*Journal of Applied Veterinary Sciences, 10 (2): 86 - 97 (April, 2025).*
**ISSN: Online: 2090-3308, Print: 1687-4072**
**Journal homepage : https://javs.journals.ekb.eg**

# Effect of the Synergism Among Nano-particles, Antibiotics and Biocides on Salmonella Typhimurium Strains, "A Comprehensive Study"

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## ABSTRACT

Microbial resistance (AMR) presents a serious hazard to the poultry sector, particularly concerning bacterial infections, as well as Food-borne microorganisms represent significant pathogens that impact food safety and contribute to human illness globally, primarily through the ingestion of food contaminated with these pathogens or their toxins, particularly in poultry products. Environmental contamination with various *Salmonella* serotypes is common in commercial laying hen farms and is a major concern for the global poultry industry. Understanding the interrelationships between these agents at the molecular level could help elucidate cross-resistance or co-resistance mechanisms, aiding in the design of effective intervention strategies. A total of 20 isolates of S.T. were obtained from poultry layer flocks. Approximately 80% of these isolates exhibited multidrug resistance (MDR). The minimum inhibitory concentration (MIC) was determined using broth microdilution methods. We investigated the synergistic effects of various nanoparticles, including silver nanoparticles (Ag-NPs), zinc oxide nanoparticles (ZnO-NPs), chitosan nanoparticles (CH-NPs), and zeolite nanoparticles (ZE-NPs), on 15 antibiotic-resistant strains of S.T. The antibacterial properties of these nanoparticles, both individually and in combination with selected antibiotics and biocides, were assessed against the tested S.T. isolates. The findings indicated a significant enhancement in antibiotic efficacy when combined with all tested nanoparticles, with the exception of nalidixic acid, where synergy was observed only with ZnO-NPs. The incorporation of nanoparticles with antimicrobial agents may provide a strategy to combat antibiotic resistance and improve their effectiveness. Furthermore, the results demonstrated a significant increase ($p < 0.05$) in the antibacterial activity of nanoparticles combined with biocides against S. T compared to the use of antibiotics and biocides alone against S.T., attributed to a notable reduction in MIC50. It can be concluded that the application of nanoparticles as efflux pump inhibitors not only aids in restoring the bactericidal effects of existing antibiotics but also diminishes the capacity of microorganisms to develop biofilms.

***Keywords:*** Antibiotics, Anti-Microbial-Resistance, Biocides, Nano-particles, Synergism.

***Original Article:***
**DOI: 10.21608/javs.2025.354340.1518**
***Received : 20 January, 2025.***
***Accepted: 08 March, 2025.***
***Published in April, 2025.***



***J. Appl. Vet. Sci., 10(2): 86-97.***

## INTRODUCTION

Salmonella is an enteric pathogen capable of infecting a wide range of animals, including humans. In poultry, salmonellosis is attributed to Gram-negative bacteria belonging to the genus *Salmonella.* This genus comprises two primary species, enterica and bongori**(Lin-Hui and Cheng-Hsun, 2007),** yet it encompasses nearly 2,700 serotypes (serovars), with approximately 10% having been identified in avian populations. Generally, many *Salmonella* serotypes can infect multiple animal species **(Gast, 2008),** including Salmonella typhimurium and Salmonella Enteritidis. Bacterial infections within commercial poultry operations lead to considerable economic losses in the poultry sector. Among these infections, *Salmonella* species are linked to various clinical manifestations in chickens and are recognized as significant food-borne pathogens for humans **(Bezerra *et al.*, 2016).** Avian salmonellosis is regarded as one of the predominant bacterial diseases affecting the global poultry industry. Furthermore, salmonellosis is the most prevalent avian disease that can be transmitted to humans **(Kabir, 2010).** Over the past decade, the rise of bacterial antimicrobial resistance has emerged as a significant

challenge in combating bacterial infections. In poultry production, there is an urgent necessity to reduce the levels of bacterial resistance to commonly used antibiotics and disinfectants **(Mohamed *et al.*, 2017).**

*Salmonella* is responsible for a higher incidence of food-borne illnesses than any other bacterial pathogen, with chicken being a significant contributor to these infections. Research indicates that roughly 1 in 25 packages of chicken available in grocery stores is tainted with *salmonella* **(CDC, 2023).** Over recent decades, there has been a notable rise in microbial resistance, largely attributed to the extensive and often inappropriate use of antibiotics. The medical community has expressed considerable interest in various applications of nanotechnology. In the realm of veterinary medicine, much of the antimicrobial resistance seen in human healthcare can be linked to improper antibiotic use among human patients; however, there is a strong argument that the use of antimicrobials in veterinary practices and food animal production exacerbates the issue for several bacterial pathogens **(Witte, 1998; National Research Council, Institute of Medicine, 1998; Barton, 1998).**

