Review

Abdullah Scott, Krishna Prasad Vadalasetty, André Chwalibog* and Ewa Sawosz

Copper nanoparticles as an alternative feed additive in poultry diet: a review

https://doi.org/10.1515/ntrev-2017-0159
Received July 2, 2017; accepted October 5, 2017; previously published online November 21, 2017

Abstract: Copper (Cu) is a vital trace element involved in various physiological and biochemical processes. However, animals can only absorb a small fraction of Cu and the most is excreted, contaminating soil and aquatic environment. Hence, the use of this mineral as a growth promoter is today one of the crucial health and environmental concerns. In recent years, many studies have reported Cu nanoparticles (Cu-NP) as a promising alternative to antibacterial reagents and a growth promoter. Depending on the size, shape, dose and animal species, Cu-NP exhibit a variety of effects on animal performance. Apart from being highly bioavailable, reports have already pointed out the growth-promoting, antibacterial and immune-modulatory effects of Cu-NP. Toxicological studies provide varied results in animal models. However, other studies being undertaken in different animal species have shown the promise of Cu-NP supplementation. Therefore, there is a need to optimise the dose and duration of Cu-NP supplementation for livestock, depending on their biological effects. Moreover, the bioavailability of Cu-NP in livestock still needs further confirmation. In this review, we summarise the benefits and hazardous effects of Cu-NP and the possibility of using Cu-NP as a feed supplement in different animals, in general, and in poultry particularly.

Keywords: copper; growth; immunity; nanoparticles; toxicity.

***Corresponding author: André Chwalibog,** Department of Veterinary and Animal Sciences, University of Copenhagen, Groennegaardsvej 3, 1870 Frederiksberg C, Denmark, e-mail: ach@sund.ku.dk
Abdullah Scott and Krishna Prasad Vadalasetty: Department of Veterinary and Animal Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark
Ewa Sawosz: Department of Animal Nutrition and Biotechnology, Warsaw University of Life Sciences, 02-786 Warsaw, Poland

1 Introduction

Copper (Cu) is a crucial trace element in animals; however, it cannot be stored in the body, thus, a regular dietary supplement is required. In addition, feed ingredients are commonly deficient in Cu; hence, the commercial diet should provide the essential amount of Cu in a biologically dynamic form, which depends on the physical and chemical properties of the form of the supplement in which the Cu is given in the diet [1].

Cu sulphate ($CuSO_4$) is the main Cu source in the diet of chickens and other animals; however, the inorganic salt shows poor bioavailability caused by the presence of ingredients that can inhibit absorption. Thus, inorganic mineral administration in animal feed poses a risk to the environment, as excretion of high mineral levels contaminates the soil and water.

Organic sources of Cu show a reduction in excretion compared to inorganic forms, due to greater bioavailability and stability in the upper gastrointestinal tract of chickens; however, due to high cost and lower supplementation doses than inorganic Cu, results are still not consistent [2]. Therefore, efforts have been made to find alternative sources of Cu to increase bioavailability and absorption, and to avoid causing harmful effects on the health and performance of chickens.

Different sources such as Cu chloride, Cu oxide, Cu citrate, Cu sulphate and tribasic Cu chloride at different concentrations have been applied in poultry feed, depending on Cu bioavailability [3]. However, the feed industry still prefers $CuSO_4$ for economic reasons.

Nanotechnology has modernised the commercial application of nanosized minerals, which have been used recently as a tool in the fields of biology, biotechnology, mineral nutrition, physiology, reproduction and pharmacology in animals [4].

Nanoparticles refer to a particle size of 1–100 nm, in which the physical, chemical and biological properties of materials differ fundamentally from their bulk form [5]. Furthermore, their small size increases the potency of active ingredients and potentially reduces the applied quantity [6].

The use of Cu nanoparticles (Cu-NP) has recently received much attention due to their high electrical and melting points, low electrochemical migration behaviour, high bioavailability and relatively low cost of production [7].

The reduced size and increased surface area of Cu-NP compared to the bulk material improve thermoconductivity and fluid viscosity, leading to a variety of applications like chemosensors, surfactants and antimicrobials [8]. However, little work has been done to explore the efficacy of Cu-NP as feed additives in animal feeding and particularly in poultry. It was hypothesised that Cu-NP, because of their high physical reactivity, could be used as an alternative, effective health and growth promoters in much smaller doses than bulk minerals in animal feed [9–11], in consequence, significantly reducing the excretion of these minerals into the environment. Moreover, Cu-NP are effective antibacterial, antifungal and antiviral agents [12]. However, their size might affect their toxicity by helping the cellular uptake and translocation of the particles in the animal's body [13]. Therefore, the objectives of the review are to present the current knowledge about Cu-NP physicochemical properties, nutritional characteristics, antimicrobial activity, immunological and toxic effects, and the possibility of using Cu-NP as an alternative growth-promoting supplement in animal diets.

2 The importance of Cu in the diet

Cu is a key element required for animal growth and development of bones, connective tissue, the heart and several other organs [14]. Furthermore, Cu is involved in the stimulation of the immune system to combat infections and repair injured tissues [15]. Additionally, it supports neutralisation of free radicals that cause severe injury to cells [16]. Cu is shown to be absorbed partially by the stomach; however, the majority is absorbed in the small intestine. Cu is mostly excreted via bile that is released into the gastrointestinal tract (GIT), with marginal Cu reabsorbed by intestinal cells [17].

The biological functions of Cu are associated with Cu's role in the active site of metalloenzymes. Cu is found in a large number of metalloenzymes such as cytochrome oxidase, superoxide dismutase (SOD), lysyl oxidase, dopamine hydroxylase and tyrosinase [18]. Cu is also needed for the development of antibodies and white blood cells, in addition to antioxidant enzyme production [19].

Ceruloplasmin is a Cu transport protein, which provides Cu to the cells and performs as an enzyme, which possesses oxidative activity [20]. However, organisms have developed a variety of antioxidant defences that include metal-sequestering proteins to protect against oxidative damage, using compounds such as vitamins C and E, and specified antioxidant enzymes [21].

A study conducted on mink [22] showed that Cu affects the regulation of ceruloplasmin concentration, SOD activity and the digestion of dietary fat. The SOD enzyme helps in removing the damage caused by reactive oxygen species (ROS) by catalysing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen [20].

Cu is also important in the body as a component of enzyme systems involved in iron metabolism, red blood cell formation and immune function. Moreover, Cu supports the formation of connective tissues such as collagen and elastin, and enhances nervous system development by synthesis of dopamine [23].

Cu is involved in cellular respiration, energy production, synthesis of collagen, uptake and utilisation of other trace minerals, antioxidant activity, cardiac function, bone formation, keratinisation and pigmentation of tissue and myelination of the spinal cord [24]. In addition, it contributes to the regulation of glucose and cholesterol metabolism [23].

A deficiency of Cu supplementation in the diet can cause disturbances in reproduction and development of sperm, high mortality of embryos during hatching, poor pigmentation of feathers, slow growth and a reduction in body weight [23]. Furthermore, it may result in muscle weakness, anaemia, bone alterations that resemble scurvy, defective connective tissue synthesis, impaired myelinisation of nerve tissues and neurological defects, altered lipid metabolism and cardiac malfunction [23]. However, it has been demonstrated that ceruloplasmin mRNA in the liver is not affected by a dietary deficiency of Cu [25].

An excess of Cu may also have adverse effects on chicken performance [26]. Therefore, Cu must be provided to livestock in optimal concentrations and according to requirements that change during the growth and development of the animal.

The essentiality of Cu depends on the ability of Cu atoms to gain and lose electrons to form cuprous Cu^{+1} and cupric Cu^{+2} states. Therefore, this alteration is crucial for enzymes to support the metabolism of all major substrates such as proteins, lipids and carbohydrates [27].

The oxidation of cuprous to cupric Cu can generate free radicals from the oxidation of lipids, proteins and nucleic acids, causing extensive functional damage in tissues and organs. Consequently, organisms have developed systems involving specific protein carriers, which regulate the absorption, distribution, use and excretion of

Cu, thereby, controlling the production of free Cu ions and minimising the risk of oxidative damage [27].

