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MINI REVIEW

Zinc and copper in animal feed – development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin

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Farmed animals such as pig and poultry receive additional Zn and Cu in their diets due to supplementing elements in compound feed as well as medical remedies. Enteral bacteria in farmed animals are shown to develop resistance to trace elements such as Zn and Cu. Resistance to Zn is often linked with resistance to methicillin in staphylococci, and Zn supplementation to animal feed may increase the proportion of multi-resistant *E. coli* in the gut. Resistance to Cu in bacteria, in particular enterococci, is often associated with resistance to antimicrobial drugs like macrolides and glycopeptides (e.g. vancomycin). Such resistant bacteria may be transferred from the food-producing animals to humans (farmers, veterinarians, and consumers). Data on dose-response relation for Zn/Cu exposure and resistance are lacking; however, it seems more likely that a resistance-driven effect occurs at high trace element exposure than at more basal exposure levels. There is also lack of data which could demonstrate whether Zn/Cu-resistant bacteria may acquire antibiotic resistance genes/become antibiotics resistant, or if antibiotics-resistant bacteria are more capable to become Zn/Cu resistant than antibiotics-susceptible bacteria. Further research is needed to elucidate the link between Zn/Cu and antibiotic resistance in bacteria.

Keywords: *Zinc*; *copper*; *animal feed*; *bacteria*; *antimicrobial resistance*

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Zinc (Zn) and copper (Cu) are essential trace elements for all forms of life and perform several biological functions. Both Zn and Cu have a ubiquitous cellular distribution and are important structural components or regulatory co-factors of a wide range of different enzymes in various important biochemical pathways in both plants and animals. Organisms have developed a homeostatic capacity that allows them to regulate the internal concentration of essential elements to a certain extent and to maintain it at optimal levels under varying external essential element availability. However, the capacity of this regulation is limited and when the external concentration becomes too high or too low, toxicity or deficiency can occur (1).

Farmed animals such as pig and poultry receive additional Zn and Cu in their diets via supplement of the elements in their compound feed as well as in medical remedies. The content of Zn and Cu in manure has been shown to be especially high from pigs and poultry and from other farmed animals receiving a high portion of their diet from compound feed. Zn and Cu are normally used in animal feed in concentrations in excess of the nutritional requirements of the animals and for prevention of diarrheal disease, and also as an alternative to in-feed antibiotics for growth promotion (2–4).

Enteral bacteria, both commensal and pathogenic, in farmed animals have been shown to develop resistance to trace elements such as Zn and Cu and concomitant cross-resistance to antimicrobial agents. Such bacteria may be transferred to other animals and human.

On request from the Norwegian Food Safety Authority, the Norwegian Scientific Committee for Food Safety has assessed the fate of Zn and Cu from feed and other sources, via pig and poultry intake to manure, and further to soil, and the long-term effects in the food chain and environment. The report is available at www.vkm.no. This review article is based on a part of that report regarding the development of resistance towards Zn and Cu

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in bacteria from poultry and pigs. We highlight the development of Zn/Cu resistance in bacteria, in particular because of the link with resistance to clinically relevant antibiotics among pathogenic bacteria.

Data source

A literature search using relevant terms such as 'Zn AND animal feed, bacteria, (antibiotic resistance OR antimicrobial resistance), requirement, bioavailability' using the Advanced Search Builder provided by PubMed (www.ncbi.nlm.nih.gov/pubmed) or Web of Science was performed. A similar search using the same terms, but 'Cu' instead of 'Zn' was also performed. The reference lists in the selected citation were scrutinized to identify additional articles or reports, overlooked by the searches. The titles of all hits were scanned, and for those that were of potential relevance, the abstracts were also scanned. Of these, for those of potential relevance, the full text was obtained and assessed whether it was of relevance to this paper. Original and review articles, and textbook content were included as references in this paper. A list of the articles on Zn/Cu driven co-selection of antibiotic resistance, which fulfilled the inclusion criteria with summary of the findings and main conclusion, is presented in Table 1.