The implications of microbial resistance in medicine are significant, leading to increased costs for treating infectious diseases, the ineffectiveness of established treatments, and ultimately a rise in morbidity and mortality associated with infections **(Adeniji, 2018).** Furthermore, antibiotic resistance compromises the human immune system's ability to combat infections and heightens the risk of complications for vulnerable patients undergoing chemotherapy, dialysis, surgery, and joint replacements **(Nilavukkarasi *et al.*, 2020).** An increasing number of human infections are becoming complex and challenging to manage for reasons beyond antibiotic resistance **(Mantzana *et al.*, 2023).**

This study aims to demonstrate: 1) the synergistic effects of nanoparticles on the effectiveness of antibiotics and biocides in relation to Salmonella Typhimurium, as well as the bactericidal properties of nanoparticles against this pathogen; and 2) the potential of developing new pharmaceutical technologies grounded in nanoscience as a promising approach to combat microbial resistance, particularly in light of recent advancements and insights in medical biology. This study seeks to elucidate how nanoparticles can be utilized to address infectious diseases.

## MATERIALS AND METHODS

### Materials

#### *Salmonella Typhimurium* (ST)

We selected 20 strains of *Salmonella Typhimurium* (ST) of various resistance patterns. Theses (20) were used from poultry farms in Al-Sharqia Governorate, Egypt. These strains were isolated from air sacs, lungs, liver and ovaries from poultry. The samples were sent to national laboratories for culture, isolation and characterization.

#### Identification of *Salmonella typhimurium* (ST)

The strains were cultured by incubating LB broth at 37°C overnight. Pure bacterial colonies were obtained by streaking the broth cultures onto Hektoen enteric agar (HEA) medium. Further confirmation of the cultures was achieved through traditional biochemical methods **(Cardona-Castro *et al.*, 2002).** Genomic DNA was extracted from the cultures following standard protocols (Pure Link genomic DNA isolation kit, Invitrogen, USA). The integrity of the extracted DNA was verified by electrophoresis on a 1% w/v agarose gel. Polymerase chain reaction (PCR) was performed using gene-specific primers (Forward primer: hfq- 5′ GGAAGGATCCATGGCTAAGGGGCAATCT 3′ and Reverse primer hfq: 5′GCGCGTCGACTTATTCAGTCTCTTCGCTGTC 3′) **(Behera *et al.*, 2015).** The primers of hfq gene (309bp) of Salmonella Typhimurium **(Behera *et al.*, 2015)**.

The primers targeted the hfq gene (309 bp) of ST **(Behera *et al.*, 2015).** The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) under a UV transilluminator. The strains were subjected to antibiotic susceptibility testing, with breakpoints determined by the minimum inhibitory concentration (MIC) of specific antibiotics to identify resistant bacteria **(Gnanadhas *et al.*, 2013).** For subsequent MIC testing, thawed fractions were diluted to a density of 108 colony-forming units (CFU)/mL, following the methodology outlined by Hannan (2000). This ensured that the inoculum density for the MIC plates was approximately 5 x 105 CFU/mL. Isolated ST of the hfq gene in S. T. (2021) served as a positive control to monitor the MIC. The minimum inhibitory concentration (MIC) was evaluated for a total of 15 different antibiotic classes, including: Ampicillin, Ticarcillin, Amoxicillin-clavulanic acid, Kanamycin, Streptomycin, Nalidixic acid, Ciprofloxacin, Sulfamethoxazole, Trimethoprim-Sulfamethoxazole-Tetracycline, Chloramphenicol, Norfloxacine, Enrofloxacin, Ceftazidime and Azithromycin.

### Methods

#### Preparation of Salmonella Typhimurium (ST) Isolates

Isolates were freeze-dried and stored at 20°C before being transported to the Veterinary Health Unit and Cairo University Management (VHM) Laboratory in Egypt. Upon arrival at the VHM laboratory, the growth characteristics of the culture were assessed,

which had been isolated from 10 ml of Rappaport Vassiliadis (RVS) broth (Oxoid) and incubated for 24 hours at 41°C for selective enrichment. Following this, the RVS culture was transferred to xylose lysine deoxycholic acid (XLD) agar (Oxoid) and brilliant green agar (Oxoid), with the plates incubated at 37°C for 24 hours. Potential *Salmonella* colonies that developed on the XLD and brilliant green agar (BGA) plates were then subcultured onto nutrient agar plates to isolate individual colonies.

Bacteria in the logarithmic growth phase were obtained from the culture medium. Use sterile physiological saline to prepare a 108 CFU/ml bacterial solution for 0.5 McFarland's standard concentration. ST was subcultured to obtain new cultures. Antibiotics at 108 CFU/mL were prepared with sterile 3% NaCl hypertonic saline and counted using the McFarland turbidimetric method.

### Antibiotic susceptibility testing

Minimum inhibition testing of 15 antibiotic drug concentrations (MICs) to evaluate the effectiveness against ST: MIC50 (mid MIC, indicating ≥50% of all isolates is correct represents, respectively. The mean MIC50 for each isolate was defined as the breakpoint for each drug, and the mean used as the MIC was a random variable rather than a continuous variable.

