Inactivated pentavalent vaccine against mycoplasmosis and salmonellosis for chickens

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ABSTRACT *Mycoplasma* and *Salmonella* are serious pathogens threaten the poultry industry. This study aimed to prepare and evaluate an inactivated pentavalent vaccine targeting bacteria, including *Salmonella enterica* serovar Typhimurium (**ST**), *Salmonella enterica* serovar Enteritidis (**SE**), *Salmonella enterica* serovar Kentucky (**SK**), *Mycoplasma gallisepticum* (**MG**), and *Mycoplasma synoviae* (**MS**), from locally isolated strains. The prepared vaccine was adjuvanted with Montanide ISA70 oil and then tested for safety, sterility, and potency. The vaccine efficacy was evaluated in 110 specific pathogen-free, 1-day-old chicks, which were divided into three groups as follows: 1) vaccinated group (50 birds), which was subdivided into five subgroups of ten birds each; 2) control positive (challenged) group (50 birds), which was subdivided into five subgroups of ten birds each; and 3) control negative (blank) group, which included ten birds. Chicks in group 1 were administered the first dose of vaccine at 7 d of age followed by a booster dose after 3 wk. At 3 wk after booster vaccination, the chicks who were administered the booster dose were challenged and kept under observation until the end of the experiment when the chicks were approximately 10 wk. Details of clinical symptoms, daily mortality, weights, and postmortem lesions; serum samples; cloacal swabs; and nasal swabs were collected during the experiment. The humoral immune response to the prepared pentavalent vaccine was assessed using enzyme-linked immunosorbent assay. Our findings revealed that the prepared vaccine showed high protective antibody titers against *Salmonella* and *Mycoplasma* with 100% efficacy and no mortalities (100% survival rate) were recorded in vaccinated and challenged birds. The vaccine reduced both clinical signs and bacterial shedding post challenge in vaccinated birds in comparison with control positive group. The prepared vaccine did not affect the body weight gain of the vaccinated birds in comparison with control negative birds. The current study concluded that locally manufactured inactivated pentavalent vaccine offers protection to birds and could be employed as an effective tool along with biosecurity measures to overcome mycoplasmosis and salmonellosis in layer and breeder chicken farms in Egypt.

INTRODUCTION

Diseases caused by *Salmonella enterica* serovar Typhimurium (**ST**), *Salmonella enterica* serovar Enteritidis (**SE**), *Salmonella enterica* serovar Kentucky (**SK**), *Mycoplasma gallisepticum* (**MG**), and *Mycoplasma synoviae* (**MS**), cause significant economic losses to the poultry industry worldwide, particularly in chicken and turkey populations and these diseases are linked to digestive, reproductive, locomotive, and

respiratory illnesses; poor performance; decreased egg production; reduced fertility; and increased embryonic mortality (Eissa et al., 2014; Ibrahim et al., 2018; Marouf et al., 2020, 2022).

Salmonella species is a gram-negative, facultative bacterium, which infects birds, reptiles, and mammals, including humans (El-Saadony et al., 2022). *Salmonella* species and their contamination of chicken eggs, especially by SE, has become a significant public health hazard (Guard-Petter, 2001; Cogan and Humphrey, 2003). ST is a common contaminant isolated from broiler chicken flesh and eggs. Therefore, it is necessary to address both SE and ST infections in poultry (Ibrahim et al., 2018; El-Saadony et al., 2022). As long-term use of antibiotics in poultry production has become more prevalent, antibiotic resistance in *Salmonellae* has increased dramatically (Van Boeckel et al., 2019; Alvarez et al., 2020; Rabello et al., 2020), with increased risks to both animals and humans (Michael and Schwarz 2016; Pan et al., 2018). Therefore, application of biosecurity measures with proper vaccination and the usage of antibiotic natural alternatives is a global direction to minimize the usage of antibiotics (Abd El-Hack et al., 2022a).

Avian mycoplasma is an economically important disease, primarily due to MG and MS infections (El-Naggar et al., 2022). These infections cause significant economic losses due to the impact of several factors on poultry, such as weight loss, low feed conversion efficiency, lowered egg production, lowered hatchability, increased embryo mortality, increased carcass condemnation rates, increased costs for broiler prophylaxis, layer therapy costs, and increased costs associated with breeder flocks (El-Naggar et al., 2022). Infections caused by MG and MS are respiratory infections recognized by the World Organization for Animal Health (OIE, 2012). MG causes chronic breathing illness and inflammation sinusitis in chicken, and MS causes synovitis and avian airsacculitis. Horizontal and vertical transmission of both the infections has been observed (El-Naggar et al., 2022).

