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IN VITRO ANTIMICROBIAL ACTIVITY OF PHOSPHATE-BASED ZINC NANOPARTICLES

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Abstract

In recent years, zinc nanoparticles have captivated an attention due to their antimicrobial properties. Moreover, the advantage of nanomaterials is an ability to modify their chemical composition and influence their antibacterial properties. In this study, zinc-phosphate nanoparticles (ZnNPs) were prepared via chemical route of synthesis. Their antibacterial activity was evaluated by monitoring a bacterial growth of model microorganisms: gram-negative (G^-) *E. coli*, and gram-positive (G^+) *S. aureus* as well as methicillin-resistant *S. aureus* (MRSA). Obtained results have shown, the ZnNPs are the most effective against G^+ *S. aureus* compared to MRSA or G^- *E. coli*. The inhibition concentrations for *S. aureus*, *E. coli* and MRSA was 0.16, 1.25, 2.5 mM, respectively. To conclude, ZnNPs exhibit antibacterial activity against both G^+ and G^- model microorganisms, however, G^- bacteria are more sensitive against ZnNPs.

Keywords: animal nutrition, zinc, nanomaterials, antimicrobials

INTRODUCTION

Zinc is a medium-hard brittle blue-white metal, crystalline and shiny when fractured. The location in the periodic table of elements, is in the fourth period and the twelfth group. Zinc occurs in the form of a divalent ion when its d-orbitals are filled with electrons and thus cannot undergo redox changes. Classified as a biogenic element essential for life. Physico-chemical properties determine its use and importance in biological systems (Kim *et al.*, 2017).

In the animal organism, zinc as essential element is employed in several metabolic pathways. Essential for DNA replication, transcription, and protein synthesis as well as it is important part of more than 160 enzymes and metalloproteins (Xia *et al.*, 2021). Together with vitamins and selenium, zinc prevents the formation of mediators of inflammation in the body and is essential to growth due to it employment in hormone metabolisms (Nowak *et al.*, 2002).

In animal nutrition the source of zinc is generally in form of oxides, sulfates, carbonates, or acetates etc. Zinc bioavailability differs according to its source, from ZnO bioavailability is lower than from $ZnSO_4$, Zn-methionine, and Zn-lysine (Case ate *al.*, 2002). Organic forms are in general more bioavailable for organism compared to inorganic forms (Pereira *et al.*, 2021). Bioavailability is influenced also by the relation of different minerals for example higher intake of copper and calcium decreases the bioavailability of zinc (Villagómez-Estrada *et al.*, 2021). Absorption of Zn^{2+} by mammals take place in the ileum, the absorption process is divided into four stages by (Geffeler *et al.*, 2015): intestinal cell uptake, mucosal cell transport, portal vein circulation, and endogenous zinc secreted back to intestinal cells.

Zinc deficiency lead to retarded growth, weakened immunity, and consequential pathological changes in the organism (Lichten *et al.*, 2009). Besides its indispensable functions in the organism, zinc has shown antibacterial activity. Studies propose three

mechanisms of antimicrobial effect. Bacterial resistance to already available antibiotics demands for new approaches in medicine field (Liaqat *et al.*, 2022).

It has been found that G^+ bacteria are more susceptible to Zn compared to G^- bacteria either by the disruption of bacterial integrity, damage by ROS (reactive oxygen species) or downregulation the transcription of oxidative stress-resistance genes (Siddiqi *et al.*, 2018).

In last decades, the research of antimicrobial nanomaterials has been raised. Nanotechnology is an interdisciplinary scientific field dealing with objects with a size of about nanometres, (Bayda *et al.*, 2020). One of the most perspective nanomaterials with antibacterial activity are zinc nanoparticles (ZnNPs). Studies (Hong *et al.*, 2020; Khan *et al.,* 2019) confirmed higher absorption of NPs by body tissues as well as easier transport through biological barriers. Moreover, ZnNPs exhibit attractive chemical properties such as high stability, and the ability to modify their surface or chemical composition, promising potential in many applications.

Antimicrobial activity of ZnNPs is enhanced by their small size and high relative surface area (Sirelkhatim *et al.*, 2015). The mechanism of action is through ROS formation which disrupt bacterial cell walls (Singh *et al.*, 2020).

Antibacterial effects of ZnNPs have attracted the attention of many scientists looking for a suitable alternative for ZnO in pig industry. Since 2022 in the European Union, the use of zinc oxide in medical doses has been banned. Some studies have suggested that zinc nanoparticles might replace ZnO for the prevention of diarrhea in weaning piglets (European Commission, 2003).