### Biocide susceptibility testing

The study employed various disinfectants commonly used in poultry settings: MICs of biocides were evaluated in triplicate using broth micro-dilution technique. ECOFF values (95% cutoff) were obtained from the ECOFF finder (ECOFF finder XL 2010 program)(https://clsi.org/meetings/microbiology/ecofinder/)

### Biocides

Formalin (37% w/v), Phenol: (10% w/v), Halamid: Sodium p-toluenesulfonchloramide trihydrate 100%, Virkon-S: Potassium peroxymonosulfate (20.4%)/NaCl (1.5%) in Virkon-S, TH4 plus: Each Litre contains Didecyldimethylammonium chloride 18.75g. Used at 0.5% (1:200) and its Minimum inhibitory concentration was of 6250 – 12500 mg/l; The term "break point" is not generally used in determining the effect of bacteria on biocide disinfectants. Instead, it relies on the concept of cross contamination **(ECOFF, 2010)** to classify bacteria as susceptible, intermediate or resistant (**Gnanadhas *et al*., 2013**).

### Nanoparticles Susceptibility Test (Silva *et al., 2015* ; Abidin *et al*., 2017; Bajaj *et al*. , 2017)

### Nanoparticles

Ag NPs: 0.312µg/mL, ZnONPs: 150 µL, CH: 0.016 mg/mL, (Aldrich: a clinoptilolite- zeolites with a $SiO_2/Al_2O_3$ with molar ratio of 13 and 5, USA) was used at 0.015 mg/mL.

### Procedure

The antimicrobial efficacy of nanoparticles was assessed using the micro-dilution technique on 96-well microplates **(Tajik *et al.*, 2015).** A stock solution was created by dissolving 1 mg of nanoparticle powder in 100 ml of sterile saline. This stock solution was subsequently diluted and introduced into the wells containing the bacterial cultures. The final bacterial concentration in the test wells was established at 1.5 x 108 CFU/mL, with nanoparticle concentrations of 0.5 and 1 µg/mL. The treatments were incubated at room temperature (22–24°C) for durations of 30 minutes and 1 hour, with three replicate wells designated for each treatment. Following the incubation, 100 µl from each well was serially diluted, and colony-forming units (CFU) were quantified on Ordeal's agar after 48 hours of incubation at 28°C **(Herigstad *et al.*, 2001).** The minimum bactericidal concentration (MBC) results were analyzed using ANOVA, employing descriptive statistics such as mean and standard deviation. Tukey's post-hoc test was utilized to evaluate the minimum inhibitory concentration (MIC) of silver nanoparticles against *Staphylococcus*. The significance level for all statistical tests was set at $p < 0.05$.

### Determination of the synergistic effect of Antibiotics combined with Nano-particles and the reduction value of the Antibiotics (MIC50)

Prepare a solution (1 mg/mL) in warm distilled water. Using a 96 well microtiter plate, prepare dilutions of the antibiotics in Frey broth medium. Dissolve bacteria in 5 ml sterile saline, adjust to 0.5 McFarland standard turbidity and dilute 1:100 (20) and nano-particles were added to each well with a twofold dilution of the antibiotic and without the antibiotic. Each experiment contained a negative control consisting of distilled water in one well and Frey broth in the other. Plates were incubated at 35°C and growth and color change were assessed after 24 h to determine the MIC50, indicating that the lowest antibiotic concentration was observed.

### Determination of the synergistic effect of Biocides combined with Nano-particles by and the reduction value of the Biocides (MIC50)

### Procedure

The synergistic antimicrobial effect of biocides in conjunction with nanoparticles was assessed using the micro-dilution method **(Tajik *et al.*, 2015).** A stock solution was created by dissolving 1 mg of the nanoparticle substance in 100 ml of the diluted disinfectant. This solution was further diluted with the disinfectant and inoculated into wells containing the bacterial culture. The test wells were prepared to achieve a final bacterial concentration of 1.5 x 108 CFU/ml, with nanoparticle concentrations of 0.5 and 1 µg/mL. The treatments were incubated at room

temperature (22–24°C) for durations of 30 minutes and 1 hour, with three replicate wells designated for each treatment. Following the incubation period, 100 µL from each well was serially diluted, and colony-forming units (CFU) were quantified on Ordal's agar after 48 hours of incubation at 28°C **(Herigstad *et al.*, 2001).** Statistical Analysis The minimum bactericidal concentration (MBC) results were analyzed using ANOVA, incorporating descriptive statistics such as mean and standard deviation. Tukey's post hoc test was employed to evaluate the minimum inhibitory concentration (MIC) of Comment silver nanoparticles against S. T. The significance level for all statistical tests was set at $p < 0.05$.