Biosecurity, therapy, and immunization are commonly used prevention and control strategies for addressing avian mycoplasmosis. Various avian immunization vaccines exist against both MG and MS and include inactivated bacteria, live attenuated, and recombinant live poxvirus vaccines (Yadav et al., 2021).

Mycoplasmosis and salmonellosis in hens are most frequently prevented through vaccination along with appropriate management and biosecurity techniques (Ibrahim et al., 2018; El-Naggar et al., 2022). Previous studies have suggested that when dealing with multiage commercial layer operations suffering from endemic infections, vaccination is the most practical option (Ibrahim et al., 2018). Currently, there are commercially available trivalent vaccines against *Salmonella infants,* ST, and SE in several countries (Crouch et al., 2020). Moreover, a trivalent *Salmonella* vaccine (ST, SE, and SK) is commercially available in Egypt (Ibrahim et al., 2018).

Vaccination against *Salmonella* cannot completely prevent infection in chickens (OIE, 2012). Nevertheless, vaccinations can reduce the risk of further infection, cause reduction in the excretion of the organism, and prevent vertical transmission, which can contribute to contamination of hatching or table eggs along with other negative outcomes for poultry (OIE, 2012). Due to its efficacy, the vaccination is effective in conjunction with other hygiene measures and eradication methods, such as all-out production and culling, and its benefits also extend to biosecurity and farm sanitation (OIE, 2012).

Previous studies have primarily focused on live attenuated, bacteria-based, and recombinant vaccines (Hussein et al., 2007; Rabie and Amin Girh, 2020), and previous trials were often conducted to prepare vaccines for use against mycoplasmosis (Fabricant, 1975). Subsequently, different types of MG vaccines are available as live attenuated vaccines, including 6/85 strain, ts-11, F strain, K strain, and K5054 (Leigh et al., 2019). These comprised attenuated, recombinant, and genetically modified MG vaccines (Leigh et al., 2019).

These vaccines are typically administered using spray or eye drop techniques, and some of them induce protective immunity against MG (Leigh et al., 2019). Unfortunately, they may produce lesions in immunosuppressed flocks, particularly in the presence of co-infections and stressors, and they may additionally increase the risk of harmful bacterial shedding. Enzyme-linked immunosorbent assay (**ELISA**) is an effective quantitative technique to evaluate the immune response of different vaccines (Elyazeed et al., 2020). In the prophylaxis of respiratory illnesses, inactivated vaccines are often more effective compared with live vaccines. However, an exception exists in the case of more expensive and complicated vaccines, which are difficult to administer and sometimes require two doses to be effective (Ley, 2003; Ishfaq et al., 2020).

Various adjuvants have been used in previous studies, but the Montanide ISA70 water-in-oil (w/o) emulsion is considered a good option for use in bird vaccines (Cahyani et al., 2020; El-Jakee et al., 2020). In inactivated vaccines that have proven successful against ST, SE, SK, MG, and MS, one or more purified antigens, killed pathogens, or bacterins with an oil adjuvant can successfully induce an immune response (Ibrahim et al., 2018; El-Naggar et al., 2022).

Based on this foundation, the current study aimed to prepare and evaluate the efficacy of a pentavalent *Mycoplasma* and *Salmonella* vaccine comprising locally isolated ST, SE, SK, MG, and MS strains to overcome the previously encountered problems.

MATERIALS AND METHODS

Bacterial Strains

Locally isolated and well-identified strains of MG and MS were obtained from a previous study by Marouf et al. (2020), conducted at the Microbiology Department, Faculty of Veterinary Medicine, Cairo University,

Giza, Egypt. *Salmonella* strains (ST, SK, and SE) were kindly supplied by the Veterinary Serum and Vaccine Research Institute in Abbasia, Cairo, Egypt (Ibrahim et al., 2018).

Vaccine Preparation

MG and MS were cultured separately using Pleuro-Pneumonia Like Organisms (**PPLO**) broth (Becton Dickinson, Franklin Lakes, NJ, USA) and were incubated at 37°C in a CO_2 incubator for 48 h, and the cultured bacterial colonies were adjusted to contain 1×10^8 colony-forming units (**CFU**) mL^{-1} in different groups of embryonated chicken eggs and hens. Calculations were performed using Rodwell and Whitcomb's (1983) standard techniques (Bekele and Assefa, 2018). ST, SK, and SE were each cultured on *Salmonella Shigella* (**SS**) agar (Lab M Limited, Lancashire, UK) at 37°C for 24 h. After being transferred into tryptone soya broth (Lab M Limited), the colonies were cultivated at 37°C for 24 h. After obtaining the total colony count, the bacterial solution was adjusted to contain 1×10^8 CFU mL^{-1}.