The assessment of Cu status in chickens can be examined using several methods. However, the most subtle indicator of Cu status is the concentration of Cu in the liver [28]. Liver Cu concentration in animals is correlated to the bioavailability of Cu in the diet [29]. In addition, when Cu intake is below physiological need, the stored Cu in the liver is released; thus, a reduction in liver Cu can be a sign of low Cu intake. The concentrations of Cu in feed ingredients and their bioavailability vary because of the differences in agronomic conditions and processing [30]. Thus, various sources of supplemental Cu (oxide, citrate, sulphate and tribasic Cu chloride) have been included in animal diets [31, 32].

The bioavailability of trace minerals for animals is defined as the degree to which the ingested trace minerals from a particular source are absorbed in a form that can be metabolised by the animal [33]. The emphasis on the bioavailability of Cu has become a concern of researchers because the use of $CuSO_4$ as the main source in feed brings some adverse effects like the interaction with other ingredients, some physicochemical problems owing to high hygroscopicity and chemical reactivity [34]. Furthermore, it is an acidic compound and a strong electron acceptor. Hence, these properties can lead to solidification of the mixture in addition to oxidation, destroying its value and integrity. In chicken feed, the moisture content is about 20%, which can make $CuSO_4$ crystal surfaces more concentrated for reactions that break down labile organic compounds such as vitamins, enzymes, fats and oils [35]. Many studies have compared the bioavailability of different Cu forms to $CuSO_4$. It has been suggested that Cu citrate is more efficient than $CuSO_4$ [36]. Moreover, Ledoux et al. [37] showed the relative bioavailability of cupric oxide, cupric carbonate and cupric sulphate, using supplementation levels of 150, 300 and 450 mg Cu/kg feed, based on Cu levels in the liver; the relative biological availability was estimated to be 88.5%, 54.3% and 0% for the sulphate, carbonate and oxide, respectively. Feeding supplementation up to 390 mg/kg feed [38] suggested that tribasic Cu chloride (TBCC) has a relative value of 134% compared to $CuSO_4$, while Miles et al. [39] proposed a value of 112% for TBCC. In terms of liver Cu accumulation, TBCC was 109% as valuable as sulphate [34], which suggests that TBCC is less harmful to vitamins both in the feed and within the bird. The liver and plasma vitamin E levels were higher in birds fed TBCC compared to sulphate using a diet of 36 IU/kg. Additionally, TBCC and cupric sulphate (220 and 180 mg Cu/kg feed, respectively) equally improved the carcass weight of broiler chickens after 45 days of feeding, compared to the control group (30 mg Cu/kg) [40].

Alternatively, the organic form of Cu (chelated with amino acids, peptides, or proteins) appears to provide a pathway for minimising Cu level in the excreta [41]. It has been suggested that chelated Cu is better absorbed and metabolised, and may prevent antagonism of Cu with other minerals [42]. Furthermore, it has been investigated that chelated Cu can replace $CuSO_4$ as a growth promoter for pigs [43].

Cu absorption in the stomach is lower than in the intestine and is further delayed by binding with phytic acid and forming insoluble compounds. This was reported when $CuSO_4$ added at 250 mg/kg of feed caused a reduction in phosphor retention in broilers, by forming an insoluble Cu-phytate chelate complex [44]. In addition, it was demonstrated that organic Cu is more effective than $CuSO_4$ in decreasing the plasma cholesterol of broilers [45]; however, a few studies have found that organic Cu is less efficient than $CuSO_4$ [46].

Another way of predicting Cu availability in chickens was investigated by the solubility of different Cu combinations in several solutions for their ability in chicken liver [47]; the authors found that solubility at pH 2 was the best predictor. The same authors found that Cu chelated with lysine or amino acid is more bioavailable than $CuSO_4$.

It has been demonstrated that supplementation with 4 mg/kg feed of Cu as a cupric chelate of amino acid hydrate may be sufficient for normal broiler growth to 29 days of age [48], while Jegede et al. [49] demonstrated a significantly higher daily weight gain in broilers fed Cu proteinate compared to cupric sulphate supplementation. Moreover, supplementation (4–8 mg/kg) with Cu glycine chelate did not affect the Cu concentration in the liver, but reduced the Cu concentration in the broiler faeces compared to $CuSO_4$ [50].

Assessing the bioavailability of Cu proteinate relative to $CuSO_4$ in broilers was studied by Liu et al. [51]. The estimated relative bioavailability of Cu proteinate was 78.8% and 79.3%, respectively, compared to that of $CuSO_4$; however, the differences were not significant. Furthermore, it was demonstrated that the substitution of $CuSO_4$ with Cu proteinate in chicken diet positively affects body gain and feed conversion ratio [52].

The outcome [53] indicated that Cu proteinate, compared to $CuSO_4$, may enhance the detrimental effect of aflatoxicosis on broiler chickens, improving the growth performance by feeding a diet containing a high level of aflatoxin.

Assessing 100 mg/kg feed (cumethionine or cuproteinate) as an alternative to antibiotics in broiler diet

showed that both Cu sources improved the growth performance of broilers, and the enhancement was comparable with supplementation of an antibiotic (avilamycin), which increased populations of lactobacilli while decreasing *Escherichia coli* in the intestine [54]. On the other hand, Kwiecień et al. [55] did not reveal any differences in performance indices of chickens supplemented with $CuSO_4$ or Cu glycinate chelate, but organic Cu positively affected the biomechanical properties of femur bones.

Diet supplementation with 50, 100 and 150 mg/kg levels of Cu proteinate or $CuSO_4$ had no significant effect on growth; however, Cu proteinate decreased plasma cholesterol, low-density lipoprotein and triglyceride in comparison to $CuSO_4$ in pullets [56].

Moreover, the comparison between $CuSO_4$ and Cu-lysine added to duck diet (4, 8, 12 and 150 ppm Cu) showed that the Cu source had no significant effect on weight gain, while feed intake was increased and feed efficiency was degraded by dietary organic Cu lysine. Compared to $CuSO_4$, Cu lysine increased Cu excretion, Cu concentration in the liver and plasma cholesterol concentration, but decreased plasma triglycerides. Although organic Cu showed some improvement in chicken performance compared to inorganic forms, the results were not always consistent [57].

The effect of different dietary Cu sources on serum trace mineral and cholesterol status in broiler chickens revealed that higher Cu content with lower iron and manganese content in serum was noticed by increasing Cu levels in the diet [58]. Furthermore, the Cu propionate source had a significantly higher serum Cu compared to $CuSO_4$. The provision of $CuSO_4$ brought significantly higher serum zinc compared to that of Cu propionate; however, the total cholesterol content in serum was not affected by Cu diets. Excess Cu supplementation in broiler chickens showed that Cu increased haemoglobin, decreased plasma cholesterol and triglyceride significantly, decreased plasma proteins and its fraction did not change due to Cu supplementation [59]. From the studies mentioned above, it can be determined that Cu supplementation has an effect on the blood levels and erythropoietic system of the chicken, which could be used as an indicator of the impact of its toxicity in chickens. Furthermore, changes in the peripheral blood enable more accurate evaluation and explanation of the effect of Cu on the chicken's body.

The growth-promoting function of Cu is related to the growth hormone axis [60] and hypothalamic appetite-regulating genes [61]. Moreover, it has been shown that adding Cu stimulates mitogen activity and also enhances the growth hormone (GH) mRNA level in the pituitary glands of pair-fed pigs. However, the production of GH is known to be affected by many factors. The influence of Cu on GH gene expression can be through either direct action on pituitary cells or indirect action on the factors influencing GH gene expression [62]. Therefore, stimulation of growth in chickens by GH association with Cu deficiency is still not clear.

It can be concluded that Cu is present within chicken tissues in very small and regular amounts, but it plays an essential role in chicken growth, acting as a catalyst in enzyme systems within cells [63]. However, a relatively constant concentration of Cu in the body of chickens suggests that the content of Cu increases with increasing body weight [2]. A deficiency of Cu will certainly affect chicken growth, while an excess of Cu is not recommended because either it will be excreted or will have an adverse effect on performance.

3 Physicochemical characteristics of Cu-NP

The Cu-NP possesses ultraviolet-visible sensitivity and electrical, catalytic, thermal and antibacterial properties due to their large surface-to-volume ratio [64]. Many atoms are present on the surface due to the smaller particle size. The surface-to-volume ratio of particles differs and depends on the shape and size of the nanoparticles. The characterisation of Cu-NP including the electronic energy levels, electron affinity, electronic transitions, magnetic properties, phase transition temperature, melting point and affinity for polymers, biological and organic molecules depends on the change in the surface area [65]. Consequently, functional activities such as the chemical, catalytic, or biological effects of nanoparticles are profoundly influenced by the particle size of the nanometals [66].