The aim of this article deals with the use of phosphate-based zinc nanoparticles and its impact on the model gram negative and gram positive microoganisms as an antimicrobial agent.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and Penta (Prague, Czech Republic) of p.a. purity, unless noted otherwise. The pH value was measured using inoLab® Level 3 (Wissenschaftlich-Technische Werkstatten GmbH; Weilheim, Germany). Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and it was subsequently purified using Millipore RG (Millipore Corp., Waltham, MA, USA) – 18 MΩ MilliQ water.

Preparation and Characterization of ZnNPs

$Zn(NO_3)_2 \cdot 6H_2O$ (4.46 g) was dissolved in water (50 mL) and solution was heated to 60 °C. $(NH_4)_2HPO_4$ (1.32 g in 20 mL of water) was added while stirring and white precipitate was immediately formed. The suspension was stirred for 2 h, cooled and water was added to reach 100 mL. The suspension was dried at 60 °C (drying oven, Binder, USA). ZnNPs size were measured by dynamic light scattering (NANO-ZS, Malvern, USA).

Preparation of Liquid and Solid Culture Medium

All bacterial cultures used were cultured in Muller-Hinton Broth (MHB) or Luria-Bertani Broth (LBB) medium. MHB consisted of 21 g/L MHB mixture (Oxoid, Hampshire, UK) and LBB of tryptone 10 g/L, NaCl 10 g/L and yeast extract 5 g/L. After the addition of MilliQ water, the medium was sterilized with a steam sterilizer (Tuttnauer, Holland) at a temperature of 121 °C for 15 minutes.

Preparation of Bacterial Cultures

Bacterial cultures used were all obtained from the Czech Collection of Microorganisms in Brno (Czech Republic). Preparation of biological material took place in deep-frozen bacterial cultures stored at -80 °C. Ten µL of a microbial culture of *Staphylococcus aureus* (NCTC 8511), *Escherichia coli* (NCTC 13216), or methicillin-resistant *S. aureus* (MRSA) was aseptically collected with a sterile loop and mixed with 25 mL of sterile MHB or LBB. Bacterial cultures were incubated overnight in a Hood TH 15 shaking incubator (Edmund Buhler GmbH, Germany) at 37 °C and 120 RPM.

Bacterial Viability and Growth Curves

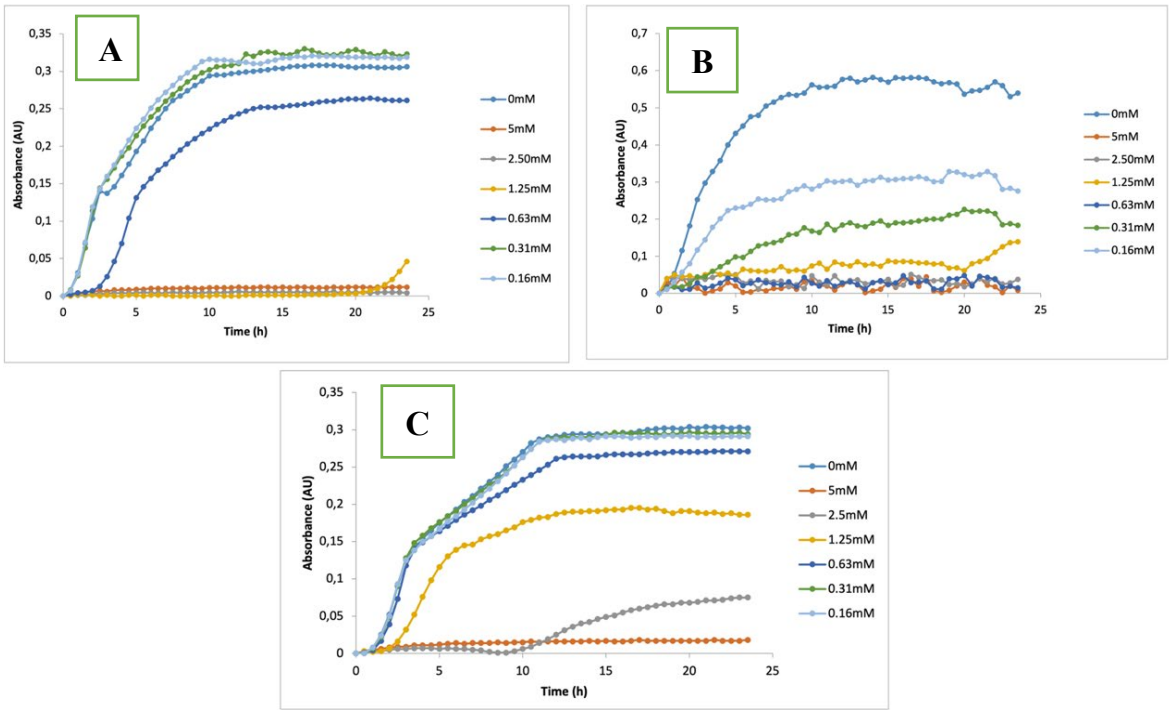
Microbial culture (1 degree McFarland) was applied to a 96-well mictrotitration plate and diluted 1:1 with a solution of zinc nanoparticles of various concentrations or, in the case of the control, only water. The absorbance of the bacterial turbidity was monitored at a wavelength of 620 nm for 24 hours at 37 °C with a 0.5 hour incubation. The concentration of selected microorganisms was determined by turbidimetric determination using Multisken EX (Thermo Fisher Scientific, Germany). The absorbance value of the individual wells of the mictrotitration plate was directly proportional to the number of bacteria.

