lg (cfu/g feces) values of the transconjugant steadily increased over time and ranged from 0.0 to 8.2.

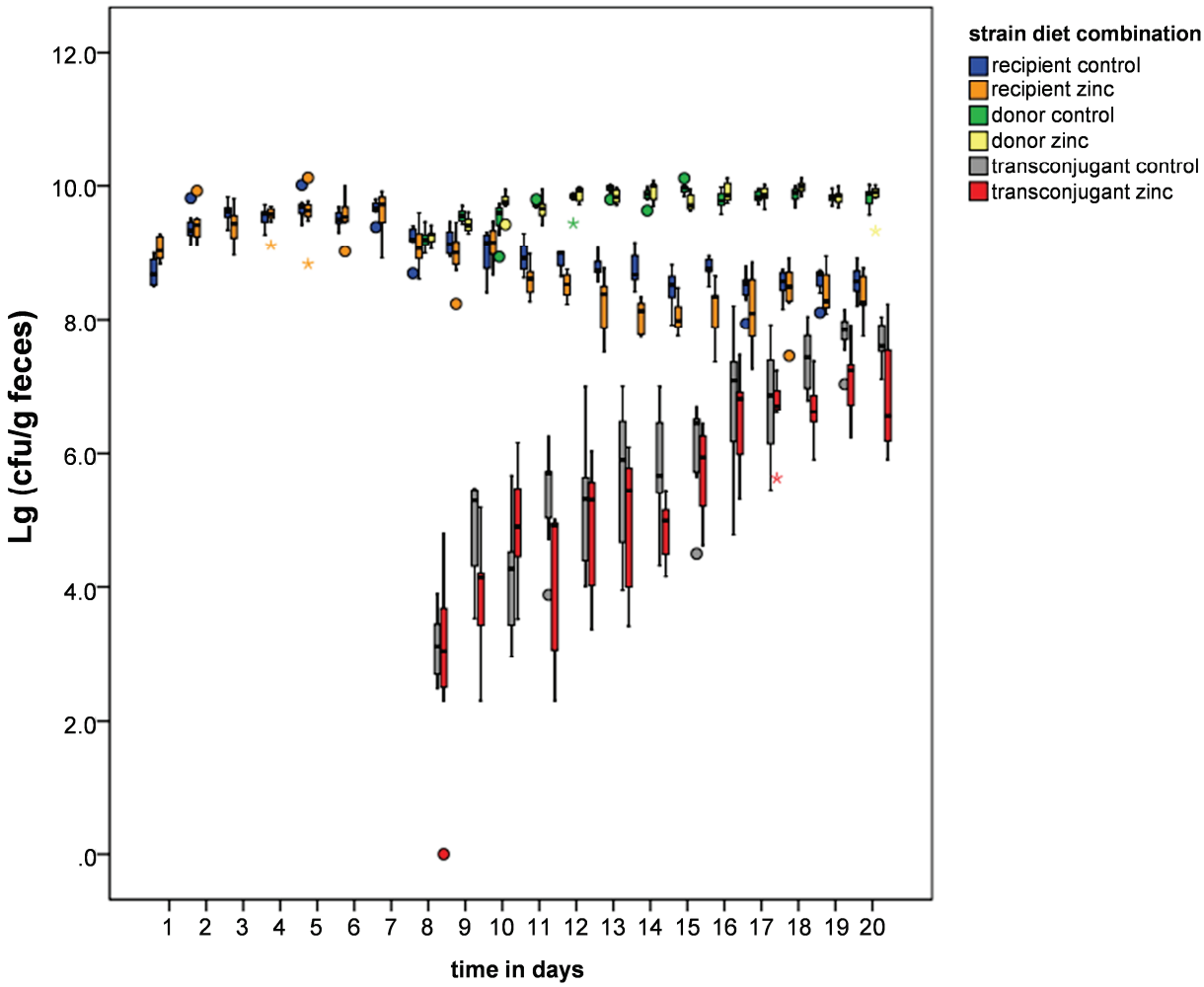

Figure 16: Lg (cfu/g feces) values for recipient, donor, and transconjugant of strain combination II (IMT30552 x VB983816.1) of the zinc mouse feeding trial
On day zero mice were associated with the recipient *E. coli* strain and on day seven with the donor *K. pneumoniae* strain. Data are separated according to the feeding group and are displayed for day one to 20. Colonies were counted on strain differentiating cultivation media and outliers are displayed separately.

The lg (cfu/g feces) data collected from day eight to 20 were integrated with the already described AUC approach (Tab. 7). The AUC values for each strain were compared between the two feeding groups. In contrary to strain combination I, this time, a significant difference between the feeding groups could be detected for the recipient AUC values with higher values for the control group (Mann-Whitney U test; p=0.002). For donor and transconjugant, AUC values no significant differences could be detected.

The recipient conjugation rates and the donor conjugation rates were calculated for each mouse (Fig. 17). Similar to strain combination I, recipient conjugation rates reached higher values as the donor conjugation rates. However, for this strain combination, conjugation rates with higher values as for strain combination I could be detected. In detail, both rates were at very low levels until day 16 and then increased notably. The recipient conjugation rate ranged from 0.0 to $8.4*10^{-1}$ and the donor conjugation rate from 0.0 to $2.5*10^{-2}$.

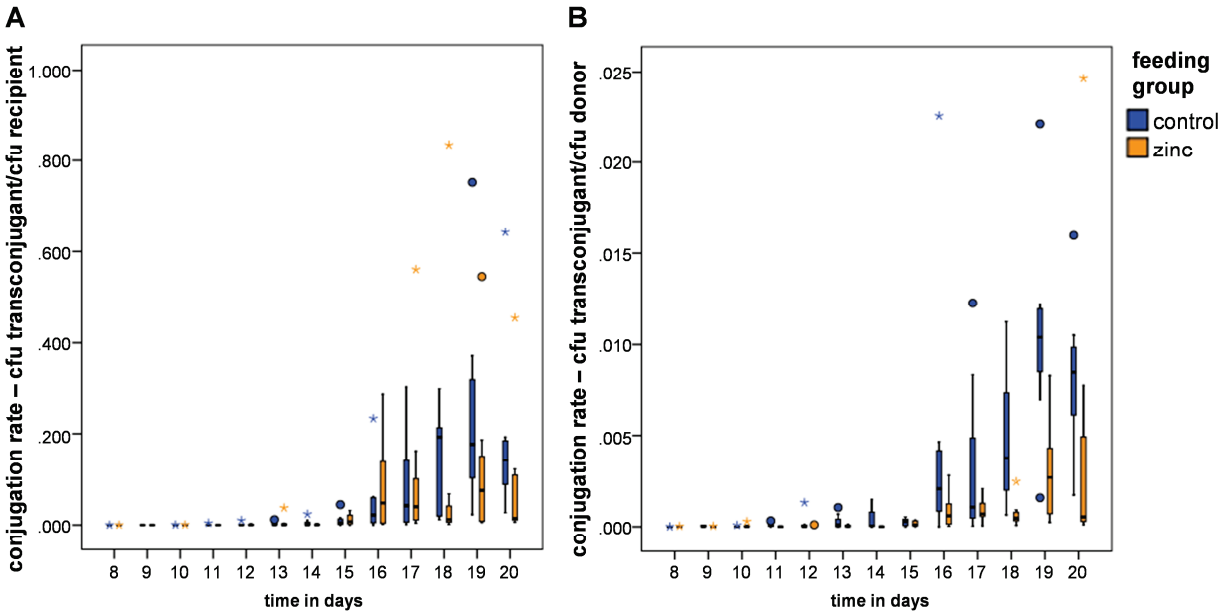

Figure 17: Recipient conjugation rate (cfu transconjugant/cfu recipient – A) and donor conjugation rate (cfu transconjugant/cfu donor – B) for feces of strain combination II (IMT30552 x VB983816.1) of the zinc mouse feeding trial
Data are separated according to the feeding group and are displayed for day eight to 20. Outliers are displayed separately

As described before, the AUC values were also calculated for both conjugation rates per mouse (Tab. 7). In accordance with the results of strain combination I, again, a significant difference between the feeding groups could be detected for the donor conjugation rate with higher values for the control group (Mann-Whitney U test; p=0.009).

Table 7: Data of the calculated AUC values for recipient, donor, transconjugant, recipient conjugation rate, and donor conjugation rate for feces of strain combination II (IMT30552 x VB983816.1) of the zinc mouse feeding trial

	Control group		Zinc group	
Strain/conjugation rate	AUC minimum – maximum	AUC median	AUC minimum – maximum	AUC median
Recipient*	104.5 – 105.8	105.4	98.4 – 103.6	101.3
Donor	115.9 – 118.2	117.4	116.3 – 118.9	117.1
Transconjugant	58.5 – 76.2	72.1	54.2 – 74.3	68.6
Recipient conjugation rate	0.135 – 1.366	0.455	0.035 – 1.913	0.123
Donor conjugation rate*	0.008 – 0.055	0.027	0.001 – 0.019	0.005

Minimum, maximum, and median values are displayed for both feeding groups. Significant differences between both feeding groups could be detected for AUC values of the recipient and of the donor conjugation rate and are displayed by underlined description and asterisked. * significant differences – Mann-Whitney U test; AUC recipient p=0.002; AUC donor conjugation rate p=0.009

3.2.3 *In vivo* colonization and gene transfer frequencies for digesta and feces at the day of sacrificing

After the collection of feces on day 20+1 of the experiment, all mice were sacrificed and caecum digesta and small intestine digesta were collected to determine the cfu/g sample of the respective strains (Fig. 18). For both strain combinations, feces lg (cfu/g sample) values of recipient, donor, and transconjugant looked similar to the caecum digesta lg (cfu/g sample) values. Comparable with the feces results gained from day eight to 20, for both samples, the donor reached the highest values followed by the recipient and the transconjugant. For strain combination II, the differences between the three strains were not so remarkable like for strain combination I, but boxplot bars of the three strains were still clearly distinuishable. For small intestine digesta, the mentioned scheme changed completely. The lg (cfu/g sample) values for all three strains were more dense and the variations between the animals increased. Besides this, for small intestine digesta, samples with cfu values equal to zero existed. As already described in this cases, the cfu was artifically defined as one so that the lg (cfu/g sample) value equaled zero.