Quantum effects are due to a combination of quantum-size and Coulomb-charging effects that impart the charge to nanoparticles. When the Coulomb-charge effect is coupled with the quantum size, a range of properties are obtained that are not observed for the same bulk material. Quantum effects are prominent in spherical particles and in particles with sharp edges [67].

The Cu-NP can be characterised by different methods such as ultraviolet-visible absorption spectroscopy, X-ray diffraction [68], directly by scanning electron microscopy [69] for relatively coarse powders or transmission electron microscopy for fine powders [70, 71], atomic force microscopy [72, 73] and infrared spectroscopy. In addition, other methods have shown convenient ways of

characterising Cu-NP such as dynamic light scattering, X-ray scattering at small angles and ultraviolet-visible spectroscopy (UVS) [12]. UVS is used because the absorption peak positions are dependent upon particle size and shape [74]. Cu-NP is normally absorbed from 280 to 360 nm. Ascorbic acid, a common reducing agent, provides a shorter peak from 240 to 280 nm. Nanoparticles are characterised by absorption spectroscopy with a peak at 580 nm. Infrared spectroscopy is used to characterise biomolecules interacting with Cu-NP [75]. Particles prepared by microwave irradiation are typically spherical and show surface plasmon resonance at 535 nm [76]. Moreover, Cu-NP prepared by microwave irradiation are influenced by the irradiation time; a linear relationship is observed between particle size and time [76]. In addition, ethers, alcohols and carbon-hydrogen bonds are responsible for the interaction of nanoparticles with biomolecules [77].

Many studies have shown different correlations between the various physicochemical properties of mineral nanoparticles linked with the health effects; the diversity of nanomaterials in terms of size, shape, aggregation and surface chemistry poses a challenge to those who are trying to characterise the animal health and environmental risks associated with incidental and unintentional exposure [78]. Evidence from several studies has revealed that several factors associated with sonication, such as temperature, sonication time, sonication methods, sonication power output, sample volume and concentration can influence the physicochemical properties of nanoparticle suspensions [79, 80]. In addition, other parameters like coating and surface roughness, solvent conditions, composition and crystalline structure will change depending on the nanoparticle preparation technique and the stage of formation [81, 82].

However, there are inconsistent reports of physicochemical properties of Cu-NP such as particulate core and outer shell chemical composition, surface oxidation state, surface charge, singlet and agglomerate sizes in relevant carriers, shape, solubility and surface area [83]. These properties could impact on the nanomaterial dose that reaches target organs. Moreover, it is believed that the surface properties are expected to change when Cu-NP enter the body and during transportation.

The morphology of Cu-NP is highly affected by the type of Cu salt used for synthesis. The Cu-NP is crystalline in nature and has strong antimicrobial activity against both, Gram-positive and Gram-negative bacteria. The different shape, size and strong antimicrobial activity of Cu-NP have higher prospective in the field of biomedical and food packaging [75].

The reducing agent affects the size of Cu-NP, as high concentrations may decrease the size while maintaining the concentration of the precursor [84]. It has been reported that an increasing concentration of a reducing agent causes a reduction in monodispersity and increases the number of nanoparticles [85]. Furthermore, it has been shown that the stabilising agent also affects the size of the nanoparticles. Micelle formation stabilises nanoparticles and makes the system monodispersive and stable in air.

The pH is the most important factor that affects the size of the nanoparticles, which increases with increasing pH of the reaction mixture. A marked increase in size appears above pH 5. It has been reported that the Cu-NP have a high solubility in an acidic environment (pH 5.5) with a high positive ζ potential [86, 87]. Cu-NP are not present at a higher pH; rather, the oxide form of the Cu-NP is present due to excess hydroxide. Further increases in pH produce Cu hydroxide without nanoparticle formation [85]. However, some works have reported the formation of Cu-NP at pH from 9 to 11. As the pH is increased, the concentration of the hydroxide increases, leading to the formation of Cu hydroxide. This explains the formation of Cu-NP in basic solution.

It has been revealed that various biological mechanisms including cellular uptake and efficiency of particle processing in the endocytic pathway are dependent on the size of the material [88–90]. The size and surface area of the particles will dictate how the system responds to, distributes and eliminates the materials [81]. However, the size of the Cu-NP could be also their main disadvantage, and it represents a challenge for the scientific community to achieve adequate physical and chemical characterisation [91].

The surface of the Cu-NP can influence cellular uptake, clearance and biocompatibility [77] and also the agglomeration of nanoparticles. Furthermore, the surface chemistry also determines *in vivo* dissolution rates and, therefore, contributes to distribution, retention and toxicity. Moreover, the surface composition of the Cu-NP can affect the generation of ROS via redox or catalytic activity [88, 92]. It has been found that the Cu-NP with longer chain ligands have surfaces that are better protected from oxidation and a corresponding lower ROS-generating capacity than the particles with shorter chain ligands. On the other hand, the Cu-NP with a greater surface oxidation also have a higher ROS-generating capacity [93]. The Cu-NP with high oxidant and cytotoxic potential may have more significant effects at the initial site of deposition and may also be cleared more rapidly due to an influx of inflammatory cells.

The surface charge of nanoparticles will influence nonspecific interactions with proteins that are present in the environment. These observations illustrate the difficulty in predicting nanoparticle uptake as a function of a single property.

The surface energy affects nanoparticle interaction with biomolecules and tissues [94]. Low-energy surfaces (hydrophobic) are particularly prone to nonspecific adsorption as proteins unfold to expose their hydrophobic core, which may disorganise the lipid components of cell membranes and enhance epithelial penetration [95]. High-energy surfaces (hydrophilic), particularly those that carry a weak negative or neutral charge, are ideal for resisting protein adsorption and cell uptake [96].

Different aspects of nanomaterials like selective adsorption of nanoparticles [97], colloidal behaviour, plasma protein binding [98], blood-brain barrier integrity and transmembrane permeability are primarily regulated by the surface charge of nanoparticles [99]. It is noteworthy that positively charged nanoparticles show significant cellular uptake compared to negatively charged and neutral nanoparticles, owing to their enhanced opsonisation by plasma proteins. As the surface charge is the main determinant of colloidal behaviour, it will affect the organism's response upon exposure to nanoparticles by changing their shape and size through agglomerate formation [97].

The biological activity of Cu-NP depends directly on the physical and chemical properties that include size, shape, concentration, surface and charge [100]. However, these parameters change depending on the nanoparticle preparation technique and the stage of formation [101].

Cu-NP can be synthesised either by the mechanical grinding of bulk metals (physical method) or via chemical reduction of metal salts (nucleation and growth of metallic atoms). Recently, the green synthesis of Cu-NP has also emerged as a novel method and is gaining more importance among researchers [102, 103]. However, the synthesis of Cu-NP needs a variety of stabilisers like donor ligands, polymers and surfactants to prevent agglomeration [71].

In this regard, it is necessary to know the physical and chemical properties and antibacterial influence of the obtained Cu-NP to standardise this nanomaterial during subsequent use as a feed supplement for animals. In addition, the physicochemical characteristics of Cu-NP may affect the epithelial barrier penetration and have emerged as important determinants, including size, surface charge and surface energy [104]. Furthermore, the Cu-NP solubility and protein-binding capacity also play a key factor in the passage of the Cu-NP across epithelial barriers, cellular uptake, cytotoxicity and biodistribution [105, 106].

4 Biological properties of Cu-NP

4.1 Nutritional and physiological characteristics

The small size of the Cu-NP can increase the uptake from the GIT and, hence, make them more effective than the bulk Cu at lower doses [107]. In the animal body, the Cu-NP interacts more effectively with organic and inorganic materials due to their larger surface area [108]. The Cu-NP has the capability to cross the small intestine and distribute into the blood, brain, heart, kidney, spleen, liver and intestine [109]. In order to reach the target organs, the Cu-NP will interact first with the protective barriers of the GIT. Hence, those nanoparticles that reach the physiological barriers are highly determined by the properties of the particles and the barriers themselves [83]. Other properties of the Cu-NP like the composition, solubility and protein-binding capacity also play a significant role in the passage of the nanoparticles across the epithelial barriers, cellular uptake, cytotoxicity and biodistribution. However, the current understanding regarding the passage of the Cu-NP across the epithelial tissue and the mechanisms of distribution and elimination is lacking.