## RESULTS

The evaluation of biocides and antibiotics against ST isolates of chicken layers revealed varied susceptibility patterns, providing insights into the effectiveness of these agents against the bacterial population. **Table 1** details the MIC50 and resistance for different classes of antibiotics against chicken ST isolates. The data revealed that certain antibiotics, such as ciprofloxacin, exhibited the lowest MIC50 (0.015 µg/mL) with 10% resistance, indicating greater potency against the bacterial population. On the other hand, enrofloxacin and Ceftazidime (0.190 µg/mL) and norfloxacin (0.246 µg/mL) showed moderate MIC50 and increased bacterial resistance (28±, 38±, and 30±, respectively). However, Tetracycline and Sulfamethoxazole had the highest MIC50 (>64 µg/mL) and (>51.2 µg/mL) values, respectively, indicating reduced effectiveness against the tested isolates (80% and 28% resistance, respectively). Also, **Table 1** presents the prevalence of antibiotic resistance in the ST isolates. Antibiotics such as Nalidixic acid, Amoxicillin-clavulanic acid Kanamycin, Trimethoprim-Sulfa and Chloramphenicol showed a higher effective pattern among the isolates (5± %, 10, %15± %,10± and 10± %).

**Table 1:** Mean ± SE of Antimicrobial resistance of *Salmonella* isolates (n = 20) against antimicrobial agents (n=15), MIC50 (µg/ml) and Breakpoints

| Antimicrobial | Resistance [a] (%) | MIC50 (µg/mL) | Break points |
|---|---|---|---|
| Ampicillin | 26±1.21 | 2 | ≥32 |
| Ticarcillin | 27±1.33 | 4 | ≥128 |
| Amoxicillin-clavulanic acid | 10±0. 12 | 0.5 | ≥32/16 |
| Kanamycin | 15±0.22 | 16 | ≥64 |
| Streptomycin | 22±1.11 | 32 | ≥64 |
| Nalidixic acid | 5±0.01 | 4 | ≥32 |
| Ciprofloxacin | 10±0.01 | 0.015 | ≥4 |
| Sulfamethoxazole | 28±1.88 | 128 | ≥512 |
| Trimethoprim-Sulfa | 10±0.04 | 2.38 | ≥4/76 |
| Tetracycline | 80±3.33 | 4 | ≥16 |
| Chloramphenicol | 10±0.02 | 8 | ≥32 |
| Norfloxacin | 30±2.22 | 0.246 | 0.38 |
| Enrofloxacin | 28±2.31 | 0.190 | 1.32 |
| Ceftazidime | 38±3.01 | 0.190 | 0.19 |
| Azithromycin | 20±0.99 | 0.335 | 0.5 |

As shown in **Table 2,** the MIC50 distributions of the biocides exhibited a diverse range of susceptibilities among the tested isolates. Formalin demonstrated consistent effectiveness, with an MIC50 value of 155 µg/mL. Phenol exhibited a narrow range of susceptibility, with an MIC50 value of 450 µg/mL. Halamid, Virkon-S, and TH4+ are the lowest of the MIC50 (26, 20.6 and 12.5 µg/mL, respectively) with varying degrees of effectiveness according to differences in MIC50 values across isolates (85 ± 0.56%, 35 ± 0.44% and 44 ± 0.66% resistance, respectively). Notably, phenol exhibited the highest MIC50 of 450 µg/mL, signifying a comparatively lower effectiveness.

**Table 3** details the MIC50 and resistance for different nanoparticles against layers of ST isolates. The data revealed that Zn ONPs exhibited the lowest MIC50 (150 µl) with 20 ± 0.12% bacterial resistance, indicating greater potency against the bacterial population. Ag NPs showed moderate MIC50 and reduced bacterial resistance (25 ± 0.43). On the other hand, CHNPs and ZENPs (0.190 µg/mL) showed higher MIC50 and increased bacterial resistance (25 ± 0.43 and 35 ± 0.33%, respectively). However, Tetracycline and Sulfamethoxazole had the highest MIC50 (>64 µg/mL) and (>51.2 µg/mL) values, respectively, indicating reduced effectiveness against the tested isolates (80% and 28% resistance, respectively).

**Table 2:** The Mean ±SE of MIC50 and range of biocides against chicken ST isolates.

| Biocides | Resist% | MIC50 (µg/ml) | Range (µg/ml) |
|---|---|---|---|
| Formalin | 25± 0.14 | 155 | 31.25 - >1000 |
| Phenol | 65± 0.22 | 450 | 300 - >1000 |
| Halamid | 85± 0.56 | 26 | 0.66 - 250 |
| Virkon;S | 35± 0.44 | 20.6 | 0.49 - 125 |
| TH4+ | 44± 0.66 | 12.5 | 0.625 – 12.500 |

TH4: Each Litre contains Didecyldimethyl ammonium chloride 18.75g. Used at 0.5% (1:200*).*

**Table 3:** The MIC50 and range of NPs against chicken ST isolates.

| NPs | Resist% | IC50 | Range |
|---|---|---|---|
| Ag | 25± 0.23 | 0.312 (µg/ml) | 0.612- >1.33 µg/ml |
| Zn O | 20± 0.12 | 150 µL | 200 - >300 µL |
| CH | 25± 0.43 | 0.016 mg/ml | 0.06 - >50 mg/ml |
| ZE Aldrich | 35± 0.33 | 0.015 mg/ml | 0.05 - >50 mg/ml |

Ag NPs 0.312µg/mL **(Bajaj *et al.*, (2017)** - ZnONPs150 µL -CH **(Silva *et al.*, (2015)** 0.016 mg/mol, Nano Chitosan. -ZE Aldrich (USA). - A clinoptilolite- zeolites with a $SiO_2/Al_2O_3$ molar ratio of 13 and 5, respectively 0.015 mg/mol.