For each strain of each habitat, the lg (cfu/g sample) values were compared between both feeding groups. For strain combination I, a significant difference could only be detected for the transconjugant of the small intestine digesta with higher lg (cfu/g sample) values for the control group (Mann-Whitney U test; p=0.009). For strain combination II, significant differences with higher values for the control group could be detected for the transconjugant

of caecum digesta and small intestine digesta (Mann-Whitney U test; caecum digesta p=0.006; small intestine digesta p=0.013).

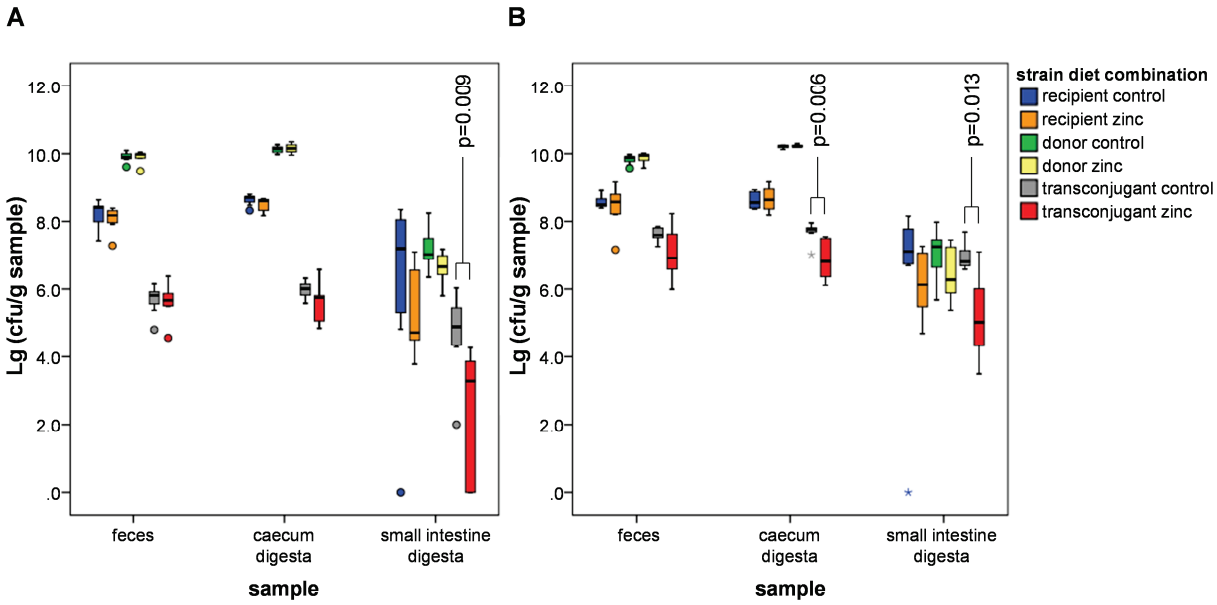

Figure 18: Lg (cfu/g sample) values for feces, caecum digesta, and small intestine digesta of strain combination I (J53 x VB983816.1 – A) and strain combination II (IMT30552 x VB983816.1 – B) for the day of sacrificing of the zinc mouse feeding trial
All mice were sacrificed at day 20+1 of the experimental period and besides feces also caecum digesta and small intestine digesta were collected. Lg (cfu/g sample) values were determined by plating on strain differentiating cultivation media and are devided for recipient, donor, and transconjugant of the control and the zinc feeding group. Significant differences are indicated by p values.

Based on the cfu values of the three strains, the recipient and the donor conjugation rates were calculated for each strain combination and the rates were compared between the two feeding groups. For both strain combinations, the highest conjugation rates were detected for the small intestine digesta. In cases that a recipient cfu equaled zero or that the calculated conjugation rate was greater than one, the corresponding conjugation rate was defined as one.

For strain combination I, minimum and maximum conjugation rates are given in table 8 and graphical presentation is given in figure 19. Significant differences between the feeding groups could be detected for the recipient and the donor conjugation rate of the small intestine digesta whereas in feces and caecum digesta no significant differences could be detected (small intestine digesta Mann-Whitney U test; recipient conjugation rate p=0.047; donor conjugation rate p=0.018). For both rates of the small intestine digesta, higher values could be detected for the control group.

Table 8: Recipient conjugation rate (cfu transconjugant/cfu recipient) and donor conjugation rate (cfu transconjugant/cfu donor) for feces and digesta of strain combination I (J53 x VB983816.1) for the day of sacrificing of the zinc mouse feeding trial

Conjugation rate	Feces minimum – maximum	Caecum digesta minimum – maximum	Small intestine digesta minimum – maximum
Recipient conjugation rate	$1.9*10^{-3} – 2.9*10^{-2}$	$2.9*10^{-4} – 2.1*10^{-2}$	0.0 – 1.0
Donor conjugation rate	$8.0*10^{-6} – 3.2*10^{-4}$	$4.0*10^{-6} – 2.0*10^{-4}$	$0.0 – 1.8*10^{-2}$

Minimum and maximum conjugation rates are displayed for feces, caecum digesta, and small intestine digesta.

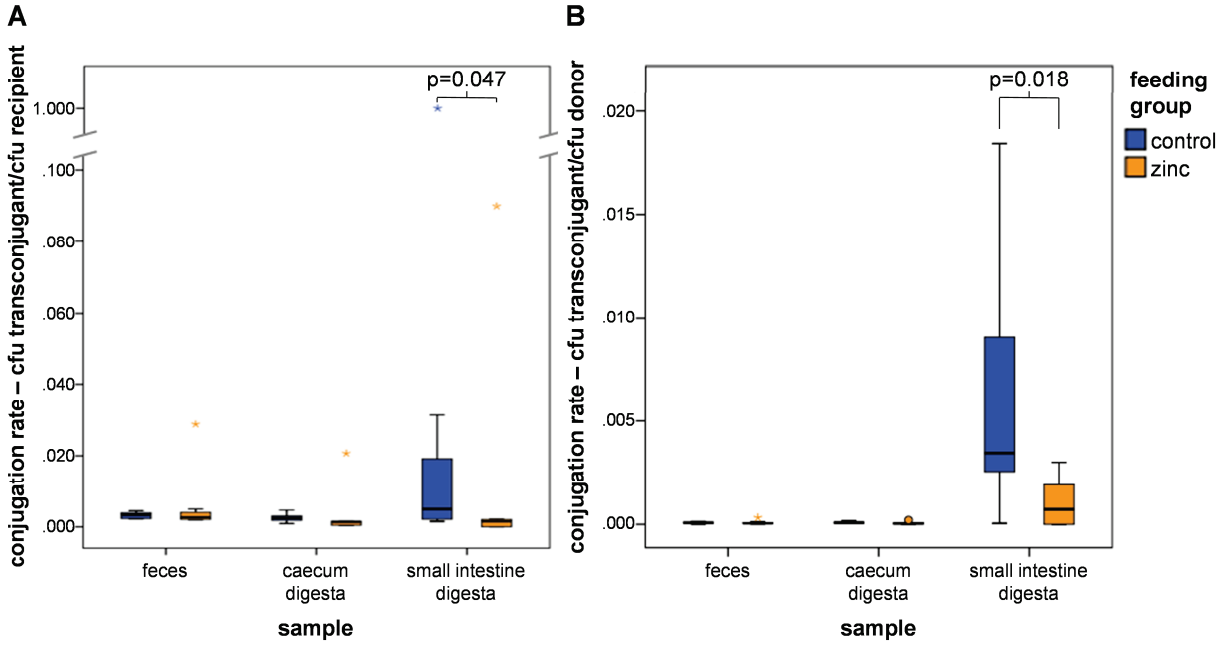

Figure 19: Recipient conjugation rate (cfu transconjugant/cfu recipient – A) and donor conjugation rate (cfu transconjugant/cfu donor – B) for feces and digesta of strain combination I (J53 x VB983816.1) for the day of sacrificing of the zinc mouse feeding trial
Seven mice per feeding group (control and zinc) were sacrificed at day 20+1 and feces, caecum digesta, and small intestine digesta were collected for cfu determination of recipient, donor, and transconjugant. With the determined cfu values the recipient (A) and the donor conjugation rate (B) were calculated. Significant differences are indicated by p values.

As observed with feces collected from day eight to 20, the conjugation rates of strain combination II (Tab. 9 and Fig. 20) reached higher values than that of strain combination I. In contrast to strain combination I, this time significant differences between the feeding groups could only be detected for the conjugation rates of the caecum digesta (Mann-Whitney U test; recipient conjugation rate p=0.018; donor conjugation rate p=0.006). Again higher values could be detected for the control group.

Table 9: Recipient conjugation rate (cfu transconjugant/cfu recipient) and donor conjugation rate (cfu transconjugant/cfu donor) for feces and digesta of strain combination II (IMT30552 x VB983816.1) for the day of sacrificing of the zinc mouse feeding trial

Conjugation rate	Feces minimum – maximum	Caecum digesta minimum – maximum	Small intestine digesta minimum – maximum
Recipient conjugation rate	$5.9*10^{-3} – 4.5*10^{-1}$	$3.2*10^{-3} – 2.6*10^{-1}$	$1.1*10^{-3} – 1.0$
Donor conjugation rate	$1.1*10^{-4} – 2.5*10^{-2}$	$8.0*10^{-5} – 6.9*10^{-3}$	$4.9*10^{-4} – 1.0$

Minimum and maximum conjugation rates are displayed for feces, caecum digesta, and small intestine digesta.