The animal associated microbiota

The microbiota associated with animals represents a complex assemblage of microorganisms covering all three domains of life (Bacteria, Achaea and Eukarya) (5). All body sites are colonized, with the lower gastrointestinal tract being the most densely populated. In the number of cells, the microbiota generally outnumbers the host by a factor of 10, while with respect to the number of genes the microbiota contains 100 times more genes than the host. Thus, the gut microbiota can be considered an organ in itself (6).

The function of the microbiota is to protect the host from pathogen invasion, to train the immune system, to extract energy from low accessible nutrients, and in addition to produce essential vitamins and metabolites needed by the host (7). Generally, lower gut microbes are strictly anaerobic due to the anaerobic conditions in that area. The anaerobic conditions are mainly created by microbial respiration (8).

The porcine gut microbiota shows a dominance of the phyla *Firmicutes* and *Bacteroidetes*, which is similar to that of humans (9, 10), while the chicken microbiota is lower in *Bacteroidetes*, and higher in *Lactobacillus* and *Proteobacteria* (11). It has been proposed that diet is the main driver for the composition and functioning (nutrient breakdown and metabolite production) of the gut microbiota since microorganisms in the gut can utilize nutrient compounds that the host cannot break down (12).

Mainly due to the complexity, we have very limited knowledge of what shapes the host associated microbiota composition (10). The main unresolved questions are if the host-associated microbiota is shaped by bacterial–bacterial competition, or if the host can shape the composition (13).

Effect of Zn and Cu on pathogenic microorganisms in pigs and poultry

In microbial ecosystems there is a delicate balance between trace metals such as Zn and Cu as limiting factors, and the toxic effect of these (14). Zn and Cu are common co-factors in enzymes, while the effects of elevated exposure are more diverse, ranging from the replacement of other trace elements, binding to enzymes, and oxidation. It has been hypothesized that the antimicrobial effect of Zn and Cu leads to growth promotion in a similar manner as for the effect of antibiotic-based growth promoters (15). For Cu, although it has been reported that there is a shift in the microbiota associated with exposure (16), it could be that its main pathogen related effect is through increased host tolerance for lipopolysaccharides (17).

A range of pathogens can exploit host responses through inflammation induction (18, 19). For instance, upon inflammation the host will produce chelating agents such as calprotectin that will limit the availability of the trace elements and thereby bacterial growth (20). For Zn, however, recent evidence suggests that pathogens can have a competitive advantage over the commensal microbiota under Zn limiting conditions, thereby being promoted under an inflamed state (14, 21). Since diarrhea in itself can lead to Zn depletion and this could also promote the pathogen survival.

Mechanisms of antibacterial activity

The potential mechanisms of growth promotional effects of Zn/Cu are attributed to their antimicrobial activities, similar to that of antibiotics, in that gut microbiota are altered to reduce fermentation loss of nutrients and to suppress gut pathogens (22). The data from (22) illustrated reduced sizes of major groups of bacteria among the porcine gastrointestinal commensals, namely, the lactobacilli and streptococci by elevated doses of dietary ZnO and $CuSO_4$. The reduced level of these commensals in the proximal part of the gastrointestinal tract may benefit the host animal by allocating more feed components for its growth performance. Furthermore, feeding the animals high dietary ZnO doses resulted in an altered pattern of organic acid accumulation, with lower levels of lactate and succinate in the stomach and small intestine and an accumulation of these compounds in the cecum and colon. How this influences the physiology of the animals needs further elucidation in detail, since lactic acid produced in the stomach is normally considered a part of the natural defense mechanism of the host, whereas lactate accumulation

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Table 1. Zn/Cu and possible antimicrobial resistance in bacterial isolates of animal origin.

