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The broader context of antibiotic resistance: Zinc feed supplementation of piglets increases the proportion of multi-resistant *Escherichia coli* in vivo

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ABSTRACT

Following the Europe-wide ban of antimicrobial growth promoters, feed supplementation with zinc has increased in livestock breeding. In addition to possible beneficial effects on animal health, feed supplementation with heavy metals is known to influence the gut microbiota and might promote the spread of antimicrobial resistance via co-selection or other mechanisms. As *Escherichia coli* is among the most important pathogens in pig production and often displays multi-resistant phenotypes, we set out to investigate the influence of zinc feed additives on the composition of the *E. coli* populations in vivo focusing on phylogenetic diversity and antimicrobial resistance.

In a piglet feeding trial, *E. coli* were isolated from ileum and colon digesta of high dose zinc-supplemented (2500 ppm) and background dose (50 ppm) piglets (control group). The *E. coli* population was characterized via pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) for the determination of the phylogenetic background. Phenotypic resistance screening via agar disk diffusion and minimum inhibitory concentration testing was followed by detection of resistance genes for selected clones.

We observed a higher diversity of *E. coli* clones in animals supplemented with zinc compared to the background control group. The proportion of multi-resistant *E. coli* was significantly increased in the zinc group compared to the control group (18.6% vs. 0%).

For several subclones present both in the feeding and the control group we detected up to three additional phenotypic and genotypic resistances in the subclones from the zinc feeding group. Characterization of these subclones suggests an increase in antimicrobial resistance due to influences on plasmid uptake by zinc supplementation, questioning the reasonability of zinc feed additives as a result of the ban of antimicrobial growth promoters.

Introduction

With the intention of decreasing the risk of infectious diseases and gaining additional growth promotion in-feed antibiotics have been used and misused for decades (Jensen, 2006; Villanueva, 2012). Driven by the emergence of multi-resistant Enterobacteriaceae (Ewers et al., 2012) in animal production in recent years, the use of antimicrobial growth promoters as feed additives has been banned in the European Union since 2006 (Casewell et al., 2003). Several alternatives have been considered to promote growth and reduce the pathogen load in animal breeding, which is required in intensive livestock production. Non-antimicrobial substances with possible beneficial effects on animal health are used as alternatives to antibiotics in livestock farming, including prebiotics, probiotics or cationic trace elements such as zinc and copper as feed supplements (de Lange et al., 2010; Heo and Opapeju, 2012; Lalles et al., 2007).

Zinc is an important trace element naturally present in feed and it is involved in various physiological functions. In addition to other heavy metals zinc is an essential micronutrient necessary

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for several cellular functions as well as components of DNA- and RNA-polymerases (Holzel et al., 2012). As higher concentrations of zinc are toxic for microbes, uptake and efflux of zinc in prokaryotes is tightly regulated (Nies, 1999). Under conditions of deficiency, zinc uptake of *E. coli* is accomplished by the high-affinity ABC transporter ZnuABC (Patzer and Hantke, 1998). The efflux of zinc, important for zinc resistance as well, involves two different families of transporters (Grass et al., 2002). First of all, P-type ATPases span the inner membrane and use ATP energy to pump metal ions from the cytoplasm to the periplasm. This is the case for ZntA, a metal-translocating P1-type ATPase from *Escherichia coli*, conferring resistance to Pb(II), Cd(II), and Zn(II) (Rensing et al., 1997; Sharma et al., 2000). ZitB, a prototype transporter of the cation diffusion facilitator (CDF) family, has also been described in *E. coli* (Anton et al., 2004). Members of this family act as chemiosmotic ion–proton exchangers (Grass et al., 2002).

In the long term however, the "zinc-alternative" to antimicrobials might be of limited value, as heavy metals, including zinc, used in animal farming might promote the spread of antibiotic resistance via co-selection and/or other mechanisms (Baker-Austin et al., 2006; Cavaco et al., 2011; Holzel et al., 2012; Seiler and Berendonk, 2012). Possible mechanisms include physiological coupling (cross-resistance), as is the case for interactions of heavy metals with efflux pumps and genetic coupling (co-resistance) (Lee et al., 2005; Nishino et al., 2007), or genetically linked resistance genes to antimicrobials and heavy metals on mobile genetic elements (Bass et al., 1999; Ghosh et al., 2000; Seiler and Berendonk, 2012). Furthermore direct interaction of heavy metals with antibiotic compounds (e.g. heavy metal cation complexes with tetracyclines (Palm et al., 2008)) and interaction of heavy metals with bacterial conjugation systems are other possible mechanisms of interaction (Holzel et al., 2012; Ou, 1973; Ou and Anderson, 1972). For many of these mechanisms, a role of zinc has already been reported, for example zinc affects the stability of ampicillin (Mukherjee and Ghosh, 1995), there are zinc-dependent beta-lactamases (Cooper et al., 1993) and zinc may influence bacterial conjugation rates (Ou, 1973; Ou and Anderson, 1972).