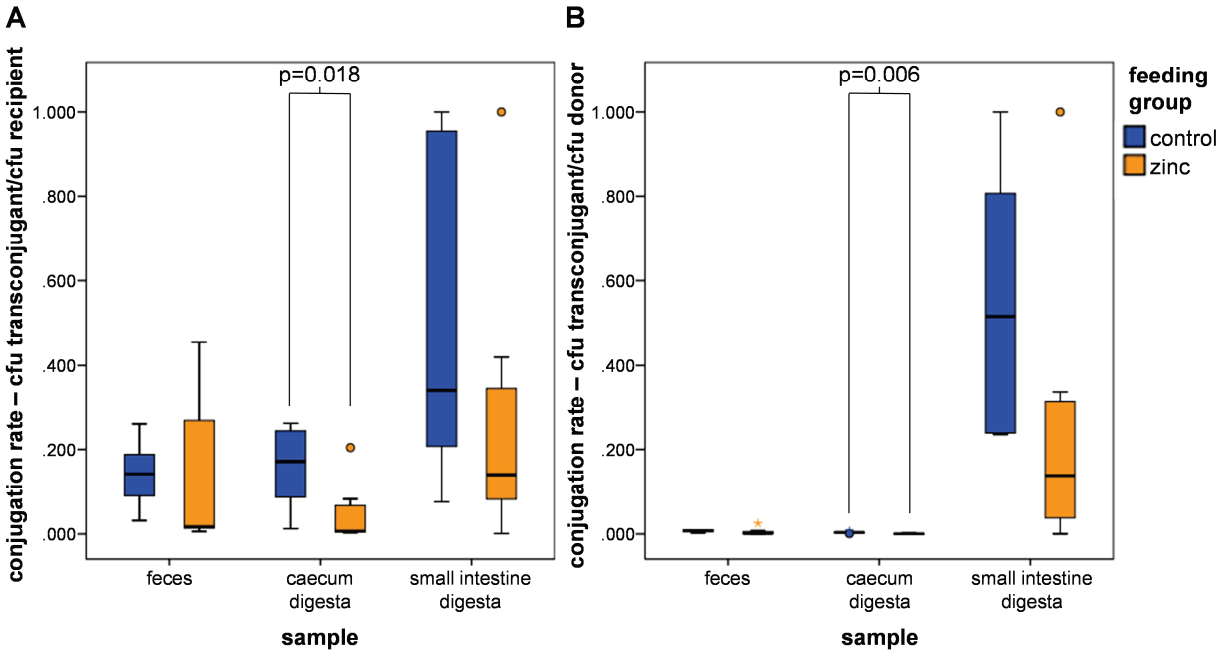

Figure 20: Recipient conjugation rate (cfu transconjugant/cfu recipient – A) and donor conjugation rate (cfu transconjugant/cfu donor – B) for feces and digesta of strain combination II (IMT30552 x VB983816.1) for the day of sacrificing of the zinc mouse feeding trial
Seven mice per feeding group (control and zinc) were sacrificed at day 20+1 and feces, caecum digesta, and small intestine digesta were collected for cfu determination of recipient, donor, and transconjugant. With the determined cfu values the recipient (A) and the donor conjugation rate (B) were calculated. Significant differences are indicated by p values.

3.2.4 Quantitative analysis and gene transfer frequencies for the *in vitro* data

To investigate if zinc influences the bacterial conjugation frequency *in vitro*, the same strain combinations as in the *in vivo* model were used. For each strain combination, five biological independent replicates were performed in which the cfu of the recipient, donor, and transconjugant were investigated after 1.5 and 24 hours of mating. Directly before mating, the cfu values of the individual grown recipient and donor were investigated. For strain combination I (J53 x VB983816.1), the lg (cfu/ml medium) values directly before mating

ranged from 7.8 to 8.1 for the recipient and from 8.1 to 8.3 for the donor. Unfortunately, for this strain combination, we were not able to quantify the transconjugant after 1.5 hours of mating. The counted colonies for the two dilutions 10^0 and 10^{-1} obviously didn't match to one another and therefore these data points were declared invalid. For the recipient and donor, the lg (cfu/ml medium) values ranged over all zinc concentrations from 6.3 to 8.1 (recipient) and 6.7 to 8.4 (donor) (Fig. 21 A). With zinc concentrations greater than 8.8 mg/l, the cfu for both strains decreased with increasing zinc concentration. After 24 hours, the lg (cfu/ml medium) values ranged from 5.8 to 9.1 (recipient), 8.5 to 8.8 (donor), and 0.7 to 5.6 (transconjugant). This time, the recipient and transconjugant decreased with an increase of zinc over 8.8mg/l whereas the donor showed stable cfu values.

For strain combination II (IMT30552 x VB983816.1), the recipient lg (cfu/ml medium) values directly before mating ranged from 8.0 to 8.3 and from 8.2 to 8.3 for the donor. For this strain combination, partly comparable results could be detected (Fig. 21 B). After 1.5 hours of mating, the lg (cfu/ml medium) values ranged from 6.5 to 8.3 (recipient), 6.8 to 8.4 (donor), and from 0 to 1.3 (transconjugant). Again, for the recipient and the donor, the lg (cfu/ml medium) values decreased with increasing zinc concentrations greater than 8.8 mg/l. For the transconjugant, only for two biological replicates colonies could be detected for 1.2 and 77.4 mg zinc/l. After 24 hours of mating, the lg (cfu/ml medium) values ranged from 7.6 to 8.8 (recipient), 8.3 to 8.6 (donor), and 2.3 to 5.0 (transconjugant). This time, the recipient and the transconjugant cfu values were not so strongly influenced by zinc as for strain combination I. The donor again showed a stable level regardless of the zinc concentration.

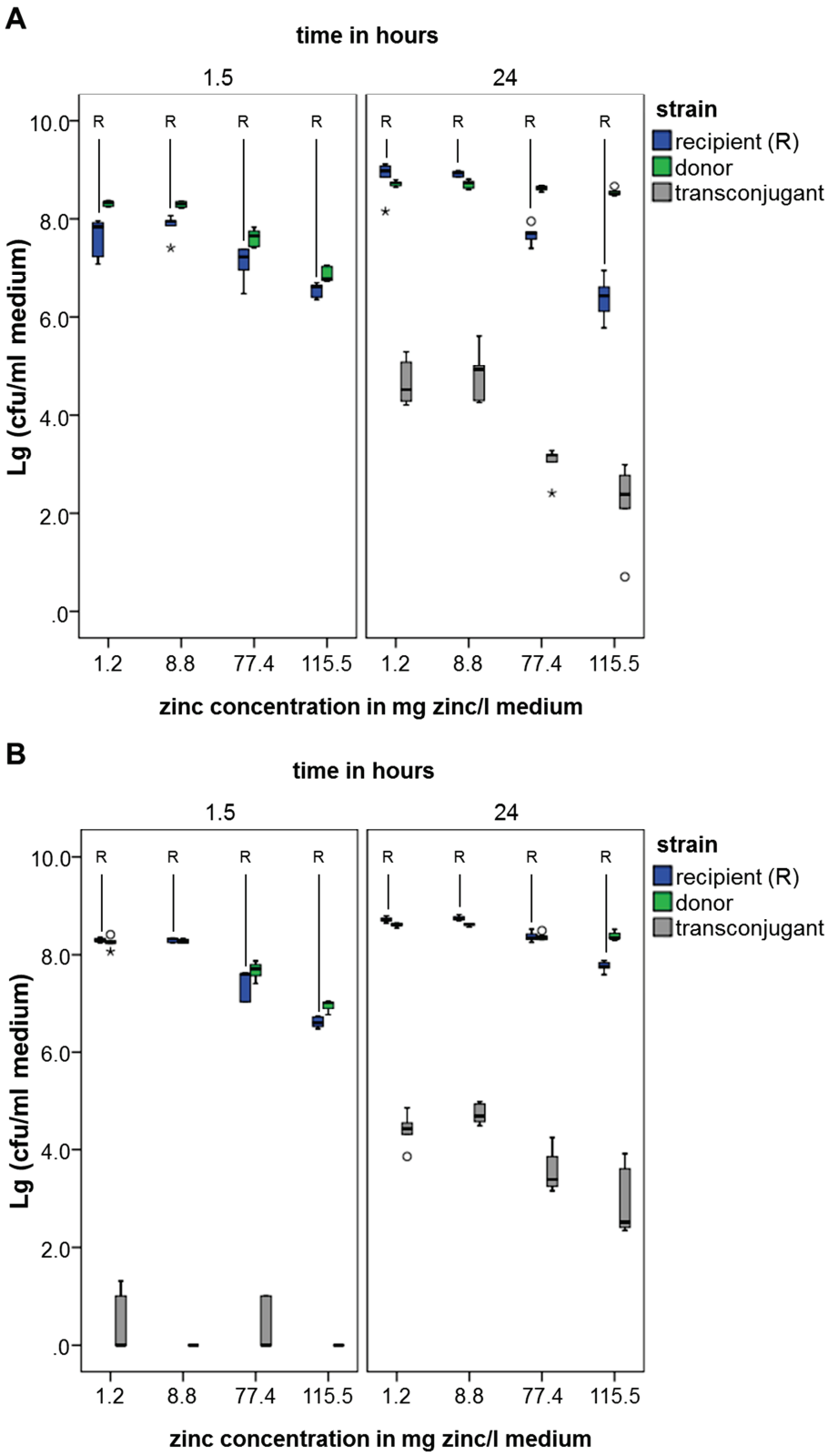

Figure 21: Lg (cfu/ml medium) values for the *in vitro* conjugation of strain combination I (J53 x VB983816.1 – A) and strain combination II (IMT30552 x VB983816.1 – B)
Lg (cfu/ml medium) values were displayed in association to two different mating time points (1.5 and 24 hours) and four different zinc concentrations (1.2, 8.8, 77.4, and 115.5 mg zinc/l medium). Recipient values were marked with R for clarity reasons. Recipient and donor were grown separately to an OD600= 0.6 and afterwards 1.5 ml of the recipient and of the donor were added to 7ml LB media of different zinc concentrations. For cfu determination samples were plated on strain differentiating cultivation media and growing colonies were counted.

For analyses of the *in vitro* data, the recipient and the donor conjugation rates were calculated for the four different mating media. As for strain combination I, the transconjugant could not be determined after 1.5 hours, only the conjugation rates after 24 hours of mating were calculated (Fig. 22). For the recipient conjugation rate, the assumption that the distribution of the conjugation rates is the same across the four categories of mating medium couldn't be rejected. However, for the donor conjugation rate a significant influence of the zinc concentrations on the conjugation rate was detected (Kruskal-Wallis test; p=0.002). Post hoc testing according to the Dunn-Bonferroni method revealed significant differences between 1.2 and 115.5 mg zinc/l (p=0.014) and between 8.8 and 115.5 mg zinc/l (p=0.008).

