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Nanocomposite of Ag-Doped Zno and Ag_2O Nanocrystals in Control of Salmonella Heidelberg Biofilms Formed in Eggs

[1]P.L.F. Nunes, [1]P.C. Almeida, [1]T.F.M. Reis, [2]L.R. Goulart Filho, [3]A.C.A. Silva, [3]N.O. Dantas, [1]D.A. Rossi, [4]P.G. Hoepers and [1]B.B. Fonseca

[1]Faculdade de Medina Veterinária, Universidade Federal de Uberlândia Uberlândia, Minas Gerais, Brasil
[2]Universidade Federal de Uberlândia, Instituto de Genética e Bioquímica, Uberlândia, Minas Gerais, Brasil
[3]Universidade Federal de Uberlândia, Instituto de Física, Uberlândia, Minas Gerais, Brasil
[4]Brasil Foods S/A, Animal Health Corporative Area, Curitiba, PR, Brazil

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Corresponding Author:
P.L.F. Nunes
Faculdade de Medina Veterinária, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brasil

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Abstract: *Salmonella* spp. is an important causal agent of salmonellosis in humans. The control of *Salmonella* spp. is important in eggs, since, the bacterium passes through shell to embryo and remains in the lots of poultry that can carry the infection to the human. Disinfection is usually done by several sanitizers but with nanotechnology advances, new and more efficient ways of controlling this agent are being studied such as nanoparticles. Preliminary studies of these nanoparticles have shown the success of their use in the control of microorganisms. The standardization of the ideal concentration for the use of this nanocomposite is fundamental for optimum efficiency in the control of *Salmonella* spp. Eggs from red commercial laying were purchased from local trade were used. In the laboratory, the eggs were treated with the nanoparticles in different concentrations and after 24 h formed the biofilm. The eggs were washed for removal of the free bacteria and performed conventional microbiology for isolation of *Salmonella* spp. and PCR for identification of colonies. The aim of this study was evaluated the effectiveness of the use of nanocomposite of silver oxide with silver oxide doped Zinc Oxide (ZnO:Ag-AgO) in different concentrations in the prevention of the formation of eggshell biofilms.

INTRODUCTION

The control of infectious diseases such as Salmonella is important for public health because it affects a large part of the population. Europe Union has reported every year about 100.000 cases in humans. Salmonellosis is a disease caused by bacteria of the genus *Salmonella* that is often associated with outbreaks of food-borne illnesses. They are also classified as zoonosis since they reach several species. In humans, the exclusive serotypes are *S. typhi* and *S. paratyphique*. The *S. gallinarum* and *S. pullorum* serotypes are poultry specific. Salmonella Heidelberg was isolated in Brazil, since, 60's but recently has been found in poultry production of various countries

(Borsoi *et al*., 2011). Salmonella has incidence in several countries, since, their control is hampered by their high contamination rate in the population and cumbersome preventive measures (WHO., 2018). In Brazil, about 2% of poultry carcasses are contaminated with this agent, causing serious financial damage to the poultry industry (Medeiros *et al*., 2011).

The egg is constantly involved in contamination by *Salmonella* spp. in humans, since its raw consumption or in food products without proper treatments may be responsible for generating toxinfection (Tauxe, 1997). In these conditions much has been invested in measures for the sanitary control of Salmonella in eggs, since, it affects the public health worldwide (Jones, 2011).

Bacteria of the genus Salmonella belongs to the family Enterobacteriaceae, is bacilli-shaped, gram-negative, facultative anaerobic and non-spore forming. Due to oscillating ambient conditions, some organisms, including *Salmonella* spp., developed the capacity of adhere on a surface and agglomerate, forming a multicellular complex filled with a matrix of polysaccharides, this process is called biofilm (Latasa *et al*., 2012). Biofilm is beneficial to bacteria because promotes certain resistance to antibiotics, disinfectants substances and the host's own immune system. Some infections in hospitals, industries are associated with this process that is why it is important to create different compounds to eliminate and control biofilm (Steenackers *et al*., 2011).

Nanotechnology is no longer just accessible for industry but is being increasingly used in our day life. This technology creates compounds with singular characteristics because of its small size that increases the contact surface and provides a greater reactivity (Gitipour *et al*., 2017). The nanomaterials have different properties according to the method used for their synthesis. Nanosilver Particles (AgNPs) have antimicrobial activity and demonstrated capacity to inhibit biofilm formation of different agents as *Escherichia coli* and *Klebsiella* spp (Dakhil, 2017).