All bacteria were pelleted after centrifugation at 12,000 x *g* for 30 min at 4°C. Using the total colony count approach, the bacterial counts of ST, SK, and SE were adjusted to 1×10^8 CFU 0.5 mL^{-1} of the final product by preparing separate final suspensions from ST, SK, and SE, respectively. Finally, all bacteria were inactivated using 0.3% formalin via agitation, and the bacterial suspension was combined with Montanide ISA70 (SEPPIC, Courbevoie, France) in a ratio of 70% volume of adjuvant to 30% volume of antigens according to Charles et al. (1994).

Quality Control of the Prepared Vaccine

Sterility Test for Oil Adjuvant and Trial Vaccine
According to Bekele and Assefa (2018), sterilization of the oil adjuvant (Montanide ISA 70) used in the immunization was achieved using a dry autoclave at 160°C for 1 h. These experiments were conducted at 37°C over a period of 7 d using thioglycolate (Lab M Limited), tryptic soy broth (Lab M Limited), tryptose agar (Lab M Limited), Sabouraud agar (Lab M Limited) and PPLO agar (Becton Dickinson). After the validation of the growth inactivation procedure, Montanide oil adjuvant was used to sufficiently emulsify the bacterial biomass. As a final step, the inactivated culture was sterilized and rendered safe.

Purity Test
The prepared vaccine was tested for bacterial and fungal contamination according to the OIE (2012).

Safety Test
In accordance with the OIE (2012), 20-day-old specific pathogen-free (**SPF**) chicks were administered a double field dose (1 mL) of the manufactured vaccine for two consecutive weeks. The chicks were observed daily for the appearance of any local responses, clinical symptoms, or mortality.

Experimental Design (Potency Test)

Experimental Birds
All experimental conditions and birds handling procedures were approved by the Ethics Committee, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt with number Vet CU12/10/2021/350.

All birds used in the study were initially examined to ensure that they were free of antibodies against *Salmonella* and *Mycoplasma* as well as their antigens. A total of 110 SPF 1-day-old chicks were obtained from Koom Osheem, Fayuom, Egypt. These chicks were housed in an area with a concrete floor with fresh wood shavings as bedding in separate pens on a deep litter system. The chicks were reared under optimal temperature, humidity, and ventilation, and were kept on a 24-h constant light schedule for a 10-wk observation period. Overall, they were kept under the same conditions as described by Evans et al. (2012).

Also, birds were provided with fresh, clean water and a balanced diet (containing starter and growth rations) without any interventions, that met or surpassed NRC (1994) recommendations.

Experimental Design
From 110 SPF 1-day-old chicks, three groups were formed, which were divided into

Table 1. Experimental design.

Groups	Subgroup (10 birds each)	First vaccine dose (0.5 mL via subcutaneous injection)	Booster dose (0.5 mL via subcutaneous injection)	Challenged bacteria (7 weeks = 49 days old)	
A total 50 bird (Vaccinated with pentavalent vaccine & challenged group)	MG MS ST SE SK	7 days old	After 21 days (4 weeks = 28 days old)	0.5 mL via eye dropping in both the eyes 0.5 mL via crop gavage	MG MS ST SE SK
A total 50 bird control positive (Challenged group)	MG MS ST SE SK	- -	- -	0.5 mL via eye dropping in both the eyes 0.5 mL via crop gavage	MG MS ST SE SK
Control negative (Unvaccinated & unchallenged group)	-	-	-	-	-

Salmonella enterica serovar Typhimurium (ST), *Salmonella enterica* serovar Enteritidis (SE), *Salmonella enterica* serovar Kentucky (SK), *Mycoplasma gallisepticum* (MG), and *Mycoplasma synoviae* (MS).

subgroups, as summarized in Table 1. The first group comprised 50 birds, which were subdivided into five subgroups (ten birds for each strain). Each chick in all subgroups was subcutaneously (S/C) injected at mid-neck region with 0.5 mL of the locally prepared inactivated pentavalent vaccine at 7 d of age. A booster dose was administered 3 wk after the administration of the first dose (28 days old); then, each subgroup was challenged 3 wK after the booster dose (49 days old) by administering 0.5 mL of each strain, MG and MS, which contained 1×10^8 CFU mL^{-1} of cells, via eye dropping in both the eyes as per the study by Bekele and Assefa (2018); moreover, 0.5 mL of each strain, ST, SE, and SK, which contained 1×10^8 CFU mL^{-1} of cells, was administered via crop gavage.