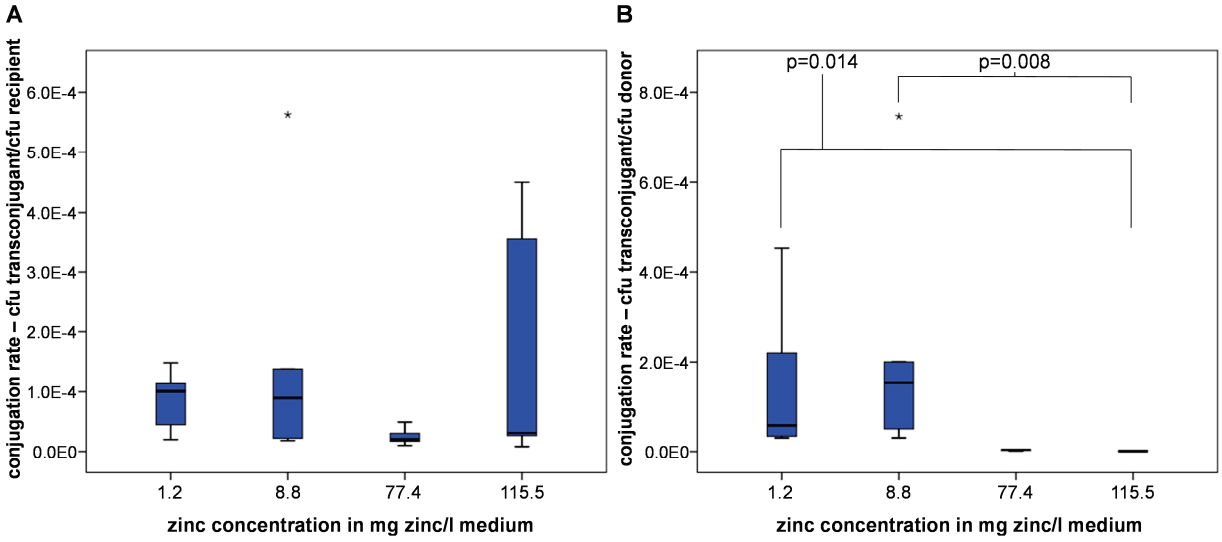

Figure 22: *In vitro* recipient conjugation rate (cfu transconjugant/cfu recipient – A) and donor conjugation rate (cfu transconjugant/cfu donor – B) after 24 hours of mating for strain combination I (J53 x VB983816.1)
Data are shown for four different zinc concentrations (1.2, 8.8, 77.4, and 115.5 mg zinc/l medium) and significant differences are indicated by p values.

For strain combination II, after 1.5 hours of mating, only for two biological replicates colonies could be detected on the transconjugant plates after mating in 1.2 and 77.4 mg/l medium. Therefore, the recipient conjugation rates ranged only from 0.0 to $2.5*10^{-7}$ and the donor conjugation rates only from 0.0 to $1.9*10^{-7}$ and were not displayed graphically. For both rates, no significant differences between the different mating media could be detected applying the Kruskal-Wallis test. After 24 hours of mating, higher conjugation rates could be detected (Fig. 23). This time, an influence of the zinc medium on the conjugation rate could be detected for the recipient and the donor conjugation rate (Kruskal-Wallis test; recipient conjugation rate p=0.049; donor conjugation rate p=0.003). For the recipient conjugation rate, no significant difference could be detected by pairwise comparison of the zinc media by Dunn-Bonferroni post-hoc testing. For the donor conjugation rate, a significant difference could be detected between 8.8 and 115.5 mg zinc/l (p=0.004).

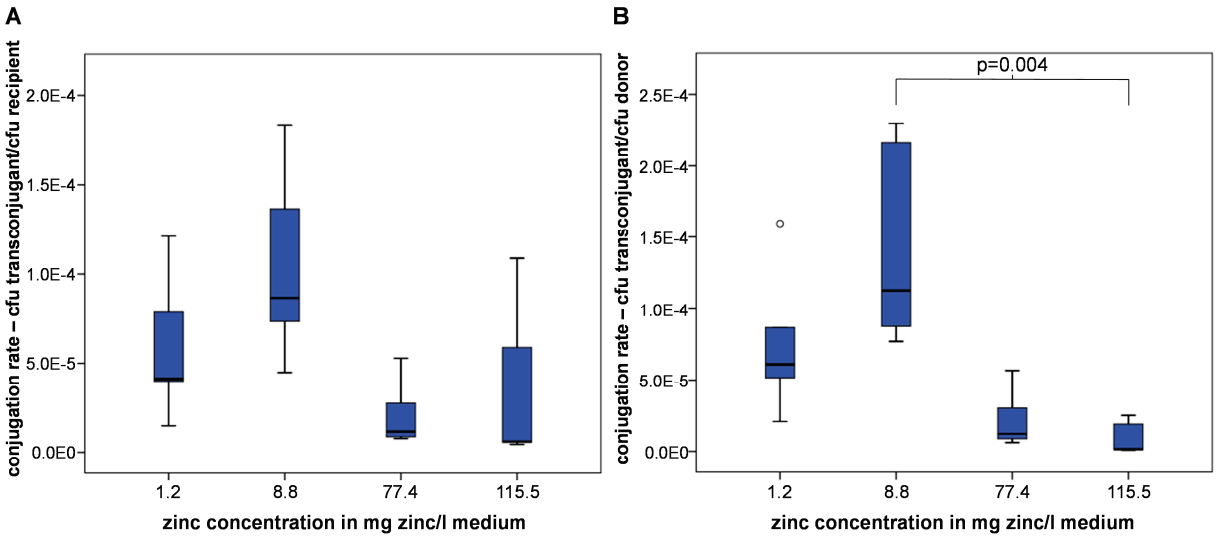

Figure 23: *In vitro* recipient conjugation rate (cfu transconjugant/cfu recipient – A) and donor conjugation rate (cfu transconjugant/cfu donor – B) after 24 hours of mating for strain combination II (IMT30552 x VB983816.1)
Data are shown for four different zinc concentrations (1.2, 8.8, 77.4, and 115.5 mg zinc/l medium) and significant differences are indicated by p values.

3.2.5 Verification of transconjugants

To verify the counted transconjugants, several randomly picked isolates of the transconjugant plates were subcultured on cefotaxime plates and growth was evaluated visually. For the zinc mouse feeding trial, 202 isolates were subcultured for strain combination I and for 200 of them well growing dark pink to reddish colonies could be detected on the cefotaxime plates. For the *in vitro* conjugation of this strain combination, 110 isolates were picked and 109 showed well growing colonies.

For strain combination II, 161 isolates of the zinc mouse feeding trial and 188 isolates of the *in vitro* conjugation were picked. All 349 subcultures grew well as dark pink to reddish colonies.

4 Discussion

4.1 A zinc pig feeding trial was performed to investigate the influence of high dietary zinc feeding on the resistance status of the *E. coli* population

Since the European ban of antimicrobial growth promoters, the research for different alternatives was further enforced. For weaning piglets, the alternative of feeding high dietary zinc oxide is widely used. Different studies confirm its positive effects on gut health and growth performance [139, 195-197]. However, concerns of this feeding strategy arise as several hints exist that high dietary zinc feeding increases the abundance of antimicrobial resistant bacteria [166-170].

One recent study regarding this topic was performed at our institute and revealed a dramatic increase of the intestinal proportion of multi-resistant *E. coli* under the influence of high dietary zinc feeding for piglets [169]. As especially multi-resistant bacteria are problematic for the treatment of infections, this finding of Bednorz et al. could have a massive influence on the strategy of high dietary zinc feeding.

Consequently, it was necessary to confirm the results of this recent zinc pig feeding trial with an independent second study using a more complex sampling approach which focuses on resistance phenotypes rather than the clonal diversity of *E. coli*. The adapted study design also allows more insights into the temporal population dynamics of *E. coli* and was therefore not only able to substantiate recent findings but also increase the impact of these findings.

4.1.1 High dietary zinc feeding promotes the persistence of multi-resistant *E. coli*

As during this zinc pig feeding trial again an increasing effect of high dietary zinc on the proportion of multi-resistant *E. coli* was detected, our study substantiates the basic findings of the prior study by Bednorz et al. [169]. In addition, the increasing effect was detected in the three gut habitats tested (mucosa, digesta, and feces), which strongly corroborates a major effect of high dietary zinc feeding on antimicrobial resistance.

By using our adapted study design, we were able to show that the proportional increase of multi-resistant *E. coli* in the piglets gut is an effect of persistence of resistant *E. coli* populations over time. The effect of high dietary zinc feeding on multi-resistant isolates was observable at the age of 52±2 days, where significant differences between both feeding groups were detected in each of the three habitats. However, the proportion of multi-resistant isolates did not increase in the zinc group over time. Rather, multi-resistant isolates were able to maintain a constant level while in the control group the proportion of multi-resistant isolates decreased as time progressed.

These results indicate that, under the influence of high dietary zinc feeding, multi-resistant *E. coli* could adapt better to the stress of weaning and the following changes in the intestinal

microbiota. In combination with the results of the absolute *E. coli* numbers, where a general decrease over time was detected, this increased survival/persistence of multi-resistant isolates is rather surprising as it points towards fitness advantages of the resistant population.

Multi-resistance is often conferred via large resistance plasmids. These plasmids have been linked to a reduction of bacterial fitness in non-selective surroundings [198, 199] which can be assumed for the piglets intestine as the animals did not receive antimicrobial substances. If multi-resistant *E. coli* have an immanent disadvantage and the absolute *E. coli* number decreases during the trial, one would expect a vanishing of the multi-resistant population right from delivery. Interestingly, this phenomenon is only detected in the control group after weaning. It might be that high dietary zinc feeding antagonizes the fitness costs of antimicrobial resistance. The mechanisms of this counteraction of zinc feeding remain largely unknown but, in general, could be due to additional factors carried on resistance plasmids or might be linked to the core genome and the phylogenetic background of the persisting strains [200-202].

It could be that co-selection, which includes co- and cross-resistance [163], leads to the selection of resistant bacteria under the influence of zinc. During co-resistance, different genes conferring antimicrobial or metal resistance are located together on one mobile genetic element such as a plasmid, integron, or transposon [203]. Therefore, the presence of the respective metal or antimicrobial substance positively selects for strains harboring the encoding genetic element. For MRSA the co-localization of the czrC gene imparting zinc resistance and the mecA gene conferring resistance to beta-lactam antimicrobials on the Staphylococcus Cassette Chromosome mec (SCCmec) is described [204, 205]. Zinc resistance is therefore often associated with methicillin resistance in *Staphylococcus aureus* from pigs and the occurrence of zinc may select for MRSA [168, 206, 207].