The results in **Table 4** showed that the antibacterial activity of antibiotics combined with AgNPs compared to ST when used alone against ST was significantly increased ($P < 0.05$), except for nalidixic acid and trimethoprime-sulfa. The increased antibiotic activity was due to the synergistic effect of AgNPs. Amoxicillin and chloramphenicol did not show any synergistic effect with ZnONPs, CHNPs and ZENPs.

**Table 4:** The synergistic effect of Antibiotics combined with Nano-particles and the reduction value of the antibiotics MIC50.

| Antimicrobial | Resis (%) before combination with NPs | Resis (%) after combination with NPs | | | |
|---|---|---|---|---|---|
| | | Ag | Zn O | CH | ZE |
| Ampicillin | 26±01.21 | 12* | 0* | 16* | 16* |
| Ticarcillin | 27±1.33 | 17* | 0* | 17* | 16* |
| Amoxicillin- | 10±0. 12 | 0* | 10 | 10 | 10 |
| Kanamycin | 15±0.22 | 5* | 0* | 15 | 5* |
| Streptomycin | 22±1.11 | 12* | 0* | 12* | 12* |
| Nalidixic acid | 5±0.01 | 5 | 0* | 5 | 5 |
| Ciprofloxacin | 10±0.01 | 5* | 10 | 2* | 10 |
| Sulfamethoo | 28±1.88 | 12* | 8* | 18* | 18* |
| Trimeth-sulfa | 10±0.04 | 10 | 0* | 10 | 10 |
| Tetracycline | 80±3.33 | 20* | 20* | 20* | 30 * |
| Chloramphenicol | 10±0.02 | 0* | 10 | 10 | 10 |
| Norfloxacin | 30±2.22 | 10* | 10* | 12* | 10* |
| enrofloxacin | 28±2.31 | 10* | 8* | 18* | 18* |
| Ceftazidime | 38±3.01 | 8* | 10* | 8* | 18* |
| Azithromycin | 20±0.99 | 8* | 8* | 10* | 10* |

As shown in **Table 5,** the results indicated that the antibacterial activity of antibiotics combined with AgNPs against S. Typhimurium was significantly increased ($P < 0.05$) compared to ST when used alone due to a significant decrease in MIC50 of all antibiotics except nalidixic acid. On the other hand, a synergistic effect between antibiotics

and NPs was not found in the case of azithromycin and CH NPs & ZE NPs, as well as between nalidixic acid and both ZnO NPs & CH NPs.

**Table 5:** The synergistic effect of Antibiotics combined with Nano-particles and the reduction value of the antibiotics MIC50.

| Antibiotics | MIC50 (µg/mL) of antibiotics | | | | |
|---|---|---|---|---|---|
| | Antibiotics | After combination with NPs | | | |
| | Before | Ag NPs | Zn O NPs | CH NPs | ZE NPs |
| Ampicillin | 2 | 0.312* | 0.015* | 0.16* | 0.33* |
| Ticarcillin | 4 | 0.6* | 1.25* | 1.84* | 1.2* |
| Amoxicillin-clavulanic acid | 0.5 | 0.312* | 0.03* | 0.25* | 0.16* |
| Kanamycin | 16 | 2.08* | 0.55* | 2.56* | 2.08 |
| Streptomycin | 32 | 16* | 4.88* | 5.23* | 7.33* |
| Nalidixic acid | 4 | 4 | 4 | 4 | 2.24* |
| Ciprofloxacin | 0.015 | 0.005* | 0.005* | 0.005* | 0.005* |
| Sulfamethoxazole | 128 | 78.5* | 90.40* | 70.40* | 78.5* |
| Trimethoprim-Sulfasulfa | 2.38 | 0.312* | 1.23* | 0.312* | 1.23* |
| Tetracycline | 4 | 2.08* | 2.08* | 0.25* | 0.25 |
| Chloramphenicol | 8 | 2.08* | 1.84* | 5* | 6 |
| Norfloxacin | 0.246 | 0.025* | 0.0 46* | 0.246 | 0.086* |
| Enrofloxacin | 0.190 | 0.09* | 0.11* | 0.11* | 0.12* |
| Ceftazidime | 0.190 | 0.11* | 0.10 * | 0.021* | 0.11* |
| Azithromycin | 0.335 | 0.225* | 0.135* | 0.335 | 0.335 |

*: Significant at $P < .05$

In **Table 7,** results showed that the antibacterial activity of biocides combined with NPs against ST was significantly increased ($P < .05$) compared to ST when used alone due to a significant decrease in MIC50 of all biocides as shown in **Table 6.** At the same time, the resistance of ST isolates was also significantly reduced ($P < 0.05$).