The Zinc Oxide (ZnO) nanocrystals is biocompatible material, is classified as Generally Recognized as Safe (GRAS) material by the United States Food and Drug Administration. Then, in this work we investigated this nanocrystal doped with Silver (Ag) and Silver Oxide (Ag_2O) nanocrystals (ZnO:Ag-AgO). The doping technique is used to increase the catalytic activity in ZnO nanocrystals and the ability to induce oxidative stress in bacteria, being one of the main mechanisms of bactericidal action (Pati *et al*., 2014).

The aim of this article is use the nanocomposite of silver oxide with silver oxide doped Zinc Oxide (ZnO:Ag-AgO), synthesized by researchers of the present study, with different concentrations to disinfect the surface of eggshell to control the biofilm formation of Salmonella Heidelberg.

MATERIALS AND METHODS

Nanoparticles are synthesized in the "Laboratório de Novos Materiais Isolantes e Semicondutores (LNMIS)" from "Instituto de Física", "Laboratório de Epidemiologia Molecular and Laboratório de Incubação from Faculdade de Medicina Veterinária" were used to biological study and incubation process of the eggs, respectively. All laboratories are from "Universidade Federal de Uberlândia".

There are many physical and chemical processes of nanoparticles synthesis. Was used a X-Ray Diffraction (XRD) to analyse the physical properties of nanoparticles. For the synthesis, the method used was the coprecipitation (patent number BR 10 2018 0077147). Nanocomposite of ZnO:Ag-AgO nanocrystal were synthesized at room temperature via. aqueous solutions of Zinc Chloride (ZnCl 2, 99.9%, 2M) and silver nitrate (99% $AgNO_3$, ranging from 0.1-11% Ag in relation Zn). The pH of the solution was adjusted to 11 using an aqueous solution of sodium hydroxide (98% NaOH). The nanoparticles formed were purified via. centrifugation at 6000 rpm/1 min. All reagents were purchased from Sigma-Aldrich. We synthesized nanocomposite ZnO:Ag-AgO-doped with three different concentrations of silver.

To determine the level of silver doping used in the experiment, antibiograms were prepared as described by Bauer *et al*. (1966), inoculating a suspension of bacteria on Mueller Hinton agar plates and adding filter paper disks impregnated with nanopacomposite at three different dosages of silver doping (5, 9 and 11). Sulphonamide disks (300 µg) (LABORCLIN®) were used as a control. The plates were incubated for 20 h at 37°C and the halos were measured for the determination of the inhibition spectrum.

The eggs used in this research came from a supermarket being commercial laying hens of a farm in the region of Minas Gerais, Brazil. SH was isolated in our laboratory where they were characterized and made genetically typed.

To evaluate nanocomposite of ZnO:Ag-AgO nanocrystal antibacterial properties in control SH, we used eggs. A total of 51 eggs were divided into seven groups each group consisting of 8 eggs with the exception of the negative control that was composed of 3 eggs and each sample unit consisting of two eggs. The eggs were treated with different concentrations of nanoparticles as follows: (G1) Spraying approximately 1.0 mL of a solution containing 1.4 µg mL^{-1} of Nanocomposite of ZnO:Ag-AgO; (G2) Spraying approximately 1.0 mL of a solution containing 1.4 µg mL^{-1} of nanocomposite of ZnO:Ag-AgOmore Tween 201% (surfactant substance); (G3) Spraying approximately 1.0 mL of a solution containing 14 µg mL^{-1} of nanosilver particles doped with zinc ZnO:Ag-AgO; (G4) Spraying approximately 1 .0 mL

of a solution containing 1.4 mg mL^{-1} of nanocomposite of ZnO:Ag-AgO; (v) Spraying approximately 1.0 mL of a solution containing peracetic acid (400 ppm) before the contact with bacteria; (G6) (positive control) Spraying ultrapure water with bacteria; (vii) (positive control) Spraying ultrapure water.