The major requirement for the occurrence of cross-resistance is that different antimicrobial agents (e.g. antimicrobial substances and toxic metals) attack the same target, trigger the same pathway to cell death, or share the same route of access to their targets [203]. Thus, the development of resistance against one antimicrobial agent leads also to resistance against the other one. One example is the membrane-bound DsbA-DsbB disulfide bond formation system in *Burkholderia cepacia* which is suggested to be involved in a metal efflux- and a multi-drug resistance system [208].

However, during our recent zinc pig feeding trial, no general association of antimicrobial and zinc resistance was detected [169] and further investigations with isolates from both zinc pig feeding trials of our institute also revealed no significant association (Ghazisaeedi, unpublished data). Therefore, we hypothesize that co-selection has no major effect on the observed results.

However, for evaluation of the hypothesis that high dietary zinc feeding antagonizes the fitness costs of antimicrobial resistance, it is necessary to mention that at least for some plasmids and certain successful bacterial lineages no fitness cost is detectable for antimicrobial resistance [90, 209, 210]. Also, earlier pig studies have proven that resistance had no significant influence on the colonization ability of *E. coli* whereby a positive association of single resistance genes and virulence associated genes was measured in parallel [211].

As already mentioned, the effect of high dietary zinc feeding on multi-resistance was only detectable at the age of 52±2 days. This might be due to nutritional changes after weaning. These lead to alterations in the intestinal microbiota composition and therefore it is possible that the effect of high dietary zinc feeding is only detectable after the microbiome stabilizes over time.

At the time point of weaning, which is in Europe often around the age of 21 to 28 days, the porcine gastrointestinal tract is still underdeveloped [212]. The microbiota in the gastrointestinal tract is unstable during the first week after weaning and the fermentative capacity in the hind gut will have been developed only after two to three weeks [213]. For lactobacilli a decrease directly after weaning is described which was followed again by an increase of the bacterial counts above the pre-weaning level. In contrast coliform bacteria increase directly after weaning and drop afterwards [212, 213]. Therefore, an inverse connection between lactobacilli and coliform bacteria was suggested [213]. With this and the detected decrease of the *E. coli* population after weaning in mind, the effect of high dietary zinc feeding on multi-resistant *E. coli* may only be detectable after the coliform bacteria counts decreased as a consequence of an increasing colonizing barrier through lactobacilli. However, lactobacilli were not investigated during this study and for feces at the age of 38±2 days no effect of high dietary zinc feeding on multi-resistant *E. coli* was detected even though the *E. coli* counts were already decreased after weaning. Beside this, it is necessary to consider that the composition of the microbiome is influenced by different external and internal host factors [8] and that different studies observed time dependent increasing, decreasing, or stable *E. coli* populations after weaning [214-216].

Whether the observed effects of multi-resistance are due to the constant feeding of high dietary zinc over the whole period of four weeks has to be debated. It is possible that even a shorter duration of zinc feeding causes an increase on multi-resistant *E. coli*, as it might only be detectable after the microbiome has stabilized and the zinc feeding is already terminated (see also 4.1.3 Possible mechanisms behind the observed effects). However, it also is possible that, if the high dietary zinc feeding would not start directly after weaning but rather at a later time point, the effect of zinc on multi-resistance would still be detectable. This is of relevance as most zinc oxide formulations with an approval as veterinary medical product in

the European Union are only approved for 14 days [154]. However, in different countries, zinc oxide feeding is not clearly regulated and also longer periods of high dietary zinc feeding can occur.

In general, proportions of multi-resistant isolates were higher than in our recent study. This could be explained by the differences in the animal trial setup between the studies with this study using a setup with emphasis towards the resistance aspect. In addition, this study included streptomycin in the resistance screening. As streptomycin resistance has become very abundant in *E. coli* [79], this may also have led to a higher detection rate for multi-resistant *E. coli*. However, in general, the comparison between resistance rates of different studies is difficult as often variances exists for sampling target, sampling point, sampling procedure, experimental method, investigated antimicrobial substances, and resistance breakpoints [83]. In case of multi-resistance, the applied definition is also of interest [180]. For example, differences between 6.7% (multi-resistant as resistant against four antimicrobial substances) [81] and 58% multi-resistant isolates (multi-resistance as resistant against at least two antimicrobial substances) [217] can easily be explained by the different definitions. The major source of bacteria colonizing the newborn piglet is the maternal feces [213]. It therefore is likely that the piglets of this study gained the high proportion of multi-resistant *E. coli* directly from their mothers.

Beside the mentioned effect on multi-resistance, also the analysis of isolates sensitive against all tested antimicrobial substances vs. isolates resistant against at least one antimicrobial substance revealed an increasing effect of zinc on resistance for some time points for bacteria isolated from feces and digesta. In contrast, the analysis of clones from digesta being resistant against at least one antimicrobial substance of the recent trial revealed only slight changes between the feeding groups [169]. Again, this is possibly due to the differences discussed before.

Our observations also corroborate previous investigations gained in livestock. Real time PCR investigations of the digesta isolated during the recent trial [169] reveal an increase of the copy numbers for tetracycline and sulfonamide resistance genes for the zinc group over time after weaning [170]. For pig manure, a positive association with the phenotypic resistance of *E. coli* against the beta-lactame piperacillin, the tetracycline doxycycline, and the multi-resistant phenotype is also described [167]. In manure and compost of pig farms and compost-connected soil samples a positive association of the zinc concentration with aminoglycosides and tetracyclines resistance genes and the overall tested resistance genes was found [218]. Furthermore, for manure and soils collected from a pig, a poultry, and a cattle feedlot a positive association of sulfonamide resistance genes with the zinc concentration was detected [219]. In a zinc pig feeding trial, an increased prevalence and persistence of nasal MRSA was detected for the high zinc group [168].

In contrast to the already mentioned data, one study investigating the feces of heifers found no effect of elevated zinc feeding on the phenotypic resistance of *E. coli* and on the abundance of one tetracycline resistance gene [220]. It might be that interspecies or regional differences in the microbiota composition lead to the circumstance that the resistance level of the microbiota of cattle is not or only negligibly affected by high dietary zinc feeding.

4.1.2 The effect on resistance is independent of the *E. coli* population density

The detected association between high dietary zinc feeding and increasing resistance is furthermore independent of the total numbers of *E. coli*, as was shown by the parallel quantitative determination of the *E. coli* population. Why is this analysis of importance?
A higher population density of *E. coli* causes a closer contact between the bacterial cells, which can increase conjugational transfer of resistance plasmids between strains [221], thus leading to increased resistant rates. Also the scheme for the selection of isolates for the antimicrobial resistance screening might be influenced by higher *E. coli* numbers. If the general amount of *E. coli* increases, the probability increases that colonies can be detected and isolated from the antimicrobial substance containing CHROMagar Orientation plates. Subsequently, higher amounts of resistant isolates could be used for the resistance screening.
As no significant differences in the total *E. coli* numbers between the control and the zinc group (in all three habitats as well as sampling time points) were detected, the two abovementioned aspects should have had no influence on differences in the proportion of resistant isolates between both feeding groups. However, it might be that a different bacterial population than *E. coli*, which may include possible donor strains for conjugation of resistance plasmids is influenced in its numbers by high dietary zinc feeding (see also 4.1.3 Possible mechanisms behind the observed effects).
For isolates of the family Enterobacteriaceae, no significant differences between both feeding groups were detected in this study. Therefore, an increased potential as donors for the transfer of resistance plasmids because of higher cell numbers is unlikely for the zinc group, although it cannot be ruled out.
The result that high dietary zinc feeding did not influence the abundance of viable *E. coli* cells is in accordance with previous studies [216, 222]. However, 16SrRNA gene quantification indicated that high dietary zinc feeding increases the relative abundance of *E. coli* in the microbiota of ileum digesta in piglets [162]. As 16SrRNA gene quantification does not allow to distinguish between viable and dead bacteria, the different methods used may be an explanation for these deviations.
Beside the determination of the absolute *E. coli* numbers, this study also investigated the quantitative data for resistant *E. coli*, where also no significant differences were detected

between the zinc and the control group. The highest amount of *E. coli* colonies were detected on ampicillin, tetracycline, and sulfamethoxazole-trimethoprim containing plates, whereas no *E. coli* colonies grew on plates containing cefotaxime, gentamicin, and chloramphenicol. These results are in accordance with various studies investigating resistance rates of *E. coli* isolated from animals [81, 83, 169, 223]. However, it is interesting that no significant differences in the amount of isolates grown on the different antimicrobial substance containing plates were detected between the feeding groups, although we detected differences when analyzing multi-resistant and sensitive vs. resistant isolates. This underlines that resistance against one antimicrobial substance is widely distributed [224].

To sum up, for all investigated populations of the microbiome (absolute *E. coli* numbers, resistant *E. coli*, and isolates of the family Enterobacteriaceae), all different samples, and all different time points, high dietary zinc feeding had no influence on their absolute abundance. However, although the quantitative data did not change the diversity within one population might be strongly influenced by high dietary zinc feeding as the results on antimicrobial resistance indicate.

4.1.3 Possible mechanism behind the observed effects

To get a deeper insight into possible mechanisms leading to the increased rates of multi-resistant isolates in the zinc group the resistance patterns of the multi-resistant isolates were investigated. A highly important finding was that, although we detected for all three habitats the same zinc effect on multi-resistance, differences for the resistance patterns exist with regards to the separate intestinal niches investigated.

The observed changes in the resistance patterns between the feeding groups and over time could be mainly explained by two effects:

(I) one population outcompetes others due to an advantage of its specific resistance pattern or

(II) the exchange of mobile genetic elements encoding for resistance patterns is altered

As the changes for the patterns of the different habitats were very versatile, whole genome sequencing and MLST were performed for random samples to get further insights into the phylogenetic structure. Isolates of all three habitats were taken at the time point 52±2 days of age, where the zinc effect on multi-resistance was detectable.

The depicted 180 isolates were assigned to 20 different Sequence Types (STs) whereas most isolates belonged to ST10 and ST34, both of sequence type complex 10. Interestingly, 68 out of 71 multi-resistant isolates belonged to ST10 and ST34 only. Therefore, the increasing effect of high dietary zinc feeding on multi-resistance for all three habitats is based

on phylogenetic related isolates of these two STs implicating that the phylogenetic background of the isolates is of importance.

Further investigations of ST10 and ST34 revealed that the proportion of multi-resistant isolates within each ST was not influenced by high dietary zinc feeding. Therefore, it might be that under the influence of high dietary zinc feeding isolates of these STs either expand or are able to persist while they diminish in the control group. This than causes a higher absolute number of multi-resistant isolates in the zinc group. However, at the age of 52±2 days, no significant differences were detectable between both feeding groups when investigating the distribution of the isolates belonging to the multi-resistance associated STs (ST10 plus ST34) or to the remaining 18 STs.