**Table 6:** The synergistic effect of biocides combined with Nano-particles and the decreased value of the resistance % of ST.

| MIC50 (µg/ml) | | | | | |
|---|---|---|---|---|---|
| Biocide | | After combination with NPs | | | |
| | Before | Ag | ZnO | CH | ZE |
| Formalin | 155 | 72* | 79* | 82.56* | 78.21* |
| Phenol | 450 | 250* | 240* | 237* | 240* |
| Halamid | 26 | 12* | 12* | 13.2* | 14* |
| Virkon'S | 20.6 | 10* | 8.66* | 10* | 10* |
| TH4plus | 12.5 | 8* | 8* | 8* | 10.4 |

**Table 7:** The Resistance of chicken ST isolates against the biocides before and after combining with the nano-particles.

| Biocides | Resist % | AgNPs | ZnONPs | CHNPs | ZENPs |
|---|---|---|---|---|---|
| | | Resist %≠ | Resist %≠ | Resist %≠ | Resist %≠ |
| Formalin | 25± 0.14 | 15* | 12* | 15* | 16* |
| Phenol | 65± 0.22 | 15* | 15* | 18* | 25* |
| Halamid | 85± 0.56 | 25* | 20* | 22* | 26* |
| VirkonS | 35± 0.44 | 5* | 10.* | 8* | 9.25* |
| TH4plus | 44± 0.66 | 16* | 18* | 18* | 20* |

## DISCUSSION

The evolution of bacteria has resulted in the emergence of various intricate mechanisms that counteract the bactericidal effects of antibiotics. These mechanisms include drug modification, alteration of drug targets, decreased membrane permeability, and the expulsion of drugs via efflux pumps. Efflux pumps are characterized by their broad substrate specificity and their efficiency in extruding drug molecules from bacterial cells. By disrupting the function of these efflux pumps, it is possible to restore the bactericidal efficacy of conventional antibiotics. Additionally, efflux pumps are significant in regulating the transport of quorum-sensing biomolecules, which are essential for biofilm formation in bacterial cells. The movement of these biomolecules into and out of bacterial cells can be hindered by inhibiting the activity of efflux pumps.

Bacteria employ a variety of strategies to develop resistance against both natural and synthetic antibiotics, which diminishes the effectiveness of these treatments and underscores the urgent need for new alternatives. However, the process of developing new antibiotics is both costly and complex, resulting in a limited number of new antibiotics being introduced in recent years. Consequently, there is a pressing need to focus on the creation of new antimicrobial agents that are less susceptible to bacterial resistance. Antimicrobial resistance genes are widely disseminated among Salmonella isolates, which is critical for devising innovative strategies to manage this zoonotic disease issue **(Ammar *et al.*, 2019).** Chloramphenicol, previously a common treatment for diarrhea by veterinarians in Egypt, has been shown to induce a multidrug resistance (MDR) response by stimulating the production of specific regulatory mRNA or genes, as observed by **Davin-Regli *et al.*, (2008).** While point mutations in DNA gyrase genes or the activation of efflux pumps can lead to increased resistance **(Meakins *et al.*, 2008),** the horizontal transfer and clonal expansion of resistance genes can occur between food-producing animals and humans **(Hawkey, 2008).** *Salmonella* isolates have been found to possess virulence factors (invA, ompA, and stn) and exhibit resistance (qnrS, qnrA, blaTEM and blaCTX) genes.

### Antimicrobial Effect of Nanoparticles on Bacteria

Numerous studies have demonstrated both direct and indirect antibacterial properties of nanoparticles against antibiotic-resistant pathogenic bacteria **(Kobayashi and Nakazato, 2020).** Our findings indicate that the antibiotic efficacy was markedly enhanced when combined with all tested nanoparticles, with the exception of Nalidixic acid, where an increase was observed solely with Zn ONPs. Zinc oxide nanoparticles represent a promising class of nanoparticles that exhibit low toxicity to mammalian cells while delivering significant antimicrobial effects at minimal concentrations **(Iram *et al.*, 2015; Singha *et al.*, 2019).**

The effectiveness of various combinations of antibiotics and nanoparticles against vancomycin-resistant enterococci (VRE) was evaluated. Antibiotics such as vancomycin, methicillin, erythromycin, and ciprofloxacin were tested in conjunction with three distinct metal oxide nanoparticles: CaO, MgO, and ZnO. Notably, ZnO nanoparticles were found to restore the sensitivity of VRE strains, as indicated by the lowest minimum inhibitory concentration (MIC) values and the most effective combinations of ZnO and antibiotics **(Vassallo *et al.*, 2022).**