Data Evaluation and Statistics

All measurements were statistically evaluated and presented in the form of figures using Microsoft Excel (Microsoft Corporation, Redmond, USA).

RESULTS

E. coli was used as a representative of G^- microorganisms, and *S. aureus* and MRSA were used as G^+ bacteria. Phosphate based ZnNPs (472 nm) were used in the concentrations: 0.16, 0.31 0.63, 1.25, 2.5 and 5 mM.



1: *Effect of Zn-A nanoparticles at different concentrations on bacterial culture growth of (A) E. coli, (B) S. aureus and (C) MRSA*

ZnNPs decreased viability on *E. coli* in the concentrations 1.25, 2.5 and 5 mM (Fig. 1A). Lower concentrations of ZnNPs 0.16, 0.31 and 0.63 mM showed similar trend of growth curve as *E. coli* without ZnNPs treatment. ZnNPs antibacterial efficiency against *S. aureus* is shown in Fig. 1B. From obtained results is obvious that ZnNPs concentrations from 0.16 to 5 mM inhibited *S. aureus* growth. However, the full growth inhibition was reached at concentrations of 1.25, 2.5 and 5 mM. From Fig. 1C is clearly shown, the ZnNPs concentrations 2.5 and 5 mM inhibited MRSA growth, and concentration of ZnNPs 1.25 caused slower growth of bacteria.

DISCUSSION AND CONCLUSION

In our previous study (Horky *et al.*, 2019) zinc phosphate nanoparticles were reported as a promising alternative to ZnO outstanding to their antibacterial property. Increment of antibacterial activity of nano zinc compounds is being experimented by different research groups, either by reducing the particle size or by incorporating heteroatoms in the matrix. (Souad *et al.*, 2020) evaluated the antibacterial property of calcium phosphate by measuring the inhibition zone of *S. aureus*. The antibacterial efficacy of calcium phosphate functionalized with aniline was determined by evaluating its ability to prevent the growth of *E. coli* by Lorena *et al.* (2018). Yadav *et al.* (2021) in a study on antibacterial and antifungal activities of zinc phosphate incorporated with Ag (AgZnP), resulted the bacterial growth-inhibiting potential of AgZnP against *E. coli* and *S. aureus* as well as its antifungal activity against *S. cerevisiae* and *A. brasiliensis*.

Our results have shown the phosphate-based ZnNPs have various efficiency against model pathogenic bacteria. The highest effect has been observed against G^+ *S. aureus*. ZnNPs inhibited bacterial growth even at the lowest applied concentration 0.16 mM. In contrast, ZnNPs were less effective on G^- *E. coli*. The efficient inhibition concentration was 1.25 mM. The lightest antibacterial effect has been observed in the case of MRSA where the 2.5 mM concentration of ZnNPs inhibited bacterial growth. Our results are in agreement with similar studies which has confirmed higher antibacterial efficiency of ZnNPs on G^+ bacteria (Baholet *et al.*, 2022). Malagurski *et al.* (2017) observed antimicrobial effect of 50 mM Zn-phosphate nanocomposite film, using DDM (disk diffusion method). Using the same method DDM, (Alghari *et al.*, 2020) confirmed the antibacterial activity of *E. coli* and *S. aureus* using Ni NPs, 80% bacteria were killed. In a similar study (Vahedi *et al.*, 2017) on *S. aureus* acknowledged that Ni NPs possess strong antibacterial efficiency.

The Zn-phosphate nanocomposite film was more effective in elimination of G^+ bacteria. This phenomenon could be explained by differences in bacterial cell wall structure between G^+ and G^- bacteria (Silva *et al.*, 2019).

In this study, formulations of phosphate-based ZnNPs were synthetized and tested. Our formulation of zinc phosphate-based nanoparticles ZnNPs exhibited antibacterial activity against G^+ and G^- bacteria. Therefore, nanoparticles have a potential to be used as antibacterial agents, especially for reduction of coliform bacteria. Further studies, primarily focused on ZnNPs applications in livestock productions, are warranted.

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