ST10 and ST34 are both described for porcine isolates [169, 225], (University of Warwick, http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The relevance of ST10 in the multi-resistance effect is of special interest. This ST is highly diverse with more than 300 isolates comprising commensal as well as pathogenic isolates from human and animal origin (University of Warwick, http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). It was suggested that commensals of ST10 are capable to support the evolution of different pathotypes and indeed one study reveals insights into the microevolution and metabolic adaptations of UPEC and NMEC isolates of ST10 (Dematheis, unpublished data). A high colonization of pigs with isolates of ST10 can therefore represent a reservoir for the evolution and distribution of pathogenic *E. coli*. Beside this, an association of ST10 with the spread of ESBL genes is described [77]. However, during this study no bacterial growth of *E. coli* colonies was detected on cefotaxime containing plates.

In summary, results of the resistance pattern analysis and the MLST do not allow an identification of the underlying mechanism leading to the increased resistance rates under high dietary zinc feeding and we will need future whole genome sequence analyses to get further insights. Although MLST data gave interesting insights it is necessary to keep in mind that, in contrast to the different analyses of the zinc pig feeding trial, no course of time was investigated for MLST data until now. Therefore, statements to persistence, increase, and decrease of some STs in association with multi-resistant isolates can unfortunately not be given. Beside this, the number of isolates belonging to ST10 and ST34 ranged only from one to 17 and therefore only small isolate numbers were investigated in the statistical analyses of these STs.

Besides the already discussed points, several different other mechanisms could be involved in the increase of resistant *E. coli* isolates under the influence of high dietary zinc feeding and have to be considered. Co-regulation is a mechanism that occurs if transcriptional or translational responses to antimicrobial substances and metals are linked and form a response to either stress [163]. For *E. coli* strain MG1655 the *mdtABC* operon was

upregulated under the influence of excess zinc [226]. This resistance-nodulation-cell division (RND-type) efflux system is involved in imparting resistance to certain antimicrobial substances like novobiocin and deoxycholate, a bile salt component [227]. However, as piglets were raised without exposure to antimicrobial substances and the phenotypic resistance screening and MIC determination of the isolates were conducted in the absence of zinc, co-regulation is unlikely to explain the observed changes.

As antimicrobial resistance is often conferred via transferable plasmids, it could also be that zinc has a positive influence on the *E. coli* plasmid uptake rates, especially for resistance plasmids. Evidence exists that zinc influences the bacterial conjugation [171, 172] and the expression of genes involved in cell membrane structure and transport [226]. Alteration in both can lead to an increase plasmid uptake. Indeed, during our recent study differences in the resistance pattern of subclones of both feeding groups could be partially connected with differences in the plasmid profiles. Beside this, especially the strong effect of high dietary zinc feeding on multi-resistant isolates substantiates the idea that plasmids are involved in the underlying mechanisms as these often carry multiple resistance genes [62]. However, even if an increased plasmid uptake leads to the increased resistance rates in the zinc group, this uptake seems rather to be effective for isolates of the same phylogenetic background as most multi-resistant isolates at the age of 52±2 days belonged to ST10 and ST34. A possible influence of high dietary zinc on bacterial conjugation was in detail investigated in the second part of this study and will be discussed further under 4.2.

All putative involved mechanisms mentioned above directly target the *E. coli* population, but it is necessary to keep in mind the complex composition of the intestinal microbiota. High dietary zinc feeding could have, in parallel to weaning, an influence on intestinal populations other than *E. coli*. Under the influence of high dietary zinc feeding decreasing, increasing as well as no effects were described for lactic acid bacteria, lactobacilli, Enterobacteriaceae, and enterococci [153, 162, 222, 228, 229]. Although no uniform picture about the influence of high dietary zinc feeding was detected, it is obvious that the microbiota is altered by high dietary zinc feeding.

Changes on different intestinal populations may result in new niches under the influence of high dietary zinc feeding. If resistant *E. coli* populations adapt faster to changes in the intestinal microbiota they could occupy these niches and increase their relative abundance. Alternatively it might be that different populations which can act as donor strains for the conjugation of resistance plasmids increase under the influence of high dietary zinc feeding and therefore leading to an increased transfer of resistance plasmids to the *E. coli* population.

Finally, it is also possible that high dietary zinc feeding acts on the host and thereby lead to alterations in the microbiota. The trace element zinc is known to be essential for the

epigenome as it is involved in several pathways which are relevant for epigenetic mechanisms [230, 231]. For weaned piglets the DNA methylation and gene expression of the ZIP4 zinc uptake transporter of the small intestine were investigated under the influence of high dietary zinc feeding [232, 233]. However, studies with a focus on epigenetic alterations which might influence the microbiome have to be performed to get an idea if epigenetic plays a role for the increasing resistance rates under the influence of high dietary zinc feeding. If high dietary zinc feeding influences the microbiota via effects on the host this can lead to a longer delay until when alterations are detectable. This might be an additional explanation for the circumstance that the impact of high dietary zinc feeding on multi-resistance was only detectable at the age of 52±2 days.

In conclusion, it is necessary to mention that the complex microbiota composition of piglets in combination with the study design did not allow to identify the mechanisms by which high dietary zinc feeding leads to an increased resistance of intestinal *E. coli*. Therefore, the second part of this study investigated the influence of high dietary zinc feeding on bacterial conjugation as one possible mechanism leading to the observed effects on resistance.

4.2 Bacterial conjugation as a possible mechanism for an increased resistance in the porcine *E. coli* population under the influence of high dietary zinc feeding

As described above, it can be assumed that high dietary zinc feeding for piglets increases the abundance of resistant bacteria. However, the mechanisms by which high dietary zinc feeding increases especially the abundance of multi-resistant *E. coli* are still unknown.
Several hints exist that zinc influences the bacterial conjugation. Already in the early seventies the influence of zinc on bacterial conjugation was investigated with male Hfr donor and female F- recipient *E. coli* strains [171, 172, 234]. Beside this, as discussed previously, results of our recent zinc pig feeding trial lead to the suggestion that high dietary zinc feeding enhance bacterial conjugation and increase therefore the abundance of multi-resistant *E. coli* [169].
As bacterial conjugation is also one very important driving factor for the distribution of resistance genes, the second part of this work further focuses on the question if high dietary zinc feeding increases bacterial conjugation.
As the complex microbiota of piglets is not useful to investigate this question, gnotobiotic mice were used as model organisms for *in vivo* conjugation experiments. To get a comprehensive view on this topic also *in vitro* mating in liquid media under the influence of zinc was investigated.

4.2.1 Bacterial conjugation is not effected by high dietary zinc feeding

With view on all performed investigations during this study, the diverse results do not allow to identify a major effect of zinc on bacterial conjugation. Although some hints exist that zinc decrease the bacterial conjugation, not all results support this suggestion.
For *in vivo* as well as *in vitro* investigations, two different *E. coli* strains were used as recipient strains in combination with one *K. pneumoniae* donor strain to prevent the detection of a strain-specific effect.
Because of technical reasons, two different species were used as recipient and donor strain. Individual colony morphologies for both species allow to distinguish between the *K. pneumoniae* donor and the *E. coli* transconjugant on the donor agar plates, where also the transconjugant could grow. Therefore, the direct determination of donor cfu values was possible. The usage of different species and selective media for donor and recipient/transconjugant respectively was previously described [125, 126, 128] and allows an additional phenotypic verification of the strains.
E. coli was chosen as recipient to simulate the zinc pig feeding trial. In general, one can distinguish between the potential and readiness of the recipient and the donor to perform

conjugation. If zinc increases the activity of *E. coli* as donor, it might be that an increasing effect of zinc on bacterial conjugation is measured. However, this result cannot really explain the results of the pig trial as, in this case, the *E. coli* donor is already resistant from the start. For the question if zinc feeding increases the *E. coli* resistance rates via bacterial conjugation, it is therefore more important to analyze whether *E. coli* as recipient has an increased uptake of resistance plasmids.

Two different analyses were used for the evaluation if zinc influences the bacterial conjugation. On the one hand the lg (cfu/g sample) values were compared for each strain between the two feeding groups and, on the other hand, the recipient conjugation rates and the donor conjugation rates were compared between the two feeding groups or the four zinc concentrations for *in vitro* mating respectively. The recipient conjugation rate describes the percentage of transconjugant to recipient cells. In a simplified model, one recipient can only take up one plasmid. The donor conjugation rate describes the percentage of transconjugant to donor cells. Here, it is possible that one donor gives its plasmid after a recovery time [235] to multiple recipient cells. Often, studies use only one of the mentioned rates but both rates are described [123, 125, 128].

At first, feces were investigated over a period of 13 days. For both strain combinations donor cfu values reached the highest levels. Taking into consideration that the recipient was introduced first and in a way represents the established microbiota, this points toward a situation where both tested recipients had a competitive disadvantage compared to the donor. However, this phenomenon is already described for different other strain combinations [123] and does not interfere with conjugation.

Regarding the intestinal composition, one day after association with the donor an equilibrium existed between all three strains for strain combination I. This is in accordance with different other conjugation studies [123-125]. For strain combination II, the transconjugant increases steadily whereas recipient and donor almost stayed at stable levels. It might be that with a longer experimental duration also for this strain combination an equilibrium would establish. It was shown that without the presence of the antimicrobial substance positively selecting for the plasmid-encoded resistance, it can take more time for the development of the equilibrium [125].

Conjugation in the presence of such a selective pressure might serve as positive control for conjugation experiments as it increases the number of transfer events and leads to a growth advantage of the transconjugant [125]. However, the growth of our recipient strains was strongly influenced after the addition of 0.005µg/ml cefotaxime to LB media (data not shown) and as we primary focused on the zinc effect we renounced such positive control.

In general the continuous detection of transconjugants indicates a situation where the transferred plasmid leads not to a significant fitness loss or where a high rate of plasmid

transfer takes place. It was suggested that a dense colonization of the recipient prior to donor inoculation, as it was the case for both strain combinations during this study, is important for the colonization of the new transconjugants as they are already attached to the intestinal mucosa and do not need to compete for a new niche [125]. However, transconjugants can also successfully colonize if the donor is introduced first to the germfree mice [123].