The efficacy of certain antibiotics is influenced by their interactions with bacterial surface components; thus, conjugating multiple antibiotics to the surfaces of nanomaterials can yield enhanced antibacterial effects **(Masri *et al.*, 2019).** For instance, gold nanoparticles possess a stable surface that can effectively bind various antibiotics, significantly augmenting the antibacterial activity of these drugs by improving their interaction with bacterial cell walls. **Banoee *et al.*, (2010)** reported a novel role of zinc oxide nanoparticles as efflux pump inhibitors targeting Nor A efflux pumps in *S. aureus*, revealing a 27% and 22% increase in the zone of inhibition for ciprofloxacin in the presence of zinc oxide nanoparticles against *S. aureus* and *E. coli*, respectively. The emergence of antibiotic-resistant variants of *Salmonella* species has raised significant public health concerns **(Silver *et al.*, 2006; Akinyemi *et al.*, 2011)**, leading to a reevaluation of traditional antibiotic therapies. Traditional antibiotics are being replaced by new alternative technologies such as nanotechnology **(Rudramurthy *et al.*, 2016).** Silver nanoparticles are a suitable alternative among metallic nanoparticles with antibacterial activity because, in addition to possessing a strong antibacterial profile, they are also reasonably affordable to produce **(Lee *et al.*, 2007; Pal *et al.*, 2007; Zhang *et al.*, 2008; EL-sherif and Ali, 2020).**

The emergence of antibiotic-resistant variants of Salmonella species has raised significant public health concerns **(Silver *et al.*, 2006; Akinyemi *et al.*, 2011).** Consequently, traditional antibiotics are increasingly being supplanted by innovative alternatives, such as nanotechnology, which offers a broad spectrum. Among metallic nanoparticles, silver nanoparticles are particularly promising due to their robust antibacterial properties and relatively low production costs **(Silver *et al.*, 2006; Lee *et al.*, 2007; Pal *et al.*, 2007; Zhang *et al.*, 2008; EL-sherif and Ali, 2020).** In the evaluation of nanoparticles, those

exhibiting very low minimum inhibitory concentration (MIC) levels warrant particular attention, taking into account treatment concentration and bacterial genus. The antibacterial efficacy of silver nanoparticles (AgNPs) is primarily due to their capacity to adhere to bacterial cell surfaces, facilitated by the electrostatic attraction between the positively charged AgNPs and the negatively charged membranes of microorganisms. Additionally, the interaction of Ag+ ions with sulfur-containing proteins in the bacterial cell wall leads to cell wall disruption **(Rai *et al.*, 2012; Abbaszadegan *et al.*, 2015).** The large surface area of nanoparticles enables the controlled release of metal ions in response to external stimuli. These metal ions penetrate cell membranes and interact with the functional groups of vital proteins and nucleic acids, entering bacterial cells through ion channels and biological pumps, ultimately accumulating to toxic levels that result in bacterial cell death (**Chang *et al.*, 2012; Wang *et al.*, 2014; Su *et al.*, 2015).**

## Synergistic Effects of Nanoparticles with Antibiotics

Nanoparticles (NPs) can be utilized in conjunction with antimicrobial agents to address antibiotic resistance and enhance their efficacy. Notably, silver nanoparticles (AgNPs) have the potential to diminish both the dosage and toxicity associated with antibiotics. Given that NPs interact with bacteria through various mechanisms, the likelihood of microorganisms developing resistance is considerably low. The probability of simultaneous mutations required for resistance is minimal, particularly when NPs are used alongside antimicrobials **(Hutchings *et al.*, 2019).** Consequently, the integration of NPs with antibiotics is regarded as a viable strategy to mitigate the emergence of bacterial resistance **(Zhao *et al.*, 2013; Lee *et al.*, 2019).** Research on the bacterial-mediated synthesis of silver nanoparticles has highlighted their synergistic activity with amikacin, kanamycin, and streptomycin against pathogens such as *E. coli*, *S. aureus*, and *P. aeruginosa*. The findings indicated that the combined antibacterial effects could significantly reduce side effects by allowing for lower dosages. Therefore, employing nanoparticles in conjunction with antibiotics may enhance their effectiveness against a diverse range of pathogenic microorganisms **(Barapatre *et al.*, 2016).**

Antibiotics paired with NPs demonstrate increased efficacy against both Gram-positive and Gram-negative bacteria, particularly against drug-resistant strains. **Aabed and Mohammed (2021)** noted this enhanced effectiveness against *P. aeruginosa*, *E. coli*, *S. aureus*, and *Candida albicans*. Additionally, **Abo-Shama *et al.*, (2020)** illustrated that the synergistic effect of antibiotics, including azithromycin, cefotaxime, cefuroxime, fosfomycin, and chloramphenicol, against E. coli was significantly amplified in the presence of AgNPs compared to the use of antibiotics alone **(Zhang *et al.*, 2018).**

In vitro studies indicate that the concentration of silver nanoparticles (Ag-NPs) and the duration of contact significantly influence bacterial activity. A reduction in particle size enhances reactivity, which in turn increases the surface area and the number of cells that adhere, facilitating improved interaction with microorganisms **(Morones *et al.*, 2005; Lock *et al.*, 2006; Dror-Ere *et al.*, 2009; Kurmuli *et al.*, 2018).** Ag-NPs have the capability to bind to bacterial cell membranes and infiltrate the cells **(Pal *et al.*, 2007; Dror-Ehre *et al.*, 2009).** The evolution of bacteria has led to the emergence of various intricate resistance mechanisms that counteract the bactericidal properties of antibiotics. These mechanisms include enhanced drug efficacy, targeted modifications, decreased membrane permeability, and the expulsion of drugs via efflux pumps. Efflux pumps exhibit broad substrate specificity and high efficiency in removing drug molecules from bacterial cells. The impairment of these pumps may increase the bactericidal potency of existing antibiotics. Additionally, efflux pumps are crucial in regulating the entry and exit of quorum-sensing biomolecules, which are essential for the biofilm formation of bacterial cells. Disruption of efflux pumps can interfere with the migratory dynamics of these quorum-sensing molecules within or outside the bacterial cell. Our findings demonstrated that the antibacterial efficacy of nanoparticles in conjunction with biocides against S. T. was markedly enhanced compared to the use of biocides alone, as evidenced by a significant reduction in the MIC50.