Recent results have indicated that antimicrobial resistance in porcine *E. coli* might be increased by copper and zinc (Holzel et al., 2012). *E. coli*, a member of the gastrointestinal microbiota of mammals and birds, can be grouped into nonpathogenic (commensal) and pathogenic strains, causing intestinal (InPEC) or extraintestinal diseases (ExPEC) (Johnson and Russo, 2002; Kaper et al., 2004). Commensal *E. coli* contribute to the maintenance of the microbial gut balance (Gordon and Cowling, 2003). In addition to aspects regarding pathogenicity and commensalism, *E. coli* is also ubiquitous in the environment and in wildlife, and is one of the key organisms in terms of antimicrobial resistance in human and veterinary medicine (Canton and Coque, 2006; Ewers et al., 2012; Guenther et al., 2011; Martinez, 2009). As porcine intestinal *E. coli* populations are dynamic (Casewell et al., 2003; Schierack et al., 2007) and are influenced by various factors including diet (Duriez et al., 2001; Franklin et al., 2002), supplementation of zinc as a feed additive might change the composition of the *E. coli* microbiota both in terms of diversity and antimicrobial resistance (Cavaco et al., 2011; Vahjen et al., 2010, 2011).

Based on these observations, we hypothesized that zinc may have a direct effect on the *E. coli* diversity and resistance in the gut of animals. Indeed, our cumulative data show high zinc concentrations increase the diversity and the proportion of multi-resistant *E. coli*. These data question the suitability of zinc feed additives as a substitute for antimicrobial growth promoters.

Materials and methods

Animals, housing and diets

All procedures involving animal handling and treatment were approved by the local state office of occupational health and technical safety 'Landesamt für Gesundheit und Soziales, Berlin' (LaGeSo Reg. Nr. 0347/09). A total of 126 purebred landrace piglets were weaned at 26 ± 1 days of age with a mean body weight of 7.6 ± 1.1 kg and randomly allocated into two treatment groups balancing for gender, litter and body weight. Animals were housed in pens (n = 2 per pen) with straw bedding and ad libitum access to feed and water. No antibiotics were administered to the piglets before and during the experiment.

During the four week experimental period, piglets received a commercial starter diet based on wheat, barley and soybean meal which was formulated to meet the requirements according to NRC recommendations (2008). All diets were prepared in the feed mill of the Institute of Animal Nutrition, Berlin, Germany. The dietary zinc level was adjusted to approximately 50 or 2500 mg/kg by supplementation of corn starch with analytical grade zinc oxide (Sigma Aldrich, Taufkirchen, Germany). The dietary zinc levels (i.e. 57 mg/kg and 2425 mg/kg, respectively) were confirmed by atomic absorption spectrometry analysis.

Sampling

A total of 36 piglets (n = 6 per group and each time point) were euthanized on 32 ± 2, 39 ± 2, and 53 ± 2 d of age, treatment groups were balanced for litter and gender. The piglets were sedated with 20 mg/kg BW of ketamine hydrochloride (Ursotamin®, Serumwerk Bernburg AG, Germany) and 2 mg/kg BW of azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany) prior to euthanasia with intracardial injection of 10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61®, Intervet, Unterschleißheim, Germany). Following euthanasia and a midline abdominal incision, the entire intestinal tract was removed from the peritoneum and intestinal contents were collected from the ileum and ascending colon.

Isolation of E. coli

The isolation of *E. coli* followed methods described before (Schierack et al., 2009b). Digesta (0.2 g in 6 ml PBS) were plated by serial dilutions. To gain a high diversity of susceptible *E. coli*, CHROMagar orientation agar (CHROMagar, Paris, France) (Merlino et al., 1996), sheep blood and Gassner agar plates (Sifin, Berlin, Germany) were used. To obtain a maximum diversity of antimicrobial resistant *E. coli*, CHROMagar orientation agar with supplementation of the estimated breakpoint concentrations of ampicillin (≥32 mg/ml), streptomycin (≥64 mg/ml), chloramphenicol (≥32 mg/ml), gentamicin (≥16 mg/ml), tetracycline (≥16 mg/ml) and cefotaxim (≥4 mg/ml) was used (Guenther et al., 2010).

Isolates were assumed to be *E. coli* if colonies showed a typical pink color on CHROMagar orientation and a blue color on Gassner agar plates after incubation at 37 °C for 24 h. Additional basic biochemistry confirmed the results. Up to 30 colonies each per plate (each representing a single isolate) and specimen were randomly chosen and subcultivated on CHROMagar and sheep blood agar for the identification of phenotypic hemolytic isolates and incubated at 37 °C for 24 h.