For statistical analysis lg (cfu/g feces) values of this time period were integrated with the AUC approach. For strain combination I higher values for the transconjugant could be detected in the control group indicating that zinc decreases the conjugation rate. However, this result could only be supported by the donor conjugation rate and not by the recipient conjugation rate. The consideration of the lg (cfu/g feces) values alone is not as meaningful as all three parameters together. The two different zinc concentrations can have, beside their effect on conjugation, also an influence of the cfu values of the recipient and donor strain. If this is the case, both feeding groups have a different starting point for conjugation. However, for feces of strain combination I this was not the case.

The result that the recipient conjugation rate is not influenced by zinc is contrary to the previously detected increased fertility of recipients under the influence of Zn^{2+} [171]. In contrast, the decreased donor conjugation rate under high dietary zinc feeding is in accordance with the decreased fertility of donors in the surrounding of zinc [172].

For strain combination II, the lg (cfu/g feces) values of the recipient were significantly different with higher values for the control group. One might suspect that, therefore the recipient conjugation rates will be lower in the control group. However, this was not the case. Surprisingly, the donor conjugation rates were significantly higher in the control group even though no significant differences were detectable between the lg (cfu/g feces) values of the donor and transconjugant itself. This might be due to the fact that individual combinations of lg (cfu/g feces) values of donor and transconjugant of one mouse have an effect and that the conjugation rates were very low.

It is known that differences between distinct intestinal sections exist [131]. Therefore, on the day of sacrificing, besides feces, also caecum digesta and small intestine digesta were investigated. The similarity of the lg (cfu/g sample) values for feces and caecum digesta, which differ from the small intestine digesta is in accordance with a different study [129]. However, for colon material also higher conjugation rates were described compared to feces [123].

With respect to zinc, again no consistent picture about an influence of zinc feeding on bacterial conjugation was detected for digesta samples. Zinc has a negative influence on conjugation in the small intestine digesta of strain combination I and on conjugation in caecum digesta for strain combination II. However, these results were not supported by the other strain combination and with regard to the zinc pig feeding trial where a strong influence

of high dietary zinc feeding was measured for all three tested habitats, it seems unlikely that bacterial conjugation has a major effect on this result.

For the evaluation of the observed transfer frequencies of the performed *in vivo* experiments, it is necessary to keep in mind the differences between conventional colonized and gnotobiotic animals. Gnotobiotic animals display a high intestinal density of conjugative strains as they are only inhabited by recipient, donor and transconjugant. However, as the intestinal microbiota is a well-known resistance barrier against new introduced bacteria it occurs that introduced recipient and donor will be eliminated in conventional animals and no conjugation could be detected [129, 236, 237]. As for conjugation experiments, characterized recipient and donor strains have to be established in the intestine, gnotobiotic animals are therefore an adequate model used for conjugation studies.

To complete the investigations on the influence of zinc on bacterial conjugation, *in vitro* conjugation in liquid media was performed. As the zinc concentrations in the caecum digesta were on average 95.3 mg/kg for the zinc group and 11.7 mg/kg for the control, the four different zinc concentrations 1.2, 8.8, 77.4, and 115.5 mg/l medium, which enclose the digesta concentrations, were chosen. Liquid mating media were incubated statically to allow efficient mating pair formation. The *in vitro* data somehow confirm the *in vivo* results as again no clear trend was identifiable. For strain combination I after 24 hours, a higher donor conjugation rate was measured for 1.2 and 8.8 mg/l compared to 115.5 mg/l indicating a negative influence of zinc on conjugation. However, these results were not supported by the recipient conjugation rate. This can be explained by the fact that, beside the transconjugant, also the recipient cfu dropped down with higher zinc concentrations. For strain combination II, significantly higher values were detectable for the 8.8 mg/l compared to 115.5 mg/l. Again, this result was not supported by the recipient conjugation rate and beside this no significant difference between 1.2 mg/l and 115.5 mg/l was detectable.

In general, *in vivo* conjugation rates reached higher values than the *in vitro* conjugation rates. This is in accordance with different other studies which hypothesize an underestimation of intestinal conjugation rates by *in vitro* conjugation experiments [123, 125, 128, 129, 238]. However, when comparing *in vivo* and *in vitro* conjugation rates which were only obtained 24 hours after mating starts, the differences were lower and for strain combination II *in vitro* conjugation rates were even higher than *in vivo*.

Taken together, all *in vivo* and *in vitro* results of this study can give no definite answer if zinc influences the bacterial conjugation. Some results indicate that zinc might negatively influence the conjugation. However, despite having only three different strain populations in the whole intestine or the liquid media these kinds of experiments are still highly complex. Already mentioned was the fact, that zinc influences the recipient and the donor population in cell division and cell death which is especially the fact for the performed *in vitro* experiments.

The cfu values for the transconjugants are a result of real conjugation from donors and transconjugants with recipients and of cell devision of transconjugants and can be very versatile. Moreover, it should be kept in mind that the transconjugant can lose the plasmid again as well. Finally, these experiments do not allow to distinguish between bacterial transformation and conjugation as during cell death of the donor the plasmid would be released. During the performed investigations, the highly variable cfu values for the *E. coli* population underline the high flexibility of this system.

Also, it is possible that no influence of zinc on bacterial conjugation was determined because it might be that only a narrow range of the zinc concentration has an influence, which was not tested. Individual strains and plasmids could also be differently influenced by zinc. As already mentioned, the microbiota of spf piglets is highly complex. It might be that the bacterial conjugation of some isolates is enhanced during high dietary zinc feeding or that some plasmids interfere with zinc and offer a fitness advantage for transconjugants. During our investigations only one donor with one ESBL plasmid was tested, which is a limitation of this study.

Taken together, the conjugation data generated during this study support the assumption that bacterial conjugation is not the major factor driving the results of increased resistance rates under the influence of high dietary zinc feeding, which were observed in the pig trial of this work. In the light that high dietary zinc feeding for pigs promoted the persistence of multi-resistant isolates rather than leading to an increase of the multi-resistant isolates over time, the results of the pig and mice part are in accordance with each other. Beside this, also the MLST data which revealed that multi-resistant isolates mainly belong to ST10 and ST34 point toward a situation where mainly the phylogenetic background of the isolates is important for the resistance effect under the influence of high dietary zinc feeding and no high transfer of resistance plasmids takes place between *E. coli* isolates of different phylogenetic lineages.

4.3 Evaluation of high dietary zinc feeding as alternative to antimicrobial growth promoters

Taken together, the results of the zinc pig feeding trial supports the results of our recent study where a massive increase of multi-resistant *E. coli* was detected under the influence of high dietary zinc feeding [169]. The sampling schedule focuses on antimicrobial resistance and therefore ruled out any influence of the clonal classification during the first study and the additional investigation of two gut habitats increase the impact of this pig study. Although this study is not able to identify bacterial conjugation as main force leading to this increased resistance we could show that high dietary zinc feeding promoted the persistence of multi-resistant *E. coli* and that the effect is independent of the total *E. coli* population numbers.

In conclusion, the results of the pig part fully support the hypothesis that high dietary zinc feeding in no acceptable alternative to antimicrobial growth promoters.

Besides the association of zinc feeding with antimicrobial resistance found during this study and by others [166-168, 170], also different other information currently available question the usage of high dietary zinc feeding.

General data on growth performance of weaning piglets with high dietary zinc feeding are contrary. Although several studies detect a positive influence of high dietary zinc feeding on growth performance [139, 195-197], other studies were not able to determine such positive effects [168, 239, 240]. The positive effect on growth performance seems to depend on many different variables. However, this variability and unpredictability of their effects is also described for other feed additives [213].

Furthermore one has to consider that pig manure can be applied on agricultural fields. Increased amounts of resistant bacteria and resistance genes which were detected for high dietary zinc feeding could therefore be distributed [241, 242]. Furthermore the application of pig manure leads to a transfer of zinc to agricultural fields and thus to the environment [243, 244]. There, heavy metals can promote over the different mechanisms of co-selection the transfer and distribution of resistance genes [245].

Summing up, the mentioned points indicate that the usage of high dietary zinc as an alternative for antimicrobial growth promotors is highly questionable and thus inappropriate.

4.4 Outlook

To get further insights in the involved mechanisms leading to the increased resistance rates under high dietary zinc feeding, in-depth analysis of the whole genome sequencing data already generated is necessary. For example it would be of interest to investigate the relationship of the isolates on a deeper level than MLST, to search for zinc related genes which might be connected to fitness advantages, to investigate the different types of plasmids of the isolates (inc/rep typing) and to further investigate antimicrobial resistances on the genome level. Additionally, whole genome sequencing for isolates from the time points 24±2 and 38±2 days of age would be beneficial to shed light on the changes over time. Also, the investigation of differences between the three tested habitats would be of interest.

Beside this, feces of the zinc pig feeding trial were stored for 16SrRNA gene quantification. The investigation of metagenome data can give additional insights in involved mechanisms which not directly targeting the *E. coli* population.

Finally, it is important to highlight the necessity to use holistic approaches in antimicrobial resistance research to control this world-wide emerging problem. The results of our pig feeding trial clearly indicate that, beside the usage of antimicrobial substances, additional factors exists which contribute to the spread of resistant bacteria. Therefore, research should also focus on different factors to combat the spread of antimicrobial resistance for example on heavy metals like zinc, the influence of tenacity to disinfectants, virulence factors, and metabolic features to mention a few.

Summary

The influence of high dietary zinc feeding on antimicrobial resistance of intestinal *Escherichia coli*

The usage of feed additives to increase growth performance and health in livestock is very common. For pigs, zinc oxide in concentrations ranging from 2000 – 3000 mg zinc/kg diet is often used after weaning. However, more and more evidence exists that high dietary zinc feeding is associated with the emergence of antimicrobial resistant bacteria in the pigs.

As antimicrobial resistant bacteria represent a worldwide problem for human and veterinary medicine, it was necessary to shed further light into this highly important area. This study focuses on *E. coli*, a highly variable bacterial species which is ubiquitously distributed, colonizes the intestine of warm-blooded animals, birds, and reptiles and is of clinical relevance as beside commensal strains also pathogenic ones of intestinal and extraintestinal pathogenicity exist. For pigs *E. coli* represent an important infectious agent for diarrhea of suckling and weaned piglets which lead to large economic losses in the pig industry.