Reference	Country	Sample	Bacterial species /ARG	Susceptibility to Zn/Cu	Susceptibility to antimicrobial agents	Conclusion
(35)	Denmark	Pigs	MRSA, MSSA	Zn	Methicillin, erthyromycin, penicillin, tetracyclin	The use of Zn compounds may be partly implicated in the emergence of some MRSA clones.
(36)	Denmark	Livestock	*Salmonella*, *S. aureus*, *S. hyicus*, *E. faecalis* *S. faecium*	Heavy metals including Zn, Cu	Benzalkonium chloride, hydrogen peroxide, chlorhexidine	Acquired Cu resistance was only found in enterococci due to use of disinfection.
(38)	Denmark	Pigs and humans	*S. aureus*	Zn	Methicillin	Resistance to heavy metals such as Zn and cadmium may play a role in the co-selection of methicillin resistance in *S. aureus*.
(4)	Denmark	Pigs and calves	*S. aureus*	Zn, Cu	Methicillin	Zn resistance and the *czrC* gene are widespread among CC398 MRSA isolates and the use of Zn in feed might have contributed to the emergence of MRSA.
(31)	Denmark	Pigs, broilers, calves, sheep, and humans	*E. faecium*	Cu	Glycopeptides, Macrolides	The *tcrB* gene was found in *E. faecium* isolated from all animals but not in isolates from sheep. Cu resistance, and therefore the presence of the *tcrB* gene, was strongly correlated to macrolide and glycopeptide resistance in isolates from pigs.
(30)	Denmark	Pigs	*E. faecium, E. mundtii, E. casseliflavus, E. gallinarum*	Cu	Glycopeptides, Macrolides	A significant relationship between Cu resistance (*tcrB*), glycopeptide resistance (Tn1546), and macrolide resistance [*erm(B)*] in *E. faecium* isolated from pigs was found.
(39)	Denmark	Pigs	Enterococci	Cu	Glycopeptides	The *tcr* gene cluster mediates *in vitro* Cu resistance in *E- faecium*. and co-selection of macrolide- and glycopeptide-resistant *E. faecium* in the animal group fed the high level of Cu.

Table 1 (*Continued*)

Reference	Country	Sample	Bacterial species /ARG	Susceptibility to Zn/Cu	Susceptibility to antimicrobial agents	Conclusion
(37)	Germany	Piglets	*E. coli*	Zn	Ampicillin, streptomycin, chlroramphenicol, gentamicin, tetracycline, enrofloxacin, cetotaxime	The proportion of multi-resistant *E. coli* was significantly increased in the zinc group compared to the control group.
(40)	Germany	Pigs	*E. coli*	Zn, Cu, Mercury	Beta-lactams, aminoglycosides (29 different antimicrobial agents)	Antimicrobial resistance in the porcine microflora might be increased by Zn and Cu.
(26)	Portugal	Pigs and healthy persons	Enterococci	Cu	Vancomycin	The study indicates a current intra- and international spread of *E. faecium* and *E. faecalis* clones and their plasmids among pigs and humans.
(3)	USA	Piglets	*Enterococcus* sp.	Cu	Erythromycin	Supplementation of Cu in swine diets selected for resistance in enterocci.
(27)	USA	Heifers	*E. coli*, *Enterococcus* spp.	Zn, Cu	*E. coli*: Clindamycin, Erythromycin, tylosin penicillin, tiamulin, *Enterococcus*: Chloramphenicol, ciprofloxacin, gentamcin, linezolid, penicillin, streptomycin, vancomycin	Feeding elevated Cu and/or Zn to feedlot cattle had marginal effects on antimicrobial susceptibilities of fecal *E. coli* and enterococci.
(41)	China	Manures and soils collected from multiple feedlots	Antibiotic resistance genes detected by PCR	Zn, Cu	Chloramphenicol, tetracyclines, sulfonamide	Hg, Cu, and Zn, exerted a strong selection pressure and acted as complementary factors for ARG abundance.
(42)	China	Manure and compost from pig farms, Soil	Anitibiotic resistance genes detected by PCR	Heavy metals (Zn, Cu)	149 antibiotic resistance genes	Quantitative PCR arrays detected 149 unique resistance genes among all of the farm samples.
(28)	Republic of Korea	Bovine milk	*Enterococcus* spp.	Cu	Erythromycin	79.2% of the isolates displayed erythromycin resistance and 4.5% displayed Cu resistance and the Cu resistance gene*, tcr(B).*