The second control positive challenged with ST, SE, SK, MG, and MS groups included 50 birds, which were subdivided into five subgroups (ten birds each). Each subgroup was individually challenged by administering 0.5 mL of MG and MS strains containing 1×10^8 CFU mL^{-1} of each strain into both the eyes via eye dropping, whereas 0.5 mL of ST, SE, and SK strains containing 1×10^8 CFU mL^{-1} of each strain was administered via crop gavage. The third group included ten unvaccinated and unchallenged birds, which were kept as a blank control negative.

After the challenge, the inoculated chicks were observed for three consecutive weeks (10 wk = 70 days old). The degree of protection conferred by the vaccine was determined by the severity of the clinical signs; mortality; shedding of the challenge organisms from the tracheal swabs of MG and MS and cloacal samples of ST, SE, and SK.

For the estimation of bacterial shedding, cloacal swabs were collected from all birds in *Salmonella* groups which were inoculated into tetrathionate broth and examined bacteriologically for the shedding of different groups of *Salmonella*. In addition, nasal swabs and synovial fluids were collected from all birds in *Mycoplasma* groups which were inoculated into PPLO broth according to Hofstad et al. (1997).

Humoral immune responses were measured and evaluated using microagglutination ELISA using coated plates for MG and MS (BioChek ELISA Kit, Ascot, UK) as recommended by Ali et al. (2015). The humoral immune response against ST and SE antigens in the pentavalent vaccine was assessed by ELISA using coated plates (Jordan Bio-Industries Center -JOVAC, Amman, Jordan) while traditional ELISA was applied to detect antibodies against SK antigens as described by Barrow (1992). Therefore, blood samples (2 mL bird^{-1}) were collected from the wing vein to separate sera samples, before immunization then, regularly after administering each vaccine dose, and after the challenge for 3 wk (once per week) to measure and evaluate the developed humoral immune response.

Body Weight Gain of Experimental Birds The body weight gain of the birds was evaluated every week by analyzing feed intake, average body weight gain, and cumulative feed data. These were estimated to detect the efficacy of the trial vaccine in the birds individually and to compare the results among the pentavalent vaccinated, control positive challenged, and control negative groups.

Statistical Analysis

Data are represented as mean $\pm$ standard deviation. We analyzed the normal distribution using the Shapiro−Wilk test and analyzed homoscedasticity using the Levene's test. Additionally, significance was tested using an independent sample t test to compare the ELISA titers between the vaccinated and unvaccinated groups challenged with the same pathogen.

One-way ANOVA was performed to compare body weight gain among the three groups. Multiple comparisons between groups were conducted using the Fisher's Least Significant Difference post-hoc test. All statistical analyses were performed and graphs were obtained using RStudio v1.3.1093 (RStudio Team, 2020) and R programming language v4.0.3 (R Core Team, 2020). Differences between the groups were considered significant when P-values were < 0.05.

RESULTS

Clinical Findings

Birds vaccinated with the locally prepared pentavalent vaccine and the blank control negative group exhibits no clinical signs during the observation period. While the control positive MG-challenged chicks displayed respiratory signs including coughing, sneezing, nasal discharge, and conjunctivitis, as well as nasal discharge. The hens in the MS-challenged control group showed retarded development, swelling of the joints, with respiratory manifestations. The ST, SE, and SK vaccinated group showed slight brownish diarrhea with low degree of enteritis. While the chickens in the ST, SE, and SK challenged control positive group showed severe brownish diarrhea (Figure 1), depression, decreased feed intake, lowered weight, ruffled feathers, sleepy appearance, recumbent, and unable to walk.

Morbidity and Mortality Rates

Low morbidity and mortality rates were noted in both the vaccinated and negative control groups. Significant morbidity and mortality rates were observed in the positive control challenged groups, as illustrated in Figure 2.

Postmortem Lesions

Postmortem examination for ST, SE, and SK challenged positive control group revealed severe degree of enteritis, enlarged spleen, liver, nephritis, impacted gallbladder with tiny hepatic necrotic foci, pericarditis, and septicemic picture was observed in some cases as shown in Figures 3−5. The postmortem examination of freshly dead chicken in positive control challenged group with

Figure 1. (A) Chickens in blank control negative group showing apparently normal appearance. (B) Bird from *Salmonella enterica* serovar Enteritidis (SE) challenged non-vaccinated bird showing brown color dropping soiling the vent region. (C) Dropping in *Salmonella enterica* serovar Kentucky (SK) challenged non-vaccinated bird showing dark brown colored diarrhea.

MG showed slight foamy turbidity of air sacs as shown in Figure 6.