The advantage of the Cu-NP is to prevent mineral dissociation with other nutrients and subsequently avoid antagonism. It is assumed that supplementing chicken feed with an adequate level of Cu-NP will help to reduce the Cu levels in the excreta, thereby, reducing environmental contamination. Many studies have compared the availability of the Cu-NP to $CuSO_4$ when supplemented in the animal diet [110, 111], suggesting that the Cu-NP is better than $CuSO_4$ in enhancing the growth and performance of animals.

Generally, nanoparticles can enter the GIT in many ways, such as ingestion directly from food and water, administration of therapeutic nanodrugs and oral delivery into the GIT; inhaled nanoparticles can also be swallowed and enter the GIT following clearance from the respiratory tract [112]. The uptake of particles in the GIT depends on diffusion and accessibility through mucus and contact with the cells of the GIT. Smaller particles will diffuse faster through the GIT mucus to reach the cells of the intestinal lining, followed by uptake through the GIT barrier to reach the blood. Uptake occurs variously by passive diffusion across the mucosal cells, via active transport mechanisms and intercellular [113]. It has been suggested that smaller particles that are capable of being taken up by the villus epithelium may directly enter the bloodstream, thereafter, being predominantly scavenged by the liver and the spleen [114].

Depending on the mass of insoluble nanoparticles, they can be more readily taken up across the intestinal barrier; therefore, they will be more bioavailable than their micro or macro equivalents [115]. However, the mechanism by which nanoparticles enter the cell has important implications for their impact on biological systems, and the challenge is to identify how an organism responds to these nanoparticles [116].

Nanoparticles can enter cells by transcytosis; particles are engulfed by Kupffer cells, of sizes 50–100 nm, resulting in smaller (2 nm) nanoparticles being filtrated out of the liver via the kidneys, whereas bigger particles (40 nm) are retained in the Kupffer cells [117].

According to the physicochemical characteristics of Cu-NP, uptake may occur via one of the different forms of endocytosis [106]. However, there is no evidence that those nanoparticles are taken up individually, and one may doubt the technical ability to capture this event. In the case of small nanoparticles, it is expected that uptake occurs only when a critical density is reached [118]. It has been reported that nanoparticles with a diameter up to several hundreds of nanometres preferentially enter cells via pino- or macropinocytosis; in addition, clathrin-dependent uptake is a route for nanoparticles on whose surface serum proteins are adsorbed. Finally, negatively charged nano-objects enter mostly by caveolin- or clathrin-mediated endocytosis [106, 119].

Small drug molecules enter the cell through passive diffusion, whereas most nanomaterials are taken up by active processes such as phagocytosis or pinocytosis depending on a dynamic series of physicochemical properties [120].

The uptake of nanoparticles is determined by particle solubility, concentration and size. However, the passive uptake of particles has been shown to occur transcellularly via the intestinal lining and, to a lesser extent, between epithelial cells [121].

Nevertheless, it is not known whether Cu-NP remain in the intestinal tract unabsorbed and are excreted in the faeces or go into the body system readily [122]. Some evidence indicates that particles smaller than 100 nm are absorbed in various tissues and organs [123]. Uptake of Cu-NP across these defensive barriers occurs through various transport mechanisms including receptor-mediated endocytosis and adsorptive endocytosis [124].

Particles taken by the villus epithelium can enter the bloodstream and, then, mostly will be foraged by the liver and spleen [114, 125].

Increasing the uptake of Cu-NP in the GIT might have an impact on the growth and health of animals. Cu-NP have been reported to enhance growth performance and improve feed utilisation compared to $CuSO_4$ when provided as a feed supplement for piglets, poultry and fish [9, 126, 127]. The improvement was attributed to the better bioavailability of Cu-NP compared with $CuSO_4$ salts. However, the mechanism behind this improvement is still not clear. Some studies indicated that the effect of Cu-NP can be ascribed to their antibacterial properties [40], while others suggested the better digestibility of energy and fat [9, 11]. Furthermore, some studies demonstrated that the activity of SOD was enhanced with Cu-NP supplementation in animal diet [128, 129].

It was reported that adding Cu-loaded chitosan nanoparticles to broiler feed enhanced growth performance and immunological capacity, influenced intestinal microbiota and improved protein synthesis [130]. Further, it has been reported that there is a connection between arginine levels in the liver and the growth of chickens, which was improved after one intramuscular injection with Cu-NP [131]. Moreover, *in ovo* injection of Cu-NP to chicken eggs has been found to improve the performance of chickens in the finishing period [110]. A similar effect was shown in fish treated with 2 and 4 mg/kg of Cu-NP [127].

Generally, there is some alteration in blood biomarkers after Cu provision in the animal diet. Accordingly, an increase in albumin, alanine transferase (ALT) and uric acid, but a reduction in alkaline phosphatase, was shown in chickens fed 100–400 mg/kg of $CuSO_4$ [132]. Similarly, it was observed that *in ovo* injection of 50 mg/kg of Cu-NP decreased the levels of ALT, glucose and cholesterol [23]. The same author demonstrated a non-significant increase in calcium, magnesium and phosphorus levels in both Cu-NP and $CuSO_4$ groups compared to the control. However, Scott et al. [11] did not indicate any significant alteration in the blood of hatchlings after administering 50 mg/kg of both forms of Cu.

Compared to $CuSO_4$, dietary supplementation with Cu-NP causes a significant reduction in glucose levels in fish [127]. Additionally, Canli and Canli [133] revealed that 5 mg/kg of oxide Cu-NP significantly decreased glucose, total cholesterol and triglyceride levels in rats following oral administration compared to the control. This could be attributed to hyperglycaemia resulting from cortisol-mediated gluconeogenesis [134].

The reduction of cholesterol levels after adding Cu to a poultry diet [135, 136] can be explained by Cu supplementation regulating cholesterol biosynthesis indirectly by decreasing the reduced form of glutathione and increasing the oxidised form of glutathione [137, 138].

Blood urea also shows some reduction with Cu supplementation in the diet. This might be an indicator that amino acids are utilised more efficiently for growth [139].

Similarly, it has been shown that supplementation of Cu-loaded chitosan nanoparticles in broiler feed alters the concentration of urea in the blood [130], which could be an indicator of the rate of protein synthesis [140].

A study by Mroczek-Sosnowska et al. [23] showed that *in ovo* injection of $CuSO_4$ increased counts of white blood cells (WBCs), lymphocytes and eosinophils compared to Cu-NP. However, Cu-NP decreased the number of leucocytes compared to the $CuSO_4$ group. Additionally, adding Cu significantly altered the number of erythrocytes in the peripheral blood of the birds. Similar results were observed in turkeys treated with Cu-lysine chelate in drinking water; however, it caused a reduction in the levels of haematocrit and haemoglobin [141].

Additionally, the concentration of haemoglobin was increased, which allowed the birds to keep oxygen transport at an appropriate level [23]. The increased level of haemoglobin could be due to its continued synthesis by erythrocytes already circulating in the peripheral blood. It is also likely that the increased level of haemoglobin could be linked with the homeopathic function of Cu. Cu has been shown to directly stimulate erythrocyte synthesis, as it determines iron absorption into the body and its incorporation in haemoglobin [142].

On the other hand, it was demonstrated that $CuSO_4$ reduced the percentage of monocytes, which suppressed the metabolic activity of these cells. A reduction in phagocytic activity was also observed in the chickens, which indicates that $CuSO_4$ could contribute to accelerated damage of phagocytes and inhibit the metabolic activity of the cells [23], while the administration of Cu-NP caused an increase in the blood level of uric acid.

The addition of the oxide form of Cu-NP to broiler chicken feed indicated that haemoglobin and albumin concentrations were reduced in comparison to the non-treated group [143]. However, the same authors showed some protein denaturation, which suggests that the oxide form of Cu-NP might have a toxic effect on blood proteins.

4.2 Antibacterial activity of Cu-NP

Chickens frequently receive additional Cu as a growth-promoting supplement. However, it has been shown that bacteria can develop resistance to Cu inside a chicken's body, particularly enterococci, which are often associated with resistance to antimicrobial drugs like macrolides and glycopeptides [144]. Such resistant bacteria can be transferred from food-producing animals to humans. The resistance is more frequent at high Cu levels. Therefore, Cu supplementation for growth promotion is, today, one of the crucial health and environmental concerns that should be optimised. Although the misuse of antibiotics in animal feed is partially liable for the increased level of antibiotic resistance in bacteria, exposure to trace metals may also contribute to antibiotic resistance, even in the absence of the antibiotics, themselves [145]. However, information regarding mechanisms for inducing Cu resistance in bacteria and the dose-response relationship is limited.