MRSA: methicillin resistant *Staphylococcus aureus;* MSSA: methicillin sensitive *Staphylococcus aureus*, ARG: antimicrobial resistance gene

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in the large intestine has mainly been observed in connection with various disorders. $CuSO_4$ reduced the number of coliforms in the large intestine, which may be a part of other mechanisms, such as the suppression of specific pathogens and induced resistance of the animal to pathogen adhesion and invasion as well as pathogen-produced toxins (23).

Trace elements like Zn and Cu may be toxic to bacteria and this microbial toxicity may be due to their chemical affinity to the thiol groups of macro-biomolecules but also depends on the solubility of the metal compounds under physiological conditions.

To avoid cellular toxicity to elevated trace element exposure, bacteria have evolved mechanisms of metal tolerance. Both the mechanisms of toxicity and tolerance to trace elements in bacteria are discussed extensively in the review article of Seiler and Berendock (24). The authors concluded that in addition to antibiotic agents, heavy metals used in animal farming and aquaculture might promote the spread of antibiotic resistance via co-selection. It has been proved that antimicrobial agents other than antibiotics have the ability to promote a co-selection process, indirectly selecting for antibiotic resistance (25). The trace elements like Zn and Cu seems to have potential to act as a selective pressure that forces the proliferation and evolution of Zn/Cu and antibiotic resistance not only at the farm level, but also in the environment.

The total amounts and concentrations used of Zn and Cu in feed may differ among countries, due to f. ex. restrictions imposed by national legislation. As a consequence different selective pressure might be exerted within different countries. Data regarding development of resistance against Zn and Cu in bacteria of human origin is deficient. The average exposure of Zn and Cu in humans, from food, is probably usually far lower than the exposure in animals fed diets supplemented with these trace elements.

Resistance in animal microbiota

In bacterial isolates found in animals (Table 1), elevated minimum inhibitory concentration (MIC) values to Zn and Cu were detected in several opportunistic bacterial species compared to background isolates. The data presented in these studies indicate that such elevation in MIC-values may be due to elevated exposure of these trace elements in animal feed. MIC is defined as the lowest concentration of a given agent that inhibits growth of a microorganism under standard laboratory conditions. Testing for susceptibility against Zn/Cu in various bacterial species was performed using either a micro-dilution technique or an agar-dilution technique and under different methodological conditions (studies listed in Table 1). There is currently no standardized and approved method to determine the MIC values for Zn/Cu.

Among the examined bacterial species, the development of resistance to Cu in enterococci is associated with the presence of a Cu resistance gene (*tcrB*), which is often located on a plasmid (3, 26–28). In enterococci, the Cu resistance gene *tcrB* was shown to be associated with resistance to the macrolide antibiotic erythromycin (*ermB*). A conjugation study demonstrated co-transfer of *tcrB* and *ermB* genes between *E. faecium* and *E. faecalis* (3). Transferable Cu resistance *tcrB* has been reported in these enterococci isolated from piglets, calves, poultry, as well as humans in Denmark (29). Several studies performed in Denmark show a link between resistance to Cu and resistance to macrolides and also to glycopeptides (vancomycin) in enterococcal isolates of pig origin (30, 31). The authors concluded that there is a frequent occurrence of Cu resistance gene in these isolates, where Cu sulphate is being used in large amounts as feed additive. This may have contributed to co-selection resistance against macrolides and glycopeptides. Macrolides like erythromycin are commonly used in veterinary and human medicine. The glycopeptide antibiotic avoparcin has been used as a growth promoter in animal production by adding to feed, in many European countries, including Norway, in the past (from mid-1970s). However, it has been prohibited since the 1990s because of the development of vancomycin resistance in bacteria, in particular in enterococci. Vancomycin is an important human glycopeptide antibiotic. The discontinued use of avoparcin in animal feed has resulted in a reduction in the number of vancomycin-resistant organisms isolated from animals (32, 33). Because avoparcin and vancomycin are similar in structure, bacteria resistant to avoparcin are resistant to vancomycin as well.