In the first part of this study, a zinc pig feeding trial was performed, focusing on in-depth analysis of antimicrobial resistant *E. coli*. For three different habitats (feces, digesta, and mucosa), absolute *E. coli* numbers were investigated at different time points around weaning for the zinc and the control group. For 2665 isolates, phenotypic antimicrobial resistance was determined and confirmed by minimum inhibitory concentration testing for random samples. Finally, whole genome sequencing and multi-locus sequence typing were performed for random samples of porcine isolates.
In piglets of the zinc group, a substantial increase of multi-resistant *E. coli* was detected in all gut habitats tested, ranging from 28.9-30.2% multi-resistant *E. coli* compared to 5.8-14.0% in the control group. This zinc effect was independent of the total number of *E. coli,* for which a general decrease over time was determined for both groups. The higher resistance rates of the zinc group, therefore, seem to be linked with persistence of the resistant population under the influence of high dietary zinc feeding. Interestingly, mainly isolates of two different phylogenetic clusters namely ST10 and ST34 are involved in the increased resistance of the zinc group.

In the second part of this study, conjugation experiments were performed to investigate if bacterial conjugation is influenced by zinc and therefore, plays a role for the detected increased resistance under high dietary zinc feeding. Values for colony forming units and conjugation rates were investigated for two different recipient/donor combinations. For *in vivo*

experiments with gnotobiotic mice, feces were investigated daily over 20 days, and caecum digesta and small intestine digesta were examined to complement the analysis. In addition four different zinc concentrations were tested *in vitro*.
Overall, some results indicate that zinc decreases the bacterial conjugation. However, results were very versatile and not all results support this suggestion. Therefore, an increasing effect of high dietary zinc feeding on plasmid exchange is unlikely.

In conclusion, results of this study further corroborate recent reports on the association of high dietary zinc feeding with antimicrobial resistance. Both parts of this study point toward a situation where mainly the phylogenetic background of the plasmid host bacteria is of importance for the increased resistance and an increased plasmid exchange plays only a minor role.
Although the mechanism by which high dietary zinc feeding leads to the increased resistance is still not clear, this study with the clear focus on resistance confirms the hypothesis that high dietary zinc feeding is no acceptable alternative to antimicrobial growth promoters. The usage of high dietary zinc feeding should therefore be stopped.

Zusammenfassung

Der Einfluss hoher Zinkkonzentrationen in Futtermitteln auf die Antibiotikaresistenz von intestinalen *Escherichia coli*

Die Anwendung von Futterzusatzstoffen in der Landwirtschaft ist weit verbreitet um Wachstum und Gesundheit der Tiere zu unterstützen. So werden Schweine nach dem Absetzen oft mit Zinkoxid in Konzentrationen von 2000 – 3000 mg Zink/kg Futter gefüttert. Jedoch häufen sich die Hinweise, dass das Füttern dieser hohen Zinkkonzentrationen zu einem erhöhten Aufkommen von antibiotikaresistenten Bakterien führt.

Antibiotika resistente Bakterien stellen ein weltweites Problem in der Human- und auch Tiermedizin dar. Daher war es nötig den Hinweisen auf einen Zusammenhang zwischen Zinkfütterung und Antibiotikaresistenzen weiter nachzugehen und diese noch genauer zu untersuchen. Die aktuelle Studie fokussiert sich dabei auf Bakterien der Spezies *E. coli*. Bakterien dieser Spezies sind sehr variabel, ubiquitär verbreitet, besiedeln den Darmtrakt von Warmblütern, Vögeln und Reptilien und haben eine hohe klinische Bedeutung da neben den kommensalen auch pathogene Bakterien existieren die zu intestinalen und extraintestinalen Erkrankungen führen können. Bei Schweinen stellen *E. coli* einen wichtigen Krankheitserreger dar, der bei säugenden und abgesetzten Ferkeln zu Durchfällen führt und mit großen wirtschaftlichen Verlusten einhergeht.

Im ersten Teil der aktuellen Studie wurde ein Schweine-Zink-Fütterungsversuch durchgeführt, der im Versuchsaufbau klar auf die Untersuchung von antibiotikaresistenten *E. coli* ausgerichtet war. Sowohl für die Zink- als auch für die Kontroll- Gruppe wurden die absoluten *E. coli* Zahlen für drei unterschiedliche Habitate (Fäzes, Digesta und Mukosa) zu drei unterschiedlichen Zeitpunkten um das Absetzen herum untersucht. 2665 Isolate wurden auf ihre phänotypische Antibiotikaresistenz untersucht und für eine repräsentative Stichprobe dieser Isolate wurde zur Bestätigung zusätzlich die minimale Hemmkonzentration bestimmt. Abschließend wurden Isolate einer Stichprobe Gesamtgenom-sequenziert und die Sequenztypen für die entsprechenden Isolate bestimmt.
Für Schweine der Zinkgruppe wurde in allen untersuchten Habitaten ein erheblicher Anstieg der multi-resistenten *E. coli* detektiert mit 28.9-30.2% multi-resistenten *E. coli* in der Zinkgruppe und 5.8-14.0% in der Kontrollgruppe. Dieser Zink-Effekt war unabhängig von der Gesamtzahl der *E. coli*, für welche ein Absinken über die Zeit in beiden Futtergruppen beobachtet wurde. Die höheren Resistenzraten der Zinkgruppe scheinen daher durch die Persistenz der resistenten Population unter dem Einfluss der Fütterung von hohen Zinkkonzentrationen zu entstehen. Interessanterweise sind hauptsächlich Isolate aus den

zwei phylogenetischen Clustern ST10 und ST34 an der vermehrten Resistenz in der Zinkgruppe beteiligt.

Im zweiten Teil der Studie wurden Konjugationsexperimente durchgeführt um zu analysieren ob die Konjugation von Bakterien durch Zink beeinflusst wird und somit für die erhöhte Resistenz unter dem Einfluss von hohen Zinkkonzentrationen eine Rolle spielt. Die Anzahl der koloniebildenden Einheiten und die Konjugationsraten wurden für zwei unterschiedliche Akzeptor/Donor Kombinationen untersucht. In *in vivo* Experimenten mit gnotobiotischen Mäusen wurde über 20 Tage Fäzes und abschließend auch zusätzlich Caecumdigesta und Dünndarmdigesta untersucht. Zusätzlich wurden vier unterschiedliche Zinkkonzentrationen *in vitro* untersucht.
Zusammenfassend deuten einige Ergebnisse darauf hin, dass Zink die bakterielle Konjugation verringert. Jedoch waren die Ergebnisse sehr variabel und nicht alle Ergebnisse unterstützen diese Annahme. Eine Steigerung des Plasmidaustausches durch Fütterung von hohen Zinkkonzentrationen ist somit sehr unwahrscheinlich.

Die Ergebnisse dieser Studie untermauern somit den beschriebenen Zusammenhang von Fütterung hoher Zinkkonzentrationen mit erhöhter Antibiotikaresistenz. Beide Teile der Studie deuten darauf hin, dass der phylogenetische Hintergrund der plasmidtragenden Stämme bedeutend für den Anstieg der Resistenzen ist und ein erhöhter Plasmidaustausch nur eine Nebenrolle spielt.
Obwohl der Mechanismus nicht geklärt werden konnte, über welchen es zu einem Anstieg der Resistenz unter der erhöhten Zinkfütterung kommt, zeigt die Studie mit ihrem Fokus auf Resistenz, dass das Füttern hoher Zinkkonzentrationen keine akzeptable Alternative zum Einsatz von antimikrobiellen Wachstumsförderern ist. Der Einsatz von hohen Zinkkonzentrationen in der Fütterung sollte daher gestoppt werden.

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Abbreviations

aEPEC	Atypical enteropathogenic *E. coli*
AIEC	Adherent invasive *E. coli*
Amp	resistant against ampicillin in the analysis of the resistance pattern (3.1.5.)
APEC	Avian pathogenic *E. coli*
AUC	Area under the curve
BW	Bodyweight
CDC	Center for Disease Control and Prevention
Cfu	Colony forming units
DAEC	Diffusely adherent *E. coli*
E.	*Escherichia*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FDA	United States Food and Drug Administration
InPEC	Intestinal pathogenic *E. coli*
K.	*Klebsiella*
KPC	*K. pneumoniae* carbapenemase
LB	Lysogeny broth
Lg	Log_{10}
MCG	Maximum common genome
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence type
MRSA	Methicillin-resistant Staphylococcus aureus
NMEC	Newborn meningitic *E. coli*
PBS	Phosphate buffered saline
Rpm	Rounds per minute
SePEC	Septicemia associated *E. coli*
ST	Sequence type
STEAEC	Shiga toxin producing enteroaggregative *E. coli*
STEC	Shiga toxin producing *E. coli*
Strep	resistant against streptomycin in the analysis of the resistance pattern (3.1.5.)

Sul/Tri	resistant against sulfamethoxazole-trimethoprim in the analysis of the resistance pattern (3.1.5.)
T4SS	Type IV secretion system
Tet	resistant against tetracycline in the analysis of the resistance pattern (3.1.5.)
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infections

Publikationsverzeichnis

Poster

Rabes L., Guenther S., Vahjen W., Wieler L.H.: The influence of zinc on the spread of antimicrobial resistance genes. Tagung der Deutschen Veterinärmedizinischen Gesellschaft-Fachgruppe “Bakteriologie und Mykologie”, Freising, Germany, 2014

Rabes L., Guenther S., Doherr M.G., Wieler L.H., Loh G.: Impact of high dietary zinc concentrations on the horizontal antibiotic resistance gene transfer between enterobacteria in the intestine of gnotobiotic mice. (Einfluss hoher Zinkkonzentrationen im Futter auf den horizontalen Transfer von Antibiotikaresistenzgenen in Darm gnotobiotischer Mäuse).
70. Tagung der Gesellschaft für Ernährungsphysiologie, Hannover, Germany, 2016

Talk

Loh G.: Effect of dietary zinc on the horizontal transfer of antibiotic resistance genes in the intestine. 9th Seeon Conference “Microbiota, Probiotics and Host”, Seeon, Germany, 2016

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.
Abbildung 2 (Fig. 2) wurde im Original von BioMed Central herausgegeben. Die Abbildung stammt aus der Publikation „Q&A: Antibiotic resistance: where does it come from and what can we do about it?" von Wright, G.D. (BMC Biol, 2010. 8: p. 123.) und darf laut den "Nachdruck und Genehmigung" Richtlinien von BioMed Central unverändert reproduziert werden.

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