After a period of 24 h, the eggs were submerged, separately two by two, in a suspension of 100 mL of TSB containing 10^5 CFU mL^{-1} of SH for a period of 24 h at 25°C. In the seventh group, negative control, the eggs were also submerged for 24 h but in sterile TSB without the presence of bacteria. After this period of biofilm formation in eggs, the eggs were washed for 3 times in ultrapure water for removal free bacteria. After drying the eggs, the eggs were opened in laminar flow, separated and collected aseptically shell, albumen and yolk. Were weighed two samples of 10 g of each sample unit for two different analyses carried out at the same time. A total of 10 g of each sample were added 90 mL of peptone water (Isofar®) and incubated for 24 horas at 37°C. Later a 1 mL aliquot was inoculated in the culture medium Rappaport (Oxoid®) and 1 mL in culture medium Tetrathionate (Merck®) and after 24 h of growth, both were depleted in XLD.

Colonies with typical characteristics of Salmonella were selected and submitted to a conventional PCR assay (*omp C* gene) reaction to specie confirmation. The genomic DNA was extracted by the Wizard Genomic DNA Purification Kit (Promega, city, country), following the protocol provided by the manufacturer. Purified DNA (10 ng) was used as template for all PCR assays. The primers used (*omp C* gene) in this analysis were (3'ATCGCTGACTTATGCAATCG 5' and 5'CGGGTTGCGTTATAGGTCTG 3') (Alvarez *et al*., 2004) producing a fragment of 204 bp. We used GoTaq® Green Master Mix kit (Promega, city, country) according to the manufacturer's instructions. The microtubes containing the PCR products were transferred to the thermocycler (Eppendorf®) for amplification, following of the cycles: one initial denaturation cycle at 94°C for 5 min, amplified in 35 cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 1 min; extension at 72°C for 90 sec with final extension at 72°C for 10 min. As a positive control of the reactions, the strain of *S. enteritidis* ATCC 13076 was used. The amplified products were subjected to a 1.5% electrophoresis agarose gel and by using a TBE 0.5x runner buffer (Invitrogen), having as a molecular weight, the standard value of 100 pb marker (Invitrogen).

As the eggs were not sterilized, another microarray PCR assay was performed for serotype confirmation. The multiplex ligation detection reaction (LDR) generated DNA molecules collections. Such DNA molecules were subsequently amplified by means of a single pair of amplimers over a PCR. The PCR products were then sorted by hybridization to a low-density DNA microarray. Positive hybridization was detected using a biotin label incorporated in one of the PCR primers. Tubes were then inserted in the single-channel ATR03 array tube reader upon completion of the detection reaction and images were acquired and interpreted with the software supplied by the manufacturer (Check-Points, Wageningen, The Netherlands).

The analyses were performed software using GraphPad Prism, Version 7.0. We used the chi-square followed by the binomial between two proportions in relation to the positive control. Here, the confidence level was 95% for all reports.

RESULTS AND DISCUSSION

The great dispersion in water is ideal for a good performance of nanocrystals. SH was able to form biofilm in all groups tested. The Group (G4) that had the higher concentration of nanoparticle was the group that most controlled SH biofilm formation in relation to the positive control (G6).

The results of PCR confirmed that the typical colonies tested were SH. Microarray PCR assay confirmed the SH serotype. In the statistical analysis, all the groups do not differentiate in positive positivity index of the positive control, except in Group G4. The use of nanoparticles at the concentration of 1.4 was able to better control the formation of the biofilms in the egg shell. The Zinc-Oxide (Zn-O) is an antimicrobial compound that acts by different mechanisms. This compost is also used in packages of food to preserve (Espitia *et al*., 2016). When Zn is bounded with Ag to form nanoparticles, a good efficacy was noted in some studies against gram negative and gram positive bacteria (Fig. 1).

The average size of the silver-doped ZnO nanocrystal varied from 15-28 nm. The nanocomposite of ZnO:Ag-AgO nanocrystal manufactured by us had characteristics for a good nanocrystal. The same nanoparticle can be manufactured by different methods resulting in different properties. The nanoparticle synthesis methodologies are basically two categories: the physical methods (top-down) and methods chemical (bottom - up). In this work we used physical methods, with physical processes such as grinding, tempering, thermal decomposition, irradiation, among others where the nanomaterial is formed from a larger sample than it. We have already worked with other nanoparticles purchased both from Brazil and abroad that were not efficient in our studies. In previous studies we realized that the nanoparticles produced by us as ZnO and the nanocomposites were far superior to those acquired in the market (data not shown and and unpublished).