Vancomycin-resistant enterococci (VRE) spread rapidly and have become a major problem in many countries. The possibility of transfer of vancomycin resistance genes from enterococci to other Gram-positive bacteria, like staphylococci, raises significant concerns about the emergence of vancomycin-resistant *S. aureus* (34). Nowadays, vancomycin constitutes one of the last resort antibiotics for treatment of MRSA infection in humans.

Several studies in Table 1 have demonstrated an association between resistance to Zn and resistance to methicillin in staphylococci (4, 35, 38). The study performed by Cavaco et al. (38) found that MRSA strains from pigs from European countries, Canada, and China had a high prevalence of Zn resistance (mainly associated with *czrC* gene), whereas the corresponding MSSA were susceptible. Similar association between resistance to Zn and resistance to methicillin was also observed in samples from veal farms from the Netherlands. Methicillin is not the drug of choice for treatment of infection in veterinary medicine, neither in Norway nor in any countries within EU. There is a lack of knowledge regarding the source of methicillin–Zn-resistant staphylococci in animals. It is not

clear whether the methicillin-resistant staphylococci in animals are of human origin and have been resistant to Zn after exposure to feed or the Zn-resistant staphylococci have been resistant to methicillin, due to exposure to antibiotic(s).

A recent publication from Germany (37) showed a higher diversity of *E. coli* clones in piglets fed with diets supplemented with Zn compared to the background control group. The proportion of multiresistant *E. coli* was significantly increased in the Zn group compared to the control group. The authors suggested two possible mechanisms for their results: 1) co-selection via Zn resistance as some of the isolates were both Zn and antimicrobial resistant; 2) enhanced plasmid uptake under the influence of Zn, as the authors detected several resistance plasmids in isolates of the Zn feeding group. Identical clones were not present in the control group.

Data gaps

There is a lack of data which can demonstrate whether Cu/Zn-resistant bacteria may acquire antibiotic resistance genes/be antibiotic resistant or antibiotic-resistant bacteria are more capable to be Cu/Zn-resistant than antibiotic susceptible bacteria. Further studies are needed to elucidate this association.

Conclusion

Bacteria in animals may develop resistance to Zn/Cu. The resistance genes to these trace elements are identified in some bacterial species from animals. Resistance to Zn/Cu and its link to antibiotics resistance in bacterial species originated from animal are identified. Resistance genes to Zn/Cu are often located on plasmids, which may be transferable to other bacteria, intra- and inter-species. Although overuse of antibiotics in agriculture and medicine is partially responsible for the increased level of antibiotic resistance in bacteria, exposure to trace metals may also contribute to antibiotic resistance, even in the absence of antibiotics themselves. A resistance link between Zn and methicillin is identified in staphylococci of animal origin. Resistance to Cu is often linked to resistance to macrolides (e.g. erythromycin) or glycopeptides (e.g. vancomycin) in enterococci. Zn supplementation to animal feed may increase the proportion of multiresistant *E. coli* in gut microbiota. The transmission of Zn/Cu-resistant bacteria with resistance to antimicrobial agents to human microbiota cannot be discounted. Our knowledge regarding mechanisms of induction of Zn/Cu resistance in bacteria is limited and knowledge on dose-response relations is lacking.

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