Therefore, with the most concerning issue of microorganism resistance to antibiotics, developing new nanoagents with antibacterial effects is of great theoretical and practical concern.

Previously, the bactericidal effects of Cu-NP on strains of *E. coli* and *Staphylococcus aureus* were demonstrated [146]. Furthermore, the antibacterial activity of Cu-NP depends on the physical characteristics of the nanomaterial [101].

Cu-NP have been used for a long period as disinfectants due to their antibacterial properties [147]. Although the mechanism of action as an antibacterial agent has still not been clarified, reports have suggested that the mechanism of antibacterial activity of Cu-NP could be the same as that of silver nanoparticles [70].

Nanoparticles are capable of attaching to the bacterial membrane by electrostatic interaction and disturbing its integrity [148]. Thus, the antibacterial activity of nanoparticles is generally triggered by inducing ROS following the administration of Cu-NP [149]. Generally, three mechanisms of action of nanoparticles have been hypothesised. First, the accumulation of nanoparticles in the bacterial membrane, changing its permeability, with subsequent release of lipopolysaccharides, membrane proteins and intracellular biomolecules and dissipation of the proton motive force across the plasma membrane [150, 151]. Second, generating ROS and their corresponding ions from nanoparticles, with subsequent oxidative damage to cellular structures [152, 153]. Finally, the uptake of metallic ions derived from nanoparticles into the cells, followed by depletion of intracellular ATP production and disruption of DNA replication [154, 155].

The filamentation is caused by Cu-NP-mediated depolarisation of the cell membrane, while cell damage is caused by Cu-NP-mediated ROS generation in cells, resulting in lipid peroxidation, protein oxidation and DNA degradation [156].

The linoleic acid-capped Cu-NP are highly bactericidal for *S. aureus*, *E. coli* and *Bacillus subtilis*, suggesting that Cu-NP can be used as effective growth inhibitors in various microorganisms, making them applicable in diverse medical devices and antimicrobial control systems [157].

Based on oxidative stress, Chang et al. [149] discussed the coordination and non-homeostasis effects of Cu oxide nanoparticle toxicity on eukaryotic cells. The authors described that nanoparticles can diffuse into the cell directly through the pores present in the cell membrane or through ion channels and transporter proteins present on the plasma membrane, while other nanoparticles may enter the cells via endocytosis. Eventually, the nanoparticles that enter the cell may directly interact with other organelles.

The Cu-NP showed the most efficient form among the different Cu forms for antimicrobial activity due to their high surface area [158, 159]. Besides that, the crystal morphology of the Cu-NP results in several reactive sites, which simplifies the interaction between the Cu-NP and the microbial cell membrane [160]. Therefore, many studies have pointed out the antibacterial action of the metal nanoparticles [161–165].

Many investigations have focused on the bactericidal activity mechanism of the Cu-NP [156, 166]. They indicated that the antibacterial activity of the Cu-NP results from the ions released by the nanostructures. However, Ruparelia et al. [70] showed that Cu^{2+} ions are released because of a reaction with the nutrient components, suggesting that Cu ion release may be related to reaction with chloride from the nutrient or to the presence of a thin oxide layer on the nanoparticle surface.

The bactericidal activity of Cu-NP results not only from their particle size, high specific surface value and close interaction with microbial membranes but also from the formation of leached cuprum-peptide complexes that lead to an increase in ROS generation and decreased cell viability [167].

Lately, a study demonstrated that the antimicrobial properties of chitosan implanted with Cu-NP reduce gut bacteria such as *E. coli*, *Enterococcus faecalis*, *S. aureus* and, particularly, *Lactobacillus fermentum*, which is one of the primary targets of antibiotic growth promoters, suggesting that the Cu-NP could be used to minimise undesirable levels of microbial populations without causing cytotoxicity [168].

The antimicrobial activity of Cu seems to be similar to that of antibiotics, altering the gut microbiota and, hence, reducing fermentation loss of nutrients [169]. The reduced level of bacteria in the proximal part of the GIT may benefit the chicken by allocating more feed components for its growth performance. $CuSO_4$ reduces the quantity of coliform in the large intestine, which might be a part of other mechanisms such as the inhibition of specific pathogens and inducing resistance of the chicken to pathogen adhesion and invasion as well as toxins [170].

Supplementing chicken diet with additional Cu reduces the population of clostridia in the GIT of chickens. Generally, the concentration of Cu salts that affect the populations of coliform bacteria is higher (up to 200 mg/kg) than Cu-NP (below 50 mg/kg). While the population of lactobacilli in the GIT contents was stimulated by 150 mg/kg of Cu, the higher concentration did not reveal any effect [27].

Cu may be toxic to bacteria; however, this depends on the solubility of the metal compounds under physiological conditions. To avoid cellular toxicity, bacteria have developed mechanisms of metal tolerance [171]. Furthermore, antimicrobial agents have more proficiency than antibiotics to support a co-selection process, ultimately selecting for antibiotic resistance [172].

Cu-NP is effective against Gram-positive and Gram-negative bacteria [173, 174].

Further, Cu-NP inhibits the growth of *S. aureus*, *B. subtilis* and *E. coli* bacteria [175], *Micrococcus luteus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [176]. The subsistence rate of *E. coli* and *B. subtilis* bacteria is decreased by increasing Cu-NP concentrations [177]. However, higher susceptibility is shown to Gram-negative strains [178]. Furthermore, the activity of Cu-NP against *E. coli* has been demonstrated [179, 180].

The activity of Cu-NP depends on temperature, aeration, pH and concentration of nanostructures [181]. The main biocidal activity of Cu-NP is caused by oxidative damage to cellular structures and inhibition of DNA replication and amino acid synthesis in microorganisms [182]. The mechanism relates to the dissipation of the cell membrane under the influence of nanoparticle accumulation or the generation of ROS by highly concentrated Cu^{2+} ions [156].

A study by Ramyadevi et al. [176] carried out with *M. luteus*, *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*, as well as the fungi *Aspergillus flavus*, *Aspergillus niger* and *Candida albicans*, indicated that the Cu-NP is more toxic to bacteria than fungi. Another study revealed the antibacterial activity of polyethylene-Cu nanoparticles against *E. coli*. The result revealed a complete suppression of the number of live bacteria, and the nanocomposites damaged the plasma membrane of the bacteria, revealing a bacteriolytic effect [183].

The bacteria in the chicken's body may develop resistance to Cu and the resistance gene to this trace element is recognised in some bacterial species from animals. Resistance genes to Cu are often located on the plasmids, which may be transferable to other bacteria species [144].

From these findings, it can be concluded that the smallest nanoparticles, the highest antibacterial activity

Table 1: Overview of antibacterial activity of different forms of Cu.