The mechanisms of resistance to disinfectants bear similarities to those associated with antibiotic resistance, organism is subjected or develop strategies to avoid exposure to these agents. Given that mutations occur randomly and are typically propagated through vertical inheritance, horizontal gene transfer (HGT) is particularly noteworthy as it enables genes to undergo phylogenetic shifts **(Morones *et al.*, 2005; Lock *et al.*, 2006; Dror-Ere *et al.*, 2009; Kurmuli *et al.*, 2018).**Through cross-resistance, microorganisms can develop resistance to biocides when there is a certain selection pressure and, at the same time, improve resistance to antibiotics by increasing the rate of antibiotic resistance development (**Basiry *et al.*, 2022; Ricardo *et al.*, 2023).**

Resistance mechanisms exhibit varying degrees of effectiveness based on the characteristics of the substance involved, with alcohols providing less resistance compared to compounds such as quaternary

ammonium compounds (QAC), triclosan, and chlorhexidine. Prior research has indicated that when S. Typhimurium is exposed to disinfectants like benzalkonium chloride (BAC) and glutaraldehyde, there is an increase in antibiotic resistance levels. Gradual exposure of *Salmonella* to elevated BAC concentrations can lead to the selection of mutants that overexpress the AcrAB efflux pump. Furthermore, the activation of the AcrAB efflux pump has been linked to the transcriptional regulator RamA. Nonetheless, there are currently no studies indicating that BAC influences the function of RamA **(Wu-hen *et al.*, 2023).** Additionally, bacteria have developed mechanisms to adapt to substances such as phenol, boric acid, and chloride **(Russell, 2004).**

### Impact of Nanoparticles on Bacterial Resistance

Mechanisms Metal nanoparticles are promising candidates for inhibiting efflux pumps in bacterial cells. The application of these nanoparticles as efflux pump inhibitors not only aids in restoring the bactericidal efficacy of current antibiotics but also diminishes the capacity of microorganisms to develop biofilms. This research emphasizes the utilization of innovative metal nanoparticles that work in conjunction with existing antibiotics to effectively inhibit efflux pumps. Typically, nanoparticles range in size from 1 to 100 nanometers (nm) and exhibit distinct properties when compared to their bulk counterparts. As materials are reduced to the nanoscale, their characteristics undergo significant alterations.

## LIMITATION

The rising prevalence of antimicrobial-resistant bacterial pathogens poses significant challenges for the treatment and prevention of future infectious diseases in both humans and animals. Despite the wealth of scientific data available on this subject, numerous facets of antibiotic resistance development remain ambiguous. Nevertheless, gaining insight into the molecular mechanisms by which antimicrobial resistance genes are acquired and disseminated among bacterial pathogens in dairy production settings can undoubtedly pave the way for innovative antimicrobial strategies and modifications in management practices.

## CONCLUSION

The rising prevalence of antimicrobial-resistant bacterial pathogens poses significant challenges for the treatment and prevention of future infectious diseases in animals. Given that nanoparticles target bacteria through various mechanisms, when nanoparticles are utilized in conjunction with antimicrobials. The antibacterial efficacy of Ag NPs, Zn O NPs, CH NPs, and ZE NPs, both individually and in combination with selected antibiotics and biocides, showed a significant enhancement in antibiotic activity when paired with most of the tested nanoparticles. Furthermore, the antibacterial activity of nanoparticles in conjunction with biocides against S. Typhimurium was markedly greater than that of S. Typhimurium treated alone, as evidenced by a substantial reduction in MIC50.

### Acknowledgments

This research was carried out at the Laboratory of the Veterinary Hygiene and Management Department within the Faculty of Veterinary Medicine at Cairo University. The author expresses gratitude for the financial assistance received from the Researchers Supporting Project number (RSPD2024R581) at King Saud University, Riyadh, Saudi Arabia.

### Author Contributions

All the authors contributed to the study's conception and design. Material preparation, data collection, and analyzed.

### Disclosures

The authors declare no conflicts of interest.

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**How to cite this article:**

**Hussein A. E. Kaoud and Maged Mahmoud Khalil, 2025**. Effect of the Synergism Among Nano-particles, Antibiotics and Biocides on Salmonella Typhimurium Strains, "A Comprehensive Study". Journal of Applied Veterinary Sciences, 10 (2): 86-97. **DOI: 10.21608/javs.2025.354340.1518**