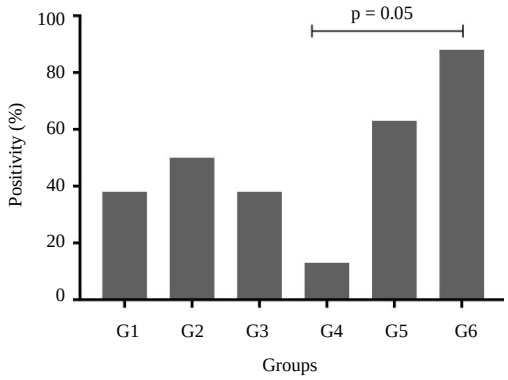

Fig. 1: Percentage of positive samples after treatment with nanocomposite of Ag:ZnO+AgO nanocrystal in the eggshell for the following groups: G1: 1.4 μg mL^{-1} Ag: ZnO+AgO, G2: 1.4 μg mL^{-1} Ag:ZnO+AgO more 1% Tween 20; G3: 14 μg mL^{-1} Ag: ZnO+AgO; G4: 1.4 mg mL^{-1} Ag: ZnO+AgO; G5: peracetic acid 400 ppm; G6: Positive Control. In G7: (Negative Control) the results were negative

The principal risk associated with the use of nanoparticles is their capacity to form free radicals in cells, like ROS (Reactive Oxygen Species) which could interfere in many intracellular processes (Fu *et al*., 2013). But ZnO:Ag-AgO nanoparticle is very stable. Silver when is bound with zinc oxide loses reactive characterizes and becomes unable to bound to intracellular oxygen. It shows that ZnO:Ag-AgO is safe to live cells of animals. Previous analyzes done by us have not shown toxicity of these nanocomposites in cell culture and chicken embryos (data still under study and unpublished).

When ambient conditions are not favorable, Salmonella has a capacity to adhere on a surface, grows and produces an extracellular matrix, a process called biofilm. Biofilm gives resistance for bacteria for many external factors that can be physical, chemical and biological (Peng, 2016). Disinfectants are not able to remove the entire matrix of biofilm, they can only reduce bacterial load (Srey *et al*., 2013). So is also associated with mechanical action to increase the process efficiency because this process can disorganize external matrix and exposed microorganisms to the disinfectant (Maukonen *et al*., 2003).

The eggshell has many pores that are large enough to allow passage of microorganisms into the egg. Studies carried out by us (data unpublished) and show that SH after a contact for a period of 24 h with the eggshell is able to infect the inside of the egg.

Because of eggshell irregular surface due to presence of pores and roughness, the egg is a great experimental model to form biofilm. The structures that are developed in the process of bioflm formation that allows the transport of nutrients have good formation in this kind of surface. Beside this, the Salmonella control in eggs is very important to the poultry industry. So, silver nanoparticles AgNPs could be an excellent alternative for Salmonella control in eggs and in ambient (Losasso *et al*., 2014). Salmonellas are responsible to cause a zoonosis, salmonellosis which affect whole world. About 94 million confirmed cases of this disease in humans are measured each year (Majowicz *et al*., 2010). The egg is one of foodstuff of animal origin that is most involved in contamination by this agent. The *Salmonella enterica* Heidelberg sorovar has been responsible for an increase in the number of cases of salmonellosis in recent years (Gieraltowski *et al*., 2016). Contamination is frequently linked with consumption of turkey and poultry products, which has generated several outbreaks in the USA (CDCP., 2018). SH is an important agent in poultry farms because Salmonella, as shown in previously studies can pass into the egg and infect embryos. Preventive treatment with nanoparticles is essential to avoid living forms of bacteria in the egg shell, since, biofilm after his formation, is very difficult to eliminate.

Although, the nanocomposites produced by us present the minimum requirements to be safe and a good nanoparticle, part still precipitates in water. This is due to lack of ideal equipment for dispersion during manufacturing. This causes a loss of particles during the sprinkling process leading to a waste of material. Even so, the nanocomposite of ZnO:Ag-AgO nanocrystal demonstrated more effectiveness than peracetic acid in high concentration to control biofilm formation in eggshell. We are working to improve the dispersion of the nanoparticles to decrease the concentration used in the next studies.

CONCLUSION

New strategies are essential for the control of microorganisms in aviculture. The nanoparticles have been shown excellent results in the control of the *S. heidelberg* biofilm. With this, the nanocomposite of ZnO:Ag-AgO nanocrystal is an alternative technology to control Salmonella in the poultry science.

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