Bacteria species	Type of Cu used	Concentration and size	Effect	Reference
E. coli, *S. aureus*	CuO-NP	50 nm	The inhibitory and bactericidal concentrations were 120 and 160 μg/ml for *E. coli* and 180 and 195 μg/ml for *S. aureus*, respectively	Chakraborty et al. [184]
B. subtilis	CuO-NP	8–10 nm	Release Cu^{2+}, electrostatic interaction, cell wall damage, rupture of the plasma membrane and disrupt biochemical process	Ruparelia et al. [70]
M. smegmatis	Cu-doped TiO_2 nanoparticles	20 mg/l, 20 nm	Release of Cu^{2+}, decreased enzymatic activity NADPH production, no cell damage, no internalisation of nanoparticles	Wu et al. [185]
Pseudomonas putida KT2442	CuO-NP	10 mg/l, 25–40 nm	Cell membrane damage and bactericidal effect	Gajjar et al. [186]
Bacillus cereus ITCC240, *K. pneumoniae* ITCC 138, *Curvularia lunata* ITCC 6257 (fungus)	Cu-NP	–	The antimicrobial activity of Cu-NP was higher in *B. cereus* and *C. lunata* than in *K. pneumoniae*	Kaur et al. [187]
E. coli	Cu_2O	500 mg/l, 40 nm	The antibacterial efficiency reached nearly 100% after 4-h exposure	Du et al. [188]
E. coli, *E. faecalis*	Cu-NP	0.86–3.43 mg/ml, 580 nm	Inhibited bacterial growth of both Gram-negative (*E. coli*) and Gram-positive (*E. faecalis*)	Dobrovolný et al. [189]
E. coli, *S. aureus*, *A. flavus* (fungus)	Cu-NP	40–60 nm	The nanocomposites are highly differentiable at low contact time and low concentration; 1% concentration of the multifunctional nanocomposite is very effective against the tested microbes at a contact time of only 10 min	Morsi et al. [190]
S. aureus, *E. coli*, *Bacillus pumilus*, *Pseudomonas fluorescens*	Cu-NP	50, 100, 300 μg/ml, 140.8 nm	Adsorption of rifampicin on the Cu-NP surface might provide a reduction in antibiotic dosage and prevent its adverse side effects	Woźniak-Budych et al. [191]
Bacillus subtilis, *P. fluorescens*	Cu-NP	100, 250 ppm 564 nm	Antibacterial activity of Cu-NP was better against biorecycling microbes under investigation. *P. fluorescens* is more vulnerable to metal nanoparticles than *B. subtilis*	Khurana et al. [164]
E. coli, *S. aureus*	Cu-NP	0.36, 0.82, 1.27 mmol/g	Maintained antimicrobial activity for at least four cycles. The material could be scored as slightly irritant, proving its biocompatibility	Villanueva et al. [192]
Enterobacter aerogenes, *S. aureus*	CuO-NP	5–45 nm	The radial diameters of the inhibition zone of *E. aerogenes* and *S. aureus* by CuO-NP are 14 and 16 mm, respectively	Abboud et al. [193]
E. coli	Cu-NP	8–15 mm	Cu-NP in an electrolysis method showed higher antibacterial activity than Cu-NP in a chemical reduction method against both Gram-negative and Gram-positive bacteria. Changes in the surface-area-to-volume ratio of Cu enhance its antibacterial activity	Theivasanthi and Alagar [162]
E. coli, *E. faecalis*, *S. aureus*, *L. fermentum*	Cu-NP	57.6 g/ml	Reduced the load of model gut bacteria under *in vitro* conditions. In particular, exhibited a higher antimicrobial effect against *L. fermentum*	Rajasekaran and Santra [168]
E. coli, *P. aeruginosa*, *B. subtilis*, *S. aureus*	CuO-NP	100 μl	The antibacterial activity was found to be size dependent. A significant increase in antibacterial activity against both Gram-positive and Gram-negative bacterial strains	Azam et al. [151]

Table 1 (continued)

Bacteria species	Type of Cu used	Concentration and size	Effect	Reference
E. coli ATCC 25922, *S. aureus* FDA 209P, *Kluyveromyces marxianus* CBS 608	Cu-NP	Dilution 1:100 and 1:1000	Cu-NP had an inhibitory effect on the growth of targeted microorganisms	Sportelli et al. [165]
M. luteus, *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*; fungi: *A. flavus*, *A. niger*, *C. albicans*	Cu-NP	50 µl, 580 nm	Cu-NP showed more inhibitory activity in bacteria than fungi, and it also showed a larger zone of inhibition in *E. coli* (26 mm) than in *C. albicans* (23 mm)	Ramyadevi et al. [176]
S. aureus, *B. subtilis*, *P. aeruginosa*, *Salmonella choleraesuis*, *C. albicans*	Cu-NP	2–350 nm	Cu-NP inhibited the growth of the targeted microorganisms	Usman et al. [194]

and Cu-NP have a better impact on bacteria than the other forms of Cu. In addition, the antibacterial activity of Cu-NP can allow more nutrient to be digested in the GIT, consequently improving the growth of chickens.

An overview of the antibacterial activity of the different forms of Cu is shown in Table 1.

4.3 Immunological effects

Here, we primarily focus on the immunological impact of the most studied Cu-NP. The interactions of Cu-NP with the immune system are a key issue to guide the future use of Cu-NP in animal feed and medicine.

Exposure to nanoparticles may induce immune stimulation or suppression; inadvertent suppression of immunological function leads to infectious diseases. In contrast, inappropriate enhancement of immune reaction can result in autoimmune diseases [195]. To date, many studies have demonstrated that Cu-NP are involved in promoting inflammatory responses, due to their physicochemical properties. However, it has been demonstrated that smaller nanoparticles (less than 70 nm) are not able to be recognised as foreign particles and are possibly later translocated through the capillary blood flow [196] and the lymphatic system [197] to the lymph nodes for antigen presentation [198]. While the recognised nanoparticles will be cleared by macrophage-mediated clearance, phagocytosed nanoparticles may be destroyed within the lysosomes of phagocytic cells [199]. If the nanoparticles exceed the size of the engulfing phagocyte, they take too long to be phagocytosed. Later, they may induce an inflammatory response, including cytokines, chemokines and ROS, which can result in inflammation [200] and DNA damage [201]. From the lymphatic and circulatory systems, nanoparticles may distribute to organs including the kidneys, from where partial or total clearance may occur. Inflammation in mice is increased when exposed to Cu-NP through inhalation [202]. Exposure to Cu-NP via inhalation and instillation in mice induces pro-inflammatory cytokines and recruitment of neutrophils, and it is expected that Cu ions at high Cu-NP doses lead to an increase in inflammatory response [54, 202]. However, *in ovo* studies with a chicken embryo model, Cu-NP injected into chicken embryos did not show immune stimulatory properties, which may be due to the Cu-NP not being recognised by antigen-presenting cells (APCs) [11, 203], as immune responses depend on nanoparticle uptake by APCs. Exposure to nanoparticles may induce toxicity, resulting in detrimental effects on the immune function. A study by Wang et al. [204] on fish reported that IL-1β and

Table 2: Toxic effect of Cu nanoparticles in different species of animals according to delivery type and dosage used.

Species	System investigated	Delivery type and time	Concentration and size	Effect and conclusion	References
Fish	Growth, digestive enzymes, histology	Semistatic exposure regime	20, 100 μl/l	Decreased growth performance with increasing level, inhibited the activity of digestive enzymes, decreased crude protein and fat, caused pathologies in liver and gills	Wang et al. [204]
Zebrafish embryo	Oxidative stress and teratogenicity	Exposure regime	40, 60 ppm	Oxidative stress in developing embryos, decreased heart rate and delay hatching time	Ganesan et al. [214]
Zebrafish embryo and larvae	Development	Exposure regime	50, 25, 12.5, 6.25, 1 mg/l, 50–60 nm	Lower doses had less effect on embryonic development; however, high doses led to hepatotoxicity and neurotoxicity	Sun et al. [215]
Rat	Gene expression	Oral administration	50, 100, 200 mg/kg, 25 nm	Alteration in expression of many genes involved in valine, leucine and isoleucine degradation	Liao et al. [216]
Rat	Liver tissue	Injection	5, 10, 100 mg/kg, 10–15 nm	Induced toxicity and histopathological changes in liver and lung tissues	Setorki et al. [217]
Fish	Histopathology	Exposure regime	0.01, 0.05, 0.15 mg/l, <50 nm	Exposure to Cu-NP demonstrated hyaline degeneration in the gills, epithelium and liver	Ostaszewska et al. [218]
Rat	Toxicity and biodistribution	Oral administration	312, 625, 1250, 2500 mg/kg, 33 ± 9.8 nm	Cu-NP-treated rats were 15–25% lower than those treated with Cu ions. Cu-NP increased significantly in soft organs. Delay in reaching the highest level of Cu-NP in the blood and tested organs compared with Cu ions. Cu-NP exerted apparent toxicological effects at higher dose levels compared with Cu ions and showed sex-dependent differences in mortality, biochemistry and histopathology	Lee et al. [219]
Mouse	Morphological changes, pathological examinations and blood biochemical indices	Oral administration	413 mg/kg, 23.5 nm	Pathological damage to kidney, liver and spleen of mice	Chen et al. [220]

TNF-α expression increased in intestine samples with an increased concentration of Cu-NP, and also, IgM concentration was decreased with Cu-NP concentration.

4.4 Toxicological effects

The predominant processes underlying nanoparticle-mediated toxicity includes oxidative stress, inflammation, DNA damage and inhibition of cell division and death. Despite the toxicity of bulk Cu being affected mainly by the salt composition, in the case of Cu-NP, additional physicochemical properties such as size, surface area, surface chemistry, surface roughness, dispersion medium and ability to agglomerate play a vital role in determining their toxicity [13].