Mycoplasma and Salmonella Shedding

Detection of Mycoplasma Shedding Both MG and MS were re-isolated from tracheal swabs and synovial fluid samples in the primary study groups (control positive challenged group and vaccinated group). The control positive group was positive for fried egg colonies of MG and MS for 2 consecutive weeks after the challenge, whereas the pentavalent group revealed *Mycoplasma* shedding only at 1-wk postchallenge.

Detection of Salmonella Shedding In the first, second, and third weeks after the challenge, the pentavalent vaccine shedding rates of ST and SE from chickens vaccinated with the inactivated vaccine were 30, 20, and 0%, respectively. In contrast, the fecal shedding rates of SK were 30, 10, and 0%, respectively. In each of the first, second, and third weeks after the challenge, the re-isolation rate in the control positive unvaccinated birds was 100% (Table 2).
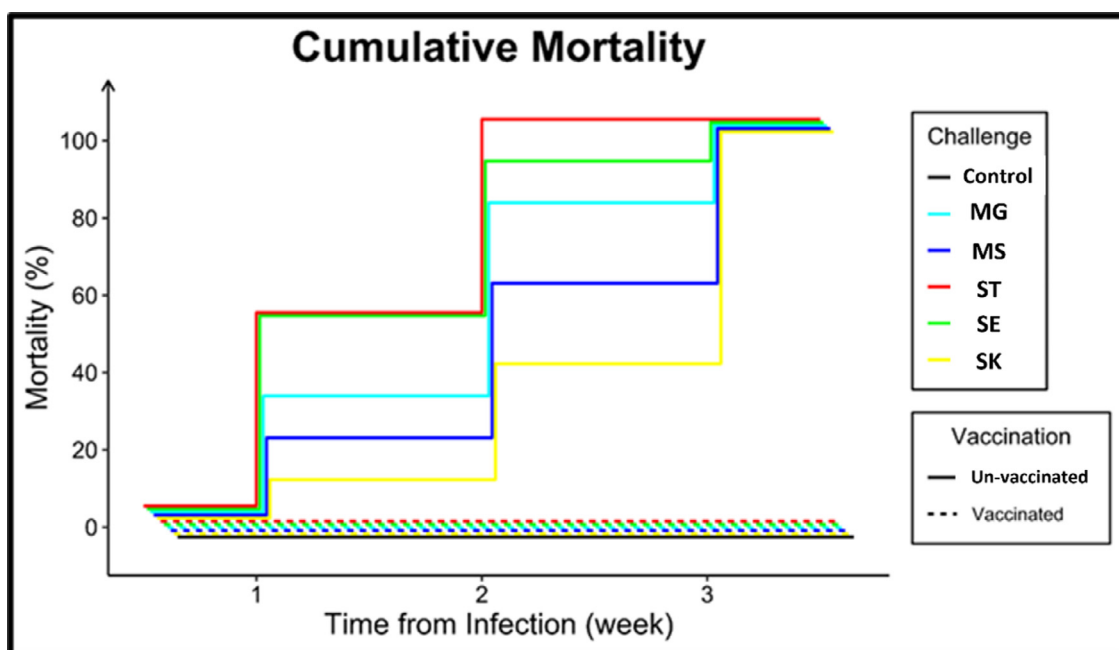
Figure 2. The mortality rates in different experimental groups; influences of different pathogenic challenges on cumulative mortality of vaccinated and not vaccinated groups. *Mycoplasma gallicepticum* (MG), *Mycoplasma synoviae* (MS), *Salmonella enterica* serovar Typhimurium (ST), *Salmonella enterica* serovar Enteritidis (SE), and *Salmonella enterica* serovar Kentucky (SK).

Figure 3. (A) Chickens in *Salmonella enterica* serovar Typhimurium (ST), challenged non-vaccinated bird showing high mortalities after challenge. (B) Postmortem examination of freshly dead chickens in *Salmonella enterica* serovar Typhimurium (ST), challenged group showing nephrosis in kidney with distended ureters with presence of enteritis with congested mesenteric blood vessels.

Humoral Immune Response in Different Groups

For antibodies against both MG and MS, the geometric mean antibody titers (**GMT**) were beyond the cutoff values of 843.0 and 737.0, respectively. The pentavalent vaccination showed an excellent protective response to the challenge posed by MG and MS. The serum ST antibody titers (GMT) of the vaccinated group increased from 190.0 before vaccination to 871.3 in the third week after the first immunization and 2225.1 in the third week after the booster administration.

The GMT increased to 2007 in the third week after the challenge. The GMT against SK increased from
