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EVALUATION OF NANOCARRIERS-BASED ADJUVANT VACCINE AGAINST CORYZA AND SALMONELLOSIS INFECTIONS IN CHICKENS

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ABSTRACT

An acute upper respiratory illness known as infectious coryza causes laying and breeding hens to produce significantly fewer eggs (10% to over 40%) and meat chickens to have greater culling rates. Vaccines are available that effectively control this disease. Control of *Salmonella* in poultry is crucial for public health, as it is a major cause of human food poisoning and a significant reservoir for *Salmonella* worldwide. Effective control of disease in chickens relies on improved biosecurity, best husbandry practices, vaccination and competitive exclusion products. The creation of vaccines has made extensive use of nanoparticles as adjuvants, antigen delivery systems, and antibacterial agents to render bacterial cultures inactive. This study examined the antibacterial capabilities of many nanomaterials, including zinc oxide (ZnO), chitosan (Cs), and chitosan-zinc oxide (Cs-ZnO), against *Salmonella* and *A. paragallinarum*. Using these nanomaterials at a 400 µg/ml concentration, combined *A. paragallinarum* and *Salmonella* vaccines were also developed to fight infectious coryza and salmonellosis. The findings showed that both pathogens were successfully inactivated at this dose (400 µg/ml). Additionally, this study showed that the highest antibody titer was created by a combination of vaccine adjuvanted with Cs-ZnO nanoparticles, followed by a combination of vaccine adjuvanted with ZnO nanoparticles and finally a combination of vaccine adjuvanted with Cs nanoparticles. It is clear that using Cs-ZnO nanocomposite as either an inactivator or adjuvant in vaccine production has a clear impact on the immune response of chickens against infectious coryza and salmonellosis.

Keywords: Chitosan; chitosan- zincoxide; infectious coryza; salmonellosis; nanomaterials; zincoxide

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INTRODUCTION

Avibacterium (A.) paragallinarum, an opportunistic pathogen, causes coryza, a severe upper respiratory tract illness in domestic chickens that has a significant financial impact on the poultry sector (Priya *et al.*, 2012). Infectious coryza is a contagious bacterial disease in chickens. It is a frequent bacterial disease in commercial poultry (Gayatri *et al.*, 2010). Birds of all ages are susceptible. In chickens, it primarily affects the upper respiratory tract. If the chicken has an *A. paragallinarum* infection, its meat is deemed to be contaminated (Blackall *et al.*, 2005). There is no natural immunity to the disease. The bacterin from *A. paragallinarum*, either by itself or in combination with other organisms, is used to prevent infectious coryza (Blackall and Reid 1987).

The current invention pertains to a combination vaccine for the protection of poultry against *Salmonella*, a persistent disease in the environment that can easily survive and propagate. *Salmonella* Enteritidis and *Salmonella* Typhimurium remain the most isolated serovars from various animal sources globally. In addition to causing gastroenteritis, they are found in asymptomatic carriers in a wide range of animal species. *Salmonella* Enteritidis is the most common, followed by *Salmonella* Typhimurium (52.3% and 23.3% of cases, respectively) (López-Martín *et al.*, 2016). *Salmonella* continues to be one of the most often found causal agents in food-borne outbreaks (26.6% of outbreaks). Eggs and egg products are frequently linked to *Salmonella* outbreaks. *Salmonella* Enteritidis, and to a lesser extent, *Salmonella* Typhimurium, are linked to egg-related epidemics (EFSA, 2004).

Combined vaccines have the advantage of providing protection against multiple diseases simultaneously, as well as lowering vaccination costs, reducing the stress of immunization for individual vaccines, reducing the number of vaccinations required, and saving time.

Much research encourages the use of nanomaterials in vaccines because of their impact on immune responses due to the special properties of nanoparticles, such as size, shape, large surface area, biocompatibility and antibacterial effects. The results showed that it is possible to stimulate the immune response using nanomaterials (Torres-Sangiao *et al.*, 2016). The purpose of this research is to investigate the effect of nanomaterial inhibition on *A. paragallinarum* and *Salmonella* growth, as well as to assess the efficiency of a developed combination vaccination against salmonellosis and infectious coryza utilizing various nanoadjuvants.

Zinc oxide (ZnO) is a versatile substance that has attracted increased attention in vaccine development due to its unique characteristics. ZnO nanoparticles can be used as a vaccination adjuvant to boost the immune response by increasing antigen absorption by antigen-presenting cells and triggering antibody and cytokine production. As a result, ZnO is a valuable choice for boosting vaccine efficacy against a variety of infectious diseases (Sharma *et al.*, 2019).

Chitosan (CS) is a natural, biodegradable, and biocompatible polymer derived from the exoskeleton of crustaceans. Because CS may stimulate immune responses, improve antigen distribution, and provide a regulated release of vaccine components, it has been thoroughly studied as a vaccine adjuvant. Because CS is cationic, it can interact with anionic antigens and pathogens to help immune cells absorb them (Meng *et al.*, 2021).

Using ZnO and CS together to create a nanocomposite (CS-ZnO) has become a promising method for creating adjuvant

nanovaccines (Prokhorov *et al.,* 2020). The combination of ZnO and CS can have a synergistic impact that improves antigen presentation, prolongs the release of vaccine components, and boosts immune system activation (Sharma *et al.*,2019). This nanocomposite can offer a flexible platform for delivering antigens and inducing both humoral and cellular immune responses, which can be especially helpful in the creation of vaccines against *Salmonella* and Coryza, a respiratory illness that affects poultry. The vaccine antigens can be efficiently delivered to the targeted immune cells by encapsulating or adsorbing them in the CS-ZnO nanocomposite. This nanocomposite's special qualities, such as its biocompatibility, biodegradability, and capacity to alter the immune response, make it a viable option for the creation of safe and efficient adjuvant nano-vaccines to prevent *Salmonella* and Coryza infections in chickens and other animals.

MATERIALS AND METHODS

1. Ethical Approval

The tests were approved and followed the ethical criteria established by the Institutional Animal Care and Use Committee at the Central Laboratory for Evaluation of Veterinary Biologics, with permission code (ARC 62429/11429). The experiments adhered to bioethical norms and the American Research Institute (ARRIVE) requirements.

2. Bacterial strains

2.1. *Avibacterium paragallinarum*

The reference strains *A. paragallinarum* strain W (serovar A) and Modesto strain (serovar C) were obtained from MSD Animal Health/Intervet International bv., Boxmeer, The Netherlands, and reference strain 0222 (serovar B) was obtained from Dr. R.B. Rimler, USDA National Animal Disease Center, Ames, Iowa, USA. The Anaerobic Vaccines Research Department, VSVRI, identified the local field strain (A) from an outbreak of infectious coryza in a laying flock in Egypt, which was then validated at the species level and serotypedusing serological testing with conventional antisera against reference serovars.

The bacteria were grown in a CO2 incubator at 37°C for 24 hours in BHI broth containing 0.01% nicotinamide adenine dinucleotide (NAD). Bacterial growth was examined aerobically and anaerobically on blood agar for contamination (Trujillo *et al.,* 2016).

2.2. *Salmonella* Typhimurium and *Salmonella* Enteritidis

These two strains are local field isolates kindly provided by the Department of Bacterial Sera and Antigens, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. These strains were used to create vaccines for testing.

3. Synthesis of the nanomaterials

3.1. ZnO Nanoparticle Synthesis

ZnO nanoparticles were manufactured using the method described by Elshoky *et al.* (2021). In brief, 2.195 g of zinc acetate dihydrate was dissolved in 230 ml of 2-propanol at 50 degrees Celsius. NaOH (0.8 g) was added to a 40 mL solution (35 mL 2-propanol + 5 mL H2O) while stirring in an ice bath. The resulting solution was agitated at 60°C for 2 hours, with the temperature regulated to avoid particle size changes. After three days of maturing at room temperature, the mixture was centrifuged at 7000 rpm for 15 minutes to remove any residual chemicals. The resulting precipitate was dried at 180°C for 8 hours before calcining at 400°C for 2 hours. DLS, zeta potential, and XRD were employed to characterize the ZnO (Elshoky *et al.,* 2021; Ivanova *et al.,* 2022).

3.2. Synthesis of Chitosan-Zinc Oxide Nanocomposites (CS-ZnO NCs)

Krumova *et al.* (2024) used the following methods to create CS-ZnO NCs: Initially, 50 mg of CS was dissolved in 40 mL of water, and then 1 mL of acetic acid was added to

improve solubility. The solution was agitated for 2 hours to ensure complete homogeneity. Separately, 75 mg of ZnO NPs were dissolved in a 1:1 combination of ethanol and water (10 mL) and sonicated for 1 hour. The ZnO NP solution was then added to the CS solution and agitated overnight to promote the production of CS-ZnO NCs, followed by the addition of 17 mg of TPP to cross-link the CS to ZnO. The resulting mixture was agitated for 30 minutes to ensure proper crosslinking and the formation of CS-ZnO NCs (Perelshtein *et al.,* 2013; Mohamed *et al.,* 2024). CS NPs were generated using the approach outlined in earlier studies (Elshoky *et al.,* 2018; Kostadinova *et al.,* 2021; Mohammed *et al.,* 2021; and Abd El-Aziz *et al.*, 2022).

4. Nanoparticle characterization

Nanoparticle homogeneity, size, and structure were assessed using a variety of characterization techniques. To establish the phase and crystal structure, an X-ray diffraction (XRD) examination was done using a Philips Panalytical X'Pert-Pro. A Zeta sizer Nano ZS device from Malvern Instruments Ltd. was used to measure dynamic light scattering (DLS) and zeta potential, and the hydrodynamic diameter and surface charge were calculated. These methods combined to provide a thorough grasp of the samples' qualities.

5. Minimal inhibition concentration (MIC) of nanoparticles:

5.1. *A. paragallinarum*

The MICs of CS (chitosan), ZnO-CS (zinc oxide-chitosan), and ZnO (zinc oxide) at different concentrations (200, 400, 600, and 800 µg/ml) were determined for the cultivation of *A. paragallinarum*. The culture mixture was cultured at 37 °C for 24 hours before being streaked over brain heart infusion (BHI) agar for confirmation and then incubated for another 24 hours. The negative control contained an *A. paragallinarum* colony.

5.2. *S.* Typhimurium and *S.* Enteritidis

The MIC at various concentrations of the three produced nanoparticles was estimated as follows: Jiao *et al.* (2017) introduced 200, 400, 600, and 800 µg/mL of each nanomaterial to 5 mL of Luria broth (LB) culture media containing 1010 CFU/mL of Tri *Salmonella* strains and incubated for 24 hours at 37 °C. Incubate 50 µL of the bacterial suspension on S.S agar for an additional 24 hours. The negative control consists of a S. Typhimurium and S. Enteritidis colony.

6. Estimation of live/dead bacteria by confocal microscopy

The vitality of *A. paragallinarum* and *Salmonella* was assessed using confocal laser scanning microscopy (LSM 710, Carl Zeiss, Germany) at nanomaterial concentrations ranging from 200 to 800 µg/ml. The live/dead ratios were measured with propidium iodide (PI) and acridine orange (AO) staining. Incubate 100 µl of *A. paragallinarum* or *Salmonella* with 10 µl of PI/AO for 15 minutes. Examine under a confocal microscope with an EC Plan-Neofluar 40x/1.3 Oil DIC M27 lens. AO emitted green fluorescence for living cells, while PI released red fluorescence for dead ones. This investigation gave insights into the viability and survival rates (Azim *et al.,* 2016; Mohamed *et al.,* 2017; Low *et al.,* 2020 and Alovisi *et al.,* 2022).

7. Vaccine preparation

Three different formulations of combined vaccines were made in accordance with (Blackall *et al.* 1992 and Charles *et al.* 1994). *Salmonella* Typhimurium and *Salmonella* Enteritidis were grown on specified media. Equal quantities of each culture (adjusted to contain 1×10^8 CFU/ml) were combined. Cultures of *A. paragallinarum* serovars A, B, and C were generated (adjusted to contain 1×10^8 CFU/ml). An equal volume of each serotype was combined. The aforesaid cultures were then merged, separated into three portions, and treated in the manner stated in Table (1).

These formulations enabled the testing of several adjuvants and formulations with *A. paragallinarum* strains A, B, and C, as well as *S.* Typhimurium and *S.* Enteritidis, demonstrating the strains' potential for vaccine development.

Table 1: Different formulations of the prepared vaccines

Vaccine no.	Formula of vaccine
Vaccine no. 1	adjuvanted with 400 µg/ml of chitosan (CS)
Vaccine no.2	adjuvanted with 400 µg/ml of chitosan with zincoxide (CS-ZnO)
Vaccine no. 3	adjuvanted with 400 µg/ml of zincoxide (ZnO)

8. Quality control testing on the generated experimental vaccinations

8.1. Sterility test.

According to the World Organization for Animal Health (OIE 2018), the manufactured vaccinations were used immediately after checking for the presence of any contaminants, such as aerobic and anaerobic bacteria, fungus, or mycoplasma.

8.2. Safety test

The produced vaccines 'safety was assessed in accordance with the WOAH protocol. To ensure vaccination safety, ten 21-day-old chickens were subcutaneously injected with a double field dosage of the produced vaccines (Gifford *et al.,* 2011; WOAH Manual, 2022). The inoculated chickens were monitored for 14 days for any signs of local reaction caused by *A. paragallinarum* or *Salmonella* infection symptoms (Roshdy *et al.,* 2023).

9. Experimental design

Two hundred and forty (240) specific pathogen-free (SPF) 4-week-old chickens vaccinated against Newcastle, Mycoplasma, and Marek's diseases were obtained from Specific Pathogen Free farm Kom Oshim in Fayoum, Egypt, and kept in isolators at the Veterinary Serum and Vaccine Research Institute's (VSVRI) animal husbandry facilities. The chickens were devoid of *A. paragallinarum Salmonella* infection and antibodies.

The chickens were fed ad libitum without any antibacterial or anticoccidial agents. These chickens were separated into five groups with twenty birds each:

-Group 1 (vaccine no. 1) received a combined vaccine adjuvanted with Cs (chitosan).
-Group 2 (vaccine no. 2) received a combined vaccine adjuvanted with Cs.- ZnO (chitosan with zinc oxide).
-Group 3 (vaccine no. 3) received a combined vaccine supplemented with ZnO.
-Group 4 was left as a negative unvaccinated control group.

10. Vaccination

The initial dose was 0.5 ml injected subcutaneously at the dorsum rear of the neck, followed by a booster dose of 0.5 ml 3 weeks later.

11. Serological characterization

Blood samples were taken on days 0 (before immunization), 1, 2, and 3 weeks after the first vaccination and 1, 2, and 3 weeks following the booster vaccination. Serum was utilized for serological testing.

12. Evaluation of the humoral immune response of the vaccinated chickens

12.1. Haeagglutination Inhibition (HI) test for *A. paragallinarum*

To assess the humoral reaction of the vaccinated chickens, hemagglutination (HA) and HI assays were used. In a microtiter plate with a U-shaped bottom, 0.025 ml of a double-fold dilution of *A. paragallinarum* antigen (1:2, 1:4, 1:8, etc.) was combined with 0.025 ml of fresh or GA-fixed RBCs. The plate remained at room temperature for

45 minutes. Titers were recorded as log10 values as the reciprocal of the greatest serum dilution, demonstrating full suppression of hemagglutinating activity; then, geometric mean antibody titers were determined (Yuan *et al.*, 2007 and Blackall 2008).

12.2. ELISA test for *Salmonella* spp.

Antibodies serve an important role in removing bacteria from the immune system, which is necessary for a protective response against *Salmonella*. The humoral immune response to *Salmonella* antigens in the produced vaccine was assessed by ELISA with a *Salmonella* antibody test kit (BioChek poultry immunoassays cat # CK117 for *S. enteritidis* and CK118 for *S. typhimurium*). The kit consists of microtiter plates coated with inactivated LPS antigens from *S.* enteritidis and *S.* typhimurium, sheep anti-chicken immunoglobulin conjugated with alkaline phosphatase in tris buffer with protein stabilizers and inert red dye sodium azide as a preservative (0.1% w/v). Substrate tablets: PNPP (p-Nitrophenyl phosphate) tablets should be dissolved in substrate buffer. Substrate buffer: Diethanolamine buffer with enzyme cofactors. Stop Solution: Sodium hydroxide in diethanolamine buffer. Sample diluent: Phosphate buffer containing protein stabilizers and a sodium azide preservative (0.1% w/v). Wash buffer: Phosphate-buffered saline with 0.05% Tween 20. As a negative control, serum from SPF chickens was diluted in phosphate buffer containing protein stabilizer and sodium azide (0.1% w/v). A positive control was antiserum containing *S.* enteritidis-specific antibodies in phosphate buffer with protein stabilizers and sodium azide (0.1% W/V).

Preparation of Reagents:

To prepare the reagent, 100 µL of 1:500 diluted serum samples were charged into microtiter plates covered with *S. entriditis* or *S. typhimurium* LPS, respectively. Positive and negative controls were used. Plates were incubated at room temperature (22-27ºC) for 30 min. The wells' contents were aspirated and washed four times with wash buffer (300 µL each). After adding 100 µL of conjugate reagent to the appropriate wells, incubate as previously and wash 5 times with washing buffer. Add 100 µL of substrate reagent to each well and incubate for 15 minutes at room temperature. Then, 100 µL of stopping solution was added. Finally, the absorbance was read at 405 nm with a microtiter plate reader. S/P ratio was computed. Then the following equation 3 was used:

$$S/P = \frac{mean\ of\ test\ sample - mean\ of\ negative\ control}{mean\ of\ positive\ control - mean\ of\ negative\ control}$$

(eq.3)

While the antibody titre was counted as equation 4:

Log10 titre= 1.13 Log (SP) + 3.156 (eq.4)

AntiLog = Antibody titre.

Where S is testing sample, P is positive control, and S/P is the test sample/positive control.

13. Evaluation of the immune response of vaccinated chickens through challenge methods

13.1. Against different serovars of *A. paragallinarum*

On day 42, the challenge test was performed by inoculating 0.2 mL of broth culture of A. paragallinarum strains into the sinus. The challenge dosage included around 6×10^8 cfu/ml. Clinical symptoms such as sneezing, nasal discharge, facial edema, snoring, and conjunctivitis were documented daily for a week as previously described (Wahyuni *et al.*, 2019). The findings are based on the presence and severity of clinical symptoms. A protected chicken was characterized as one that displayed no clinical indications.

13.2. Against *S.* Typhimurium and *S.* Enteritidis

Each group was separated into two subgroups, and each subgroup was challenged for four weeks following the booster dosage with oral administration of 1 mL containing 10^8 CFU of each strain (*S.* Typhimurium and *S.* Enteritidis) individually

(Ibrahim *et al.*, 2018). The vaccinated chickens were tracked for a month. The protection rate was calculated using the severity of the clinical symptoms, mortality, and recovery of the challenge organisms from fecal samples. Fecal samples were collected before the start of the experiment and after the challenge for four weeks (once per week) using sterile swabs that were inoculated into tetrathionate broth from all chickens, including the vaccinated and control ones, and examined bacteriologically for Salmonellae shedding according to (Cruickshank and Sim. 1987).

Protection % = (Survived chicken/ total number of chickens) X 100

14. Statistical analysis

The antibody titers from the three immunized groups were expressed as mean values. The differences between and among groups were examined using a One-Way ANOVA test in SPSS software version 26 for the samples (Snedecor and Cochran, 1980).

RESULTS

1. Characterization of the nanomaterials

Fig. (1 A and B) showed the particle sizes and zeta potential of chitosan (CS), zinc oxide (ZnO), and chitosan-zinc oxide nano-composite (CS-ZnO NCs). According to data analysis, the particle sizes of Cs, ZnO and Cs-ZnO were 43.44±19.54 nm, 78.5±47.84 nm, and 173.9±58.45 nm, respectively. The polydispersity index (PDI) values for CS, ZnO, and CS-ZnO are 0.546, 0.85, and 0.597, respectively, indicating that their size distributions are uniform. Additionally, the zeta potentials of CS, ZnO, and CS-ZnO were 47.8±3.77 mV, -20.6±5.94 mV, and 34.4±4.17 mV, respectively. The particle size, polydispersity index (PDI), and zeta potential of CS, ZnO, and CS-ZnO nanoparticles all have significant advantages in vaccine formulations.

The X-ray diffraction (XRD) patterns depicted in Fig. (1 C) reveal distinct features of the CS-ZnO nanocomposites (NCs). The discernible diffraction peaks of the CS-ZnO NCs register at 2Θ values approximately at 31.7°, 34.4°, 36.3°, 47.6°, 56.6°, 62.8°, 66.5°, 68.01°, 69.08°, and 76.9°. These peaks align precisely with the characteristic diffraction patterns attributed to zinc oxide, corroborated by the agreement with ICDD card no. 01-078-2585. This alignment unequivocally confirms the presence of a well-defined ZnO hexagonal crystalline structure (AbdElhady, 2012; Zahoor *et al.*, 2023). However, it's noteworthy that the expected peaks corresponding to CS, typically observed at 2Θ values of 10.52° and 19.62°, are subdued due to the overwhelming intensity of the ZnO peaks (Krumova *et al.*, 2024**).**

2. The minimal inhibition concentration (MIC) of nanoparticles

At a concentration of 400 µg/ml, all evaluated nanomaterials displayed synergistic effects on the development of *A. paragallinarum, S.* Typhimurium, and *S.* Enteritidis, outperforming the negative control.

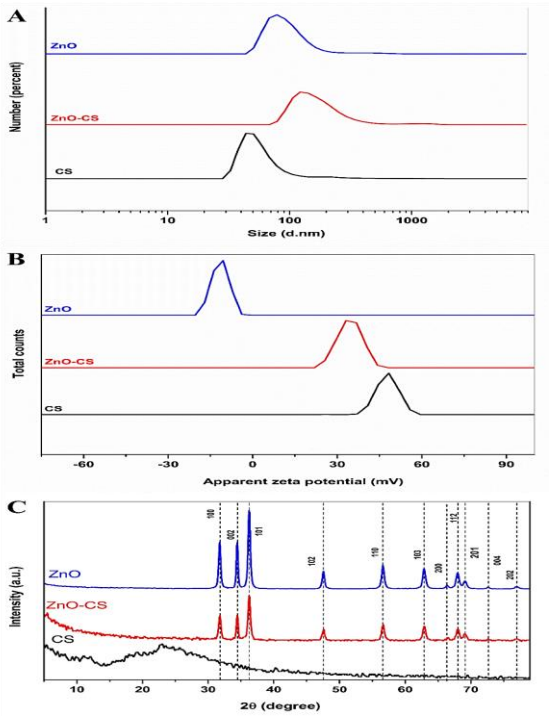

Figure 1: Nanoparticle characteristics showing the (A) particle size, (B) zeta potential, and (C) XRD pattern of chitosan (CS), zinc oxide (ZnO), and chitosan-zinc oxide nanocomposite (CS-ZnO) nanoparticles.

3. Confocal live/dead imaging

Our study focused on investigating how different nanomaterials, specifically chitosan (CS), chitosan-zinc oxide composite (CS-ZnO), and zinc oxide (ZnO) nanoparticles, affect the survivability of live/dead *A. paragallinarum* and Salmonella bacteria, as shown in Figs. (2-4). To investigate this, we exposed the bacteria to various quantities of these nanomaterials, then assessed their survival using confocal microscope imaging. This method enables us to distinguish between living and dead bacteria beneath the microscope. Using Zen software, we were able to quantify the effects by estimating the ratios of live cells in control and treated samples based on AO and PI emission intensity.

At a concentration of 200 µg/ml, the three nanomaterials had no significant impact on bacterial viability, as evidenced by comparable viable cell ratios in control and treatment samples. However, raising the concentration to 400 µg/ml resulted in a considerable decrease in bacterial viability, indicating strong antibacterial activity that corresponds with concentration, with higher concentrations leading to improved antibacterial effects. At lower concentrations (200 µg/ml), both CS and CS-ZnO showed a scaffold effect, presumably promoting the growth of beneficial bacteria.

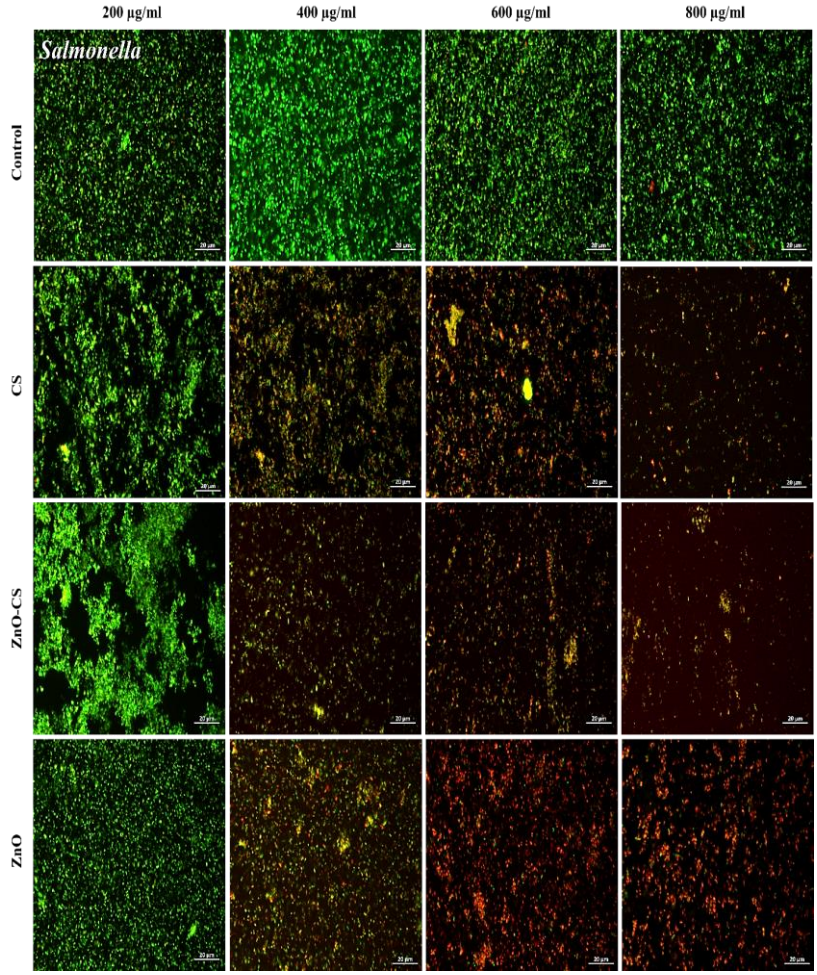

Figure 2: Confocal microscopy images showcasing the live/dead effects of chitosan (CS), chitosan-zinc oxide nanocomposite (CS-ZnO), and zinc oxide (ZnO) treatments on Salmonella bacteria over 24 h. The bacteria were subjected to concentrations of 200, 400, 600, and 800 µg / ml, and stained with AO/PI for visualization. Each image is accompanied by a scale bar indicating a size of 20 µm.

4. Quality control testing on the manufactured experimental vaccines

4.1. Sterility test.

No growth was observed in different media injected with various produced vaccinations.

4.2. Safety test

At the end of the safety study, all chickens were healthyand alive and no adverse reaction was observed and there was no change in water or feed intake.

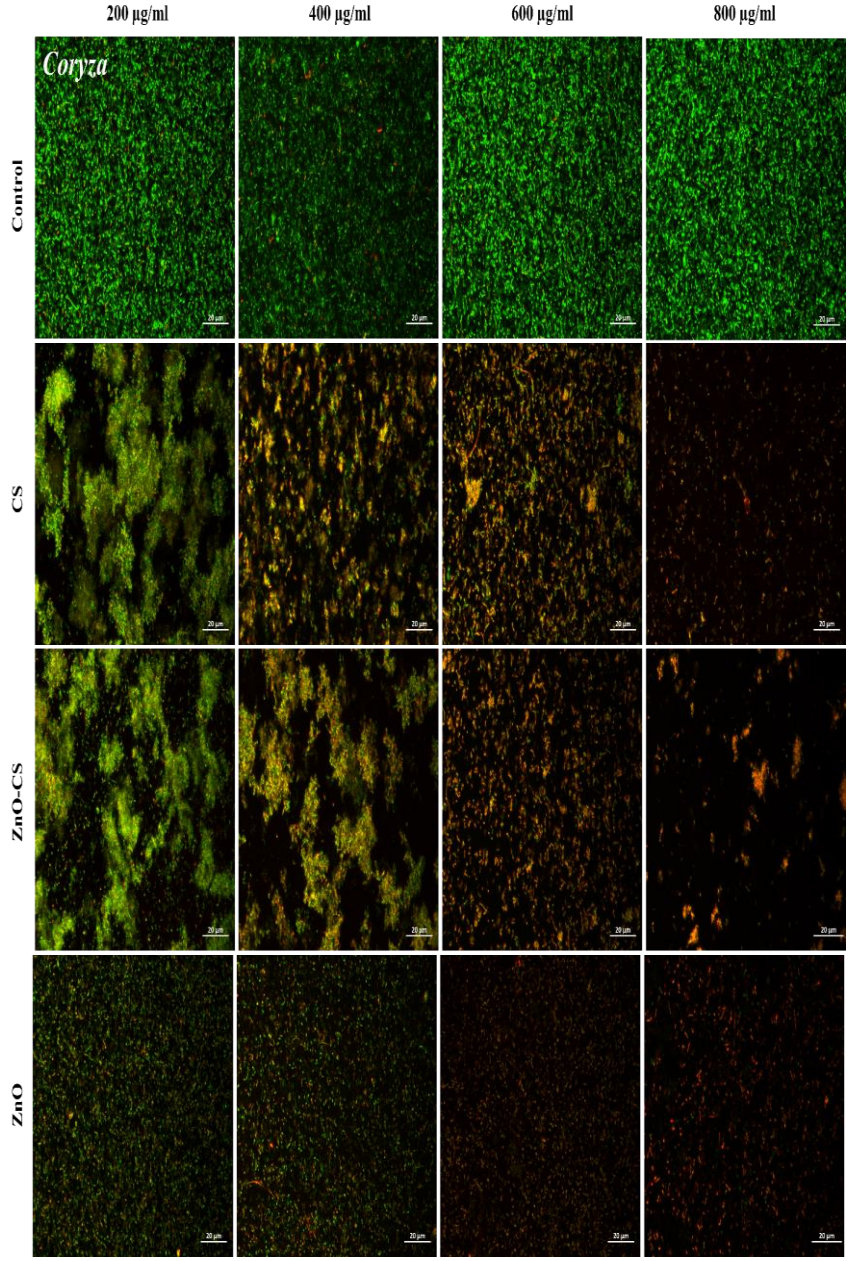

Figure 3: Confocal microscopy images showcasing the Live/dead effects of chitosan (CS), chitosan-zinc oxide nanocomposite (CS-ZnO), and zinc oxide (ZnO) treatments on A. paragallinarum bacteria over 24 h. The bacteria were subjected to concentrations of 200, 400, 600, and 800 µg /ml, and stained with AO/PI for visualization. Each image is accompanied by a scale bar indicating a size of 20 µm.

5. Humeral immune response of the vaccinated chickens

As shown in Tables (2, 3, 4, 5, and 6), the three groups of chickens developed antibody titers against each of the tested strain antigens. The data revealed that vaccine no. 2 (combined vaccine adjuvanted with Cs-ZnONPs) yielded the highest antibody titer, followed by vaccine no. 3 (combined vaccine adjuvanted with ZnONPs) and then vaccine

no. 1 (combined vaccine adjuvanted with CsNPs). The statistical analysis results show a significant difference (P≤0.05) between the three vaccine groups at 2 and 3 weeks after the first dose of vaccination and at 4, 5, and 6 weeks after the booster dose of vaccination.

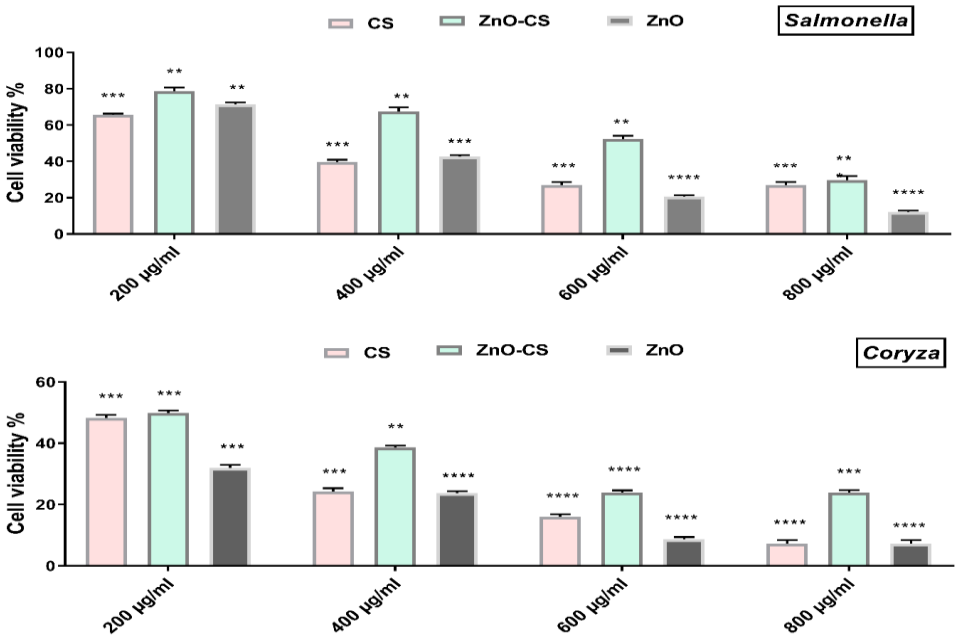

Figure 4: Live/dead ratio relative to control calculated from fluorescence intensity analyzed by Zen software. Significant at p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

Table 2: Geometric mean hemagglutinating antibody titer against *A. paragallinarum* serovar A (W strain)

Intervals / Groups	Geometric mean antibody titer						
	post 1st vaccination			Boostering	post boosting		
	1st week	2nd week	3rd week		1st week	2nd week	3rd week
1	12.9	14.9	16		18.3	22.6	24.2
2	16	21.1	24.2		32	32	36.7
3	13.9	16	18.3		24.2	27.8	32
4	0	0	0		0	0	0

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve.

Table 3: Geometric mean hemagglutinating antibody titer against *A. paragallinarum* serovar B (0222 strain)

Intervals / Groups	Geometric mean antibody titer						
	post 1st vaccination			Boostering	post boosting		
	1st week	2nd week	3rd week		1st week	2nd week	3rd week
1	11.3	12.1	12.1		14.9	18.3	19.6
2	12.1	14.9	16		21.1	24.2	29.8
3	11.3	11.3	12.9		17.1	19.6	22.6
4	0	0	0		0	0	0

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve.

Table 4: Geometric mean hemagglutinating antibody titer against *A. paragallinarum* serovar C (Modesto strain)

Geometric mean antibody titer							
Intervals / Groups	post 1st vaccination			Boostering	post boosting		
	1st week	2nd week	3rd week		1st week	2nd week	3rd week
1	12.1	12.9	14.9		19.6	21.1	24.2
2	14.9	17.1	18.3		25.9	34.2	36.7
3	12.9	14.9	16		18.3	21.1	25.9
4	0	0	0		0	0	0

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve.

Table 5: Antibody titer against *S.* Typhimurium in the sera of chickens vaccinated with combined inactivated nanovaccines as measured by ELISA.

Geometric mean antibody titer							
Intervals / Groups	post 1st vaccination			Boostering	post boosting		
	1st week	2nd week	3rd week		1st week	2nd week	3rd week
1	314	490	510		555	590	667
2	809	874	1019		1160	2120	3131
3	400	490	546		779	888	1316
4	50	80	90		120	150	160

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve.

Table 6: Antibody titer against *S.* Enteritidis in the sera of chickens vaccinated with combined inactivated nanovaccines as measured by ELISA.

Geometric mean antibody titer							
Intervals / Groups	post 1st vaccination			Boostering	post boostering		
	1st week	2nd week	3rd week		1st week	2nd week	3rd week
1	243	267	296		331	678	720
2	409	645	812		1146	1008	1612
3	222	348	369		406	783	818
4	50	80	90		120	150	160

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with Zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve.

6. Results of the challenge test

6.1. Against *A. paragallinarum* serovars

Table (7) summarizes the protective efficacy of the three vaccine formulations (vaccines No. 1, 2, and 3) utilizing various

nanoadjuvants. No mortality occurred in any of the vaccinated groups over the whole challenge time. Compared to the unimmunized control group (group 4), chickens in the immunized groups were protected. Within a week after the challenge, all of the chickens in the unimmunized control group (group 4) and very few of the birds in the immunized groups displayed the classic clinical indications of infectious coryza. Protection rates against the W strain were 70%, 90%, and 80%, respectively, against the 0222 strain were 60%, 70%, and 70% and against the Modesto strain were 60%, 80%, and 70%, respectively; when vaccines Nos. 1, 2, and 3 were used. The unvaccinated control group exhibited no protection against any of the three *A. paragallinarum* serovars.

6.2. Against *S.* Typhimurium and *S.* Enteritidis

In comparison to the unimmunized control group (group 4), chickens in immunized groups were protected, as indicated in Table (8). Within a week after the challenge, all of the chickens in the unimmunized control group (group 4) and very few of the hens in the immunized groups showed the usual clinical indications of salmonellosis. For groups 1, 2, and 3, the protection rate against both Salmonella strains was 67%, 86.67%, and 80%, respectively. The protection rate for the unvaccinated control group was 10%.

Table 7: Results of challenge test of chickens vaccinated with different combined inactivated vaccines (using different nanoadjuvants (vaccine no. 1, 2 and 3) against Infectious Coryza.

Chicken groups	Vaccine no.	Challenge strains	No. of chicken	No. of protected chickens	No. of unprotected chicken	Protection percentage %
1	1	W (serovar A)	10	7	3	70%
		0222 (serovar B)	10	6	4	60%
		Modesto (serovar C)	10	6	4	60%
2	2	W (serovar A)	10	8	2	90%
		0222 (serovar B)	10	7	3	70%
		Modesto (serovar C)	10	8	2	80%
3	3	W (serovar A)	10	7	3	80%
		0222 (serovar B)	10	7	3	70%
		Modesto (serovar C)	10	7	3	70%
Control unvaccinated (Group 4)		W (serovar A)	10	0	10	0
		0222 (serovar B)	10	0	10	0
		Modesto (serovar C)	10	0	10	0

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with Zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve.

6.3. Fecal shedding

Salmonella organisms' fecal shedding (Table 9) following challenge was 20% in the first week and dropped to 15% in the first group, 10% in the second group and then to 5% in the third, 15% until it reached 10% in the fourth group (the unvaccinated control group), and 55% in the first week after the challenge before increasing to 65%. The control unvaccinated group had a 25% shedding rate in the fourth week after the challenge, but the vaccinated group showed no shedding in groups 1, 2, and 3.

Table 8: Protection and mortality rates among vaccinated chickens against *S.* Typhimurium and *S.* Enteritidis

Groups	No. of birds	No. diseased/Weeks post challenge				Diseased/ Total	No. of Survival	Total no. Of survival	Mortality rate	Protection rate
		1st week	2nd week	3rd week	4th week					
1	10 (ST)	2	0	1	0	3/10	7	13/20	35	65
	10 (SE)	2	1	1	0	4/10	6			
2	10 (ST)	1	0	0	0	1/10	9	18/20	10	90
	10 (SE)	1	0	0	0	1/10	9			
3	10 (ST)	1	1	0	0	2/10	8	16/20	20	80
	10 (SE)	1	0	1	0	2/10	8			
4	10 (ST)	4	3	1	1	9/10	1	2/20	90	10
	10 (SE)	5	3	1	0	9/10	1			

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve.

Table 9: Re-isolation of *Salmonella* after challenge test

Groups	No. of positive birds for isolation / total No. of living birds*100 %			
	1st week %	2nd week %	3rd week %	4th week %
Group 1	20	20	15	0
Group 2	10	10	5	0
Group 3	15	15	10	0
Group 4**	55	80	65	25

Group 1: combined vaccine adjuvanted with chitosan (CS); Group 2: combined vaccine adjuvanted with chitosan with Zinc oxide (CS-ZnO); Group 3: combined vaccine adjuvanted with zinc oxide (ZnO) and Group 4: control-ve *Significant at ($P \geq 0.05$)

DISCUSION

Avibacterium paragallinarum is the cause of the acute respiratory illness known as infectious coryza in chickens. The two biggest economic losses linked to infectious coryza are a significant decrease (10–40%) in layer egg production and poor development performance in growing birds (Blackall and Soriono 2008 and Gong *et al.,* 2014).

Salmonellosis is a highly zoonotic disease that causes several diseases in both human and animal. So we have to control this disease in both human and animal through vaccination and discover new types of vaccines capable of controlling disease (Varmuzova *et al.,* 2016 and Renu *et al.,* 2020).

Several benefits of combined vaccines include protection against numerous diseases simultaneously in a single dosage, decreased costs, fewer vaccinations administered, and time savings (Awaad 2004).

Compared to a non-adjuvanted vaccine, an adjuvanted vaccine typically elicits a stronger, quicker, and more sustained immune response.

Nanoparticles are becoming more popular in veterinary vaccine manufacture because they improve immunological response. Furthermore, they can slowly release antigens, which improves vaccine performance (Kim *et al.,* 2010). The use of nanoparticles for loading antigens results in targeting the lymph cells, which leads to vaccination performance increase (Moyer *et al.*, 2016).

In this work, many nanoparticles, such as Cs, Cs-ZnO, and ZnO nanoparticles, were employed for antibacterial activity against *A. paragallinarum* and *salmonella spp*., as well as their involvement in improving infectious coryza and salmonella vaccines as adjuvants in vaccine manufacture.

Through the results of the MIC by using different concentrations (200, 400, 600 and 800 µg/ml) of Cs, Cs-ZnO and ZnO for studying their antibacterial effect against *A. paragallinarum* and *Salmonella* spp., it was found that 400 µg/ml was an appropriate concentration to inhibit the growth of *A. paragallinarum* and *Salmonella* spp. The result of confocal live/dead imaging, as shown in Figs (2-4), confirmed the result of MIC, which revealed that at a concentration of 200 µg/mL, none of the nanomaterials significantly affected bacterial viability. However, when the concentration was increased to 400 µg/mL, all nanomaterials showed a noticeable decrease in the viability of bacteria. These findings supported the hypothesis of Nagy *et al.* (2011) that the most common explanation for chitosan's antibacterial activity is that it attaches to the negatively charged bacterial cell wall, disrupting the cell and changing the permeability of the membrane. This attachment then attaches to DNA, inhibiting DNA replication and ultimately leading to cell death. Additionally, (Divya *et al.,* 2017) proposed an additional mechanism in which chitosan functions as a chelating agent, electively binding to trace metal elements to produce toxins and restrict microbial growth. These findings also corroborated those of (Seil and Webster 2012), who proposed that ZnO at the nanoscale may interact with the bacterial surface and/or core, where it enters the cell, and then display unique bactericidal processes.

Also, in this study, these nanoparticles (Cs, ZnO and Cs- ZnO) were used as adjuvants in a combined infectious coryza and salmonella vaccine by a ratio of 400 µg/mL.

The quality control testing of the prepared experimental vaccines, in which each nanoparticle (CS, CS-ZnO, or ZnO) was used as an adjuvant in the formulation of the combined vaccines, revealed no growth in different media inoculated with the prepared vaccines. During the safety test, all hens were healthy and alive, with no adverse effects. There was no difference in water or feed intake.

The humoral immune responses of the four immunized groups were assessed using the HI test to detect antibody titer for *A. paragallinarum* serovars (A, B, and C) and the ELISA for *Salmonella spp*. The data in Tables (2,3,4,5 and 6) reveal that all the comparison groups (G 1-3) show a significantly different immune response compared to the negative control group (G4), demonstrating a notable effect of the vaccines. Based on the results, vaccine no. 2 (combined vaccine adjuvanted with Cs- ZnO Nps) gave a higher antibody titer followed by vaccine no. 3 (combined vaccine adjuvanted with ZnO Nps) then vaccine no.1(combined vaccine adjuvanted with Cs Nps) and these results when correlated with the results obtained from the characterization of Cs, ZnO and Cs- ZnO Nps with zeta potential measurements, DPI and XRD patterns showed in Figure (1 A, B and C) which indicate that the smaller size, positive zeta potential, and lower PDI of CS make it favorable for targeted vaccine delivery and enhanced immunogenicity and cellular uptake, while the larger size and negative zeta potential of ZnO promote sustained release and improved vaccine dispersion. The CS-ZnO composite offers a balanced approach with intermediate size, positive zeta potential, and moderate PDI, combining the features of

both CS and ZnO for optimized vaccine performance. Overall, the interplay of particle size, PDI, and zeta potential in CS, ZnO, and CS-ZnO can significantly influence the immune response to inactivated adjuvant vaccines, impacting vaccine delivery, stability, and immunogenicity. (Abd El-Aziz *et al.,* 2022; Ibrahim *et al.,* 2024; Ivanova *et al.,* 2022 and Mohamed *et al.,* 2024). Also, the X-ray diffraction (XRD) patterns shown in Fig. (1 C) confirmed the above results.

Concerning the challenge test results, as shown in Tables (7 and 8), it was determined that chickens vaccinated with either produced vaccines were protected, compared to the unimmunized control group. Combined vaccination adjuvanted with Cs-ZnO (vaccine no. 2) was the most immunogenic for chickens and offered better protection rates, followed by vaccine no. 3 and then vaccine no. 1.

CONCLUSION

Based on the minimum inhibitory concentration data, it is possible to draw the conclusion that 400 mg/ml of chitosan (Cs), zinc oxide (ZnO), or chitosan-zinc oxide (Cs-ZnO) nanomaterials is a powerful inactivator for *A. paragallinarum* and *Salmonella* spp.

The most immunogenic vaccine, based on the chicken humeral immune response and challenge protection test, is made by combining the coryza and *Salmonella* vaccines using a chitosan-zinc oxide nanocomposite as an adjuvant.

Conflict of Interests:

The authors revealed that there are no potential conflicts of interest.

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تقييم محفز مناعي متناهي الصغر في لقاح مركب ضد مرضي السالمونيلا وزكام الطيور المعدي في الدجاج

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مرض زكام الطيور المعدي هو مرض حاد يصيب الجهاز التنفسي العلوي في الدجاج يؤدي الي ارتفاع معدلات إعدام دجاج التسمين وانخفاض ملحوظ في انتاج البيض (اكثر من ٤٠ %) في الدجاج البياض. تعد السيطرة علي السالمونيلا في الدواجن أمرا بالغ الاهمية للصحة العامة, حيث أنها سبب رئيسي للتسمم الغذائي للانسان وحامل مهم لمرض السالمونيلا في جميع انحاء العالم. تعتمد المكافحة الفعالة للامراض في الدواجن علي تحسين الامن الحيوي, وافضل طرق التربية, التطعيم والمنتجات الاستبعادية التنافسية. تم استخدام الجسيمات النانوية علي نطاق واسع في صناعة اللقاحات كمواد مساعدة, ومركبات توصيل المستضد و كتثبيط لنمو البكتريا. في هذا البحث تم دراسة الخواص المثبطة للبكتريا لبعض المواد النانوية مثل الكيتوزان (Cs) والكيتوزان وأكسيد الزنك (Cs- ZnO) وأكسيد الزنك (ZnO) ضد ميكروبي الافي باكتيريم باراجالينارم والسالمونيلا. كما تضمنت أيضًا إنشاء لقاحات مركبة من الافي باكتيريم باراجالينارم والسالمونيلا باستخدام هذه المواد النانوية بتركيز ٤٠٠ ميكروغرام/مل لمكافحة مرض الزكام المعدي وداء السالمونيلا. أشارت النتائج إلى أن هذا التركيز (٤٠٠ ميكروجرام/مل) يثبط بشكل فعال كلا البكتريا، أوضحت هذه الدراسة ايضا أن اللقاح اللمركب الممتزج بجسيمات Cs-ZnO النانوية أنتج أعلى عيار للأجسام المضادة، يليه اللقاح المركب الممتزج بجسيمات ZnO النانوية ثم اللقاح المركب الممتزج بجسيمات Cs النانوية. من الواضح أن استخدام مركب Cs-ZnO النانوي إما كمثبط للبكتريا أو كمحفز مناعي في إنتاج اللقاحات له تأثير واضح على الاستجابة المناعية للدجاج ضد مرض الزكام المعدي ومرض السالمونيلا.