There are many studies reporting on the toxicity of Cu-NP [205]. However, we could not find results regarding Cu-NP toxicity in chickens. The intranasal instillation of Cu-NP (23.5 nm) in mice resulted in the accumulation of Cu-NP in the liver and lung tissues, decreased body weight and dose-dependent lesions in the lung and liver [206]. Further, it was reported that Cu-NP induced apoptosis in the intestine of juvenile *Epinephelus coioides* via the mitochondrial pathway [207]. The toxicity may be due to the Cu-NP and released Cu ions [208]. Furthermore, it was demonstrated that the smaller Cu-NP are more toxic than the larger Cu-NP in zebrafish embryos [209]. The Cu-NP caused membrane damage and ROS formation in lung cells (A549 type II) [210] and mammalian cell lines (H4IIE and HepG2) [211]. The Cu-NP (10 μg/ml) also induced DNA strand breaks [212]. The cell viability effect of the Cu-NP embedded in the polymer matrices (hydrogel and polypropylene) strongly depends on the polymer characteristics, as hydrogels showed cytotoxicity at concentrations higher than 0.5% of that of the Cu-NP, whereas polypropylene did not show any effect even at higher concentrations (20% of Cu-NP) [213]. It was concluded that Cu-NP toxicity is elucidated by Cu ions released from the composites.

Many studies have pointed out the cytotoxicity effect of Cu-NP, depending on the animal species, delivery, concentration and size. An overview of the toxic effects of Cu-NP is shown in Table 2.

5 Potential use of Cu-NP in animal diet

The main reason for considering the Cu-NP as an alternative growth and health promoter is the overuse of Cu in animal diets, causing severe environmental pollution. In addition, the objective of using nanoparticles in animal feed is to decrease the numbers of harmful bacteria and stimulate the growth of beneficial bacteria, which may improve growth performance of animals [221]. Herein, we provide an overview of the research on the potential use of mineral nanoparticles as poultry feed additives and propose novel strategies for nanoparticle use in animal nutrition. The hypothesis behind growth improvement with Cu-NP supplementation may be related to better absorption of nutrients, i.e. with Cu-NP more nutrients will be available in the GIT for absorption. Hence, using Cu-NP as a supplement in chicken diet might have beneficial effects on growth, feed efficiency and chicken health by causing damage to pathogens, effecting a reduction in the production of bacterial toxins, increasing the synthesis of vitamins and other growth factors, improving the absorption of nutrients by reducing the thickness of the intestinal epithelium and reducing intestinal mucosal epithelial cell turnover and motility [222].

Reduction in the size of materials to the nanorange increases the absorption of nutrients, as shown for iron and selenium [223]. Selenium nanoparticles are more effective and showed better performance of broilers supplied with 0.3 ppm [224] and 0.5 ppm [225] compared to both organic and inorganic selenium sources [226]. Feeding zinc nanoparticles to chickens have produced encouraging responses in growth, immunity and reproduction. These nanoparticles enhance growth and improve feed efficiency in piglets and poultry [227–229]. Chromium nanoparticles reduce heat stress in chickens, improve feed conversion and provoke hepatic-related alterations [230]. Silver nanoparticles have antibacterial activity in chickens and pigs [231, 232]. Furthermore, Sawosz et al. [233] showed that silver nanoparticles potentially improve muscle morphology without affecting broiler performance at embryo growth. Moreover, a concentration of 50 mg/kg silver nanoparticles chelated with amino acids, threonine and cysteine improved immune competence in embryos and chickens [234]. Other metals have been synthesised as nanoparticles and supplemented to poultry feed. For example, metal oxide nanoparticles have shown antibacterial activity against *Salmonella* sp.: Al_2O_3, Fe_3O_4, CeO_2, ZrO_2 and MgO [235]. The above studies of metal nanoparticles focused on reducing antibacterial activity and, to some extent, on their influence on animal performance. However, the application of Cu-NP as a feed additive appears to be in the development phase.

Cu-NP administered through *in ovo* injection at levels of 4, 8, 12 and 16 μg/egg did not have an adverse effect on embryo growth [10]. However, a concentration of

Table 3: Advantages of using Cu nanoparticles in different animal species, according to the delivery and dosage used.

Species	System investigated	Delivery type and time	Concentration and size	Effect and conclusion	References
Chicken embryo	Growth and metabolic rate	Injection on day 1 of incubation	50 ppm, 2–15 nm	Increased metabolic rate, no harmful effect on embryo development	Scott et al. [11]
Chicken	Post-hatch performance	Injection on day 1 of incubation	50 ppm, 15–70 nm	Positively influenced chick performance and higher percentage of breast and leg muscles	Mroczek-Sosnowska et al. [110]
Chicken embryo	Hatchability and post-hatch performance	Injection on day 18 of incubation	4, 8, 12, 16 µl/egg, 72.3 nm	Not harmful to developing embryos, did not influence hatchability, increased feed efficiency, highest breast muscle with injection of 12 µl/egg	Joshua et al. [10]
Broiler chicken	Growth and metabolism	Intramuscular injection on day 14 after hatching	200 µl, 103 and 937nm	Stimulated growth and metabolic changes, increased red cell level, haemoglobin, Cu and protein in blood serum	Miroshnikov et al. [131]
Pig	Growth performance and nutrient digestibility	Feed additive	50 mg/kg, 10–100 nm	Improved growth performance and digestibility of crude fat and energy, reduced Cu in faeces	Gonzales-Eguia et al. [9]
Broiler chicken	Cu content in organs	Injection on day 1 of incubation	50 ppm	Greatest accumulation of Cu observed in the liver and spleen organs and less in the breast muscle	Mroczek-Sosnowska et al. [125]
Prawn	Growth, digestive and metabolic enzymes	Dietary supplement	10, 20, 40, 60, 80 mg/kg, 200 nm	Improved growth, digestive enzyme activity with 20 mg/kg, while 40–80 mg/kg had negative effects	Muralisankar et al. [240]
Rat	Composition and metabolism of the caecal microbiota	Dietary treatment	80, 160 mg/kg, 121.9 nm	Increased ADG, depressed activity of *B*-glucuronidase, but increased activity of *A*-galactosidase and *B*-galactosidase. Increased pH, pionate and butyrate in caecum digesta. Overall, enhanced microbiota and environment of caecum	Han et al. [242]
Rat	Small intestine morphology and digestive enzyme activity	Dietary supplement	80, 160 mg/kg, 121.9 nm	Increased villus height, but depressed crypt depth of duodenum and ileum. Improved enzyme activities in digesta	Han et al. [243]
Broiler chicken	Digestibility of nutrients and growth	Dietary supplement	100 mg/kg	No differences in digestibility of nutrients and growth performance were observed	Sarvestani et al. [244]
Chicken	Intestinal absorption of iron, zinc and calcium	Administration in drinking water	5, 10, 15 mg/l, 5 nm	The highest level of Cu-NP increased Cu content in the blood plasma of the birds. Furthermore, Cu accumulated in the intestines reduces absorption of calcium and zinc, but does not affect iron absorption	Ognik et al. [245]
Pig	Intestinal microflora and morphology	Dietary supplement	100 mg/kg, 121.9 nm	Increased ADG and feed intake and improved feed:gain ratio. Increased villus height, but depressed crypt depth of duodenum and ileum. The amount of *E. coli* decreased, but *Lactobacillus* and *Bifidobacterium* were increased	Wang et al. [246]
Broiler	Growth and immunity	Dietary feeding	50, 100, 150 mg/kg, 95 ± 10 nm	Improved growth, immune system, protein synthesis and caecal microbiota of broilers	Wang et al. [130]

Table 3 (continued)

Species	System investigated	Delivery type and time	Concentration and size	Effect and conclusion	References
Chicken embryo	Angiogenesis at systemic and molecular levels	*In ovo* injection	50 mg/kg, 37.3 nm	Cu-NP had pro-angiogenic properties at a systemic level to a greater degree than $CuSO_4$. Effect on mRNA concentration and gene expression	Mroczek-Sosnowska et al. [126]
Chicken embryo					Mroczek-Sosnowska et al. [236]
Chicken embryo	Inflammatory state	*In ovo* injection	50 mg/kg	Nanoparticles of Ag/Cu could prevent over expression of NF-kB mRNA	Sawosz et al. [247]
Broiler	Haematological and biochemical blood markers	*In ovo* injection	50 mg/kg	Cu-NP evoked an increase in blood levels of RBC, HGB, HTC, heterophils, monocytes and basophils. Reduced glucose and cholesterol levels	Mroczek-Sosnowska et al. [23]
Fish	Growth, blood profile, antioxidant and immune response	Dietary supplement	2, 4, 6, 8 mg/kg, <75 μm	2 and 4 mg/kg of Cu-NP showed better performance, lowered plasma glucose, improved immune response and antioxidant defence system	El-Basuini et al. [127]
Fish	Physiology and metal accumulation	Exposure regime	20 μg/l, 55–60 nm	Accumulation of Cu-NP was lower compared to $CuSO_4$ in gills and liver	Al-Bairuty et al. [111]
Rabbit	Performance	Feed additives	25, 50, 75 mg/kg	Higher final body weight, better feed conversion ratio, reduced abdominal fat, increased activity of superoxide dismutase enzyme, increased *Lactobacillus* counts and decreased population of ureolytic bacteria, *E. coli* and *Clostridium* spp. Increased plasma haemoglobin, red blood cell count and lymphocyte percentage	Refaie et al. [129]
Fish	Performance	Feed additives	2 mg/kg, <75 μm	Cu-NP was a significant factor on final weight, weight gain and specific growth rate, feed intake, feed and protein efficiency ratios, protein gain and protein retention, body protein and lipid content, protease and bactericidal activity and tolerance against stress	El-Basuini et al. [241]
Rat	Bone geometry and structure	Feed mixture	40 nm	Increased the ultimate load and toughness at the standard concentration. The higher values of the studied parameters prove that there was a change in bone mineralisation	Tomaszewska et al. [248]

50 mg/kg of Cu-NP showed an increase in metabolic rate with regard to oxygen consumption and heat production, which are important regulators in the developmental stages of chicken embryos [11]. The same concentration was found to exhibit pro-angiogenic properties at a systemic level, with the promotion of blood vessel development during embryogenesis and, thereafter, increase the body weight, improve feed conversion ratio and increase breast and leg muscles of broiler chickens upon administering Cu-NP *in ovo* [110, 126]. Just recently, it was shown that *in ovo* injection of Cu-NP stimulates proliferating cell nuclear antigen (PCNA)-positive cells in the long bones of broiler chickens, indicating a stimulatory effect during embryogenesis [236].

Some studies compared the inorganic forms of Cu with Cu-NP, and the latter showed an improvement in the growth performance of piglets [9]. Moreover, Cu availability was significantly improved, and the faecal Cu level was reduced compared to $CuSO_4$. Additionally, Cu-NP improved the digestibility of crude fat and energy in pigs. Furthermore, IgGγ globulin and total globulin protein levels were improved, and SOD activity increased. The same authors observed that the Cu level and cholesterol concentrations in serum were not affected by Cu-NP supplementation. The reason for these improvements may be attributed to better bioavailability and antibacterial activity of Cu-NP than $CuSO_4$.

Intramuscular injection of Cu-NP in chickens was investigated by Miroshnikov et al. [131], and they observed that Cu-NP stimulated chicken growth, increased red blood cell and haemoglobin levels, enriched Cu and protein levels in serum and also increased the arginine content in the chicken liver.

Another study reported that Cu-NP-loaded chitosan [130] improved growth performance and immune status, enhanced protein synthesis and was beneficial to the caecal microbiota of broiler chickens. Further, Zheng et al. [237] used Cu silicate nanoparticles in chicken diet and reported that adding 2 g/kg of Cu silicate nanoparticles could regulate the intestinal microflora, promote the growth of beneficial bacteria and inhibit harmful ones, enhance nitrogen metabolism and reduce ammonia emission from excreta. Recently, Nguyen et al. [238] added metal nanoparticles of iron, Cu, zinc oxide and selenium to supplement a chicken diet premix, which resulted in certain improvements of poultry farming depending on the quantity of nanocrystalline metal, which replaced the inorganic mineral component in the feed premix. The results also confirmed that nanocrystalline metals are able to decrease inorganic minerals in diet premixes by at least four times, allowing chickens to absorb feed minerals more efficiently and, consequently, decreasing the risk of environmental pollution. In addition, Cu silicate nanoparticles modified the intestinal microbiota of chicken, increasing counts of *Lactobacillus* species and decreasing *E. coli* [239].

Several studies have shown the advantages of adding Cu-NP to fish diet. A study by El Basuini et al. [127] investigated different concentrations of Cu-NP in fish diet, and the results demonstrated that 2 and 4 mg/kg of Cu-NP caused the highest final body weight, better feed efficiency, protein retention, immune response and antioxidant defence system compared to $CuSO_4$ and the control groups. Another study was performed to assess the effect of Cu-NP on freshwater prawns by Muralisankar et al. [240]; the results demonstrated that adding 20 mg/kg of Cu-NP showed an improvement in growth, digestive enzyme activity, the concentration of biochemical constituents and haemocyte count. However, the same authors reported that 40–80 mg/kg of Cu-NP had a negative effect and might be toxic to prawns. Lately, El Basuini et al. [241] determined the effects of Cu-NP and vitamin C on red sea bream and found that feed supplemented with 2 mg/kg of Cu-NP significantly influenced body weight gain, final body weight, feed intake, feed and protein efficiency ratios, body protein and lipid content, protease and bactericidal activity and tolerance against stress compared to the control diet.

There was also a study conducted on rabbits fed diets supplemented with different concentrations of Cu-NP, which showed a significantly higher body weight and better performance in comparison to the control [129].

An overview of the effects of Cu-NP on animal performance is shown in Table 3.

6 Conclusions

The main purpose of using Cu-NP as feed additives in chickens is to improve growth and performance in addition to reducing the pathogen load to improve health and reduce the excretion of Cu into the environment. Their small size and large surface-to-volume ratio make Cu-NP biologically more active than bulk Cu, allowing more efficient interaction with biological systems, which may decrease the amount of Cu used in the diet without causing an adverse effect on chicken performance and the environment.

The majority of studies have pointed out the beneficial effects on performance, antibacterial activity and immune status of Cu-NP as a feed supplement for

poultry and other animals. Furthermore, much work has been done regarding the synthesis and stability of Cu-NP.

However, it is important to control the size and concentration in order to achieve maximal benefits from Cu-NP and avoid their potential toxic effects. More studies should be conducted to describe the effects of Cu-NP on gut microbes and metabolite production. Along with these, studies should include more toxicology, histology and residue analysis of Cu-NP in the body and excreta before any wide adoption could take place.

Cu-NP incorporation in animal nutrition, which can enhance the performance and health of animals, should be conducted at a lower risk to consumers. However, a great amount of research is still required to support the safety of Cu-NP application in animal nutrition, avoiding any harm to livestock, the environment and human beings.

The inclusion of Cu-NP supplements in poultry feed seems possible in the near future; however, the use of Cu-NP is still in its infancy, but encouraging results from recent studies are driving further investigations.

Conflict of interest statement: The authors declare that they have no competing interests.
Funding: National Centre of Research and Development, Poland, (Grant/Award Number: "Gutfeed/267659").

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Bionotes

Abdullah Scott
Department of Veterinary and Animal Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark

Abdullah Scott is a PhD student under the supervision of Professor André Chwalibog at Copenhagen University in Denmark. His research focuses on the metal nanoparticles as alternative growth promoter in embryos and growing chickens.

Krishna Prasad Vadalasetty
Department of Veterinary and Animal Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark

Krishna Prasad Vadalasetty is a PhD student under the supervision of Professor Andre Chwalibog at the University of Copenhagen. Presently, he is working on the molecular influence of the graphene family nanomaterials on human breast cancer cells (MCF-7 and MDA-MB-231) under *in vitro* and *in ovo* conditions.

André Chwalibog
Department of Veterinary and Animal Sciences, University of Copenhagen, Groennegaardsvej 3, 1870 Frederiksberg C, Denmark,
ach@sund.ku.dk

André Chwalibog, Prof. DSc, is the head of the Division of Nanonutrition, University of Copenhagen. His research focuses on the application of nanomaterials in nutrition and medicine. He has been responsible for several national and international research projects and supervised numerous PhD and postdocs. He has an extensive list of publications and books.

Ewa Sawosz
Department of Animal Nutrition and Biotechnology, Warsaw University of Life Sciences, 02-786 Warsaw, Poland

Ewa Sawosz, Prof. dr hab., is the head of the Department of Animal Nutrition and Biotechnology at Warsaw University of Life Sciences. Her research focuses on different aspects of nanobiotechnology and nanomedicine. She has been responsible for several national and international research projects and supervised numerous PhD and postdocs. She has an extensive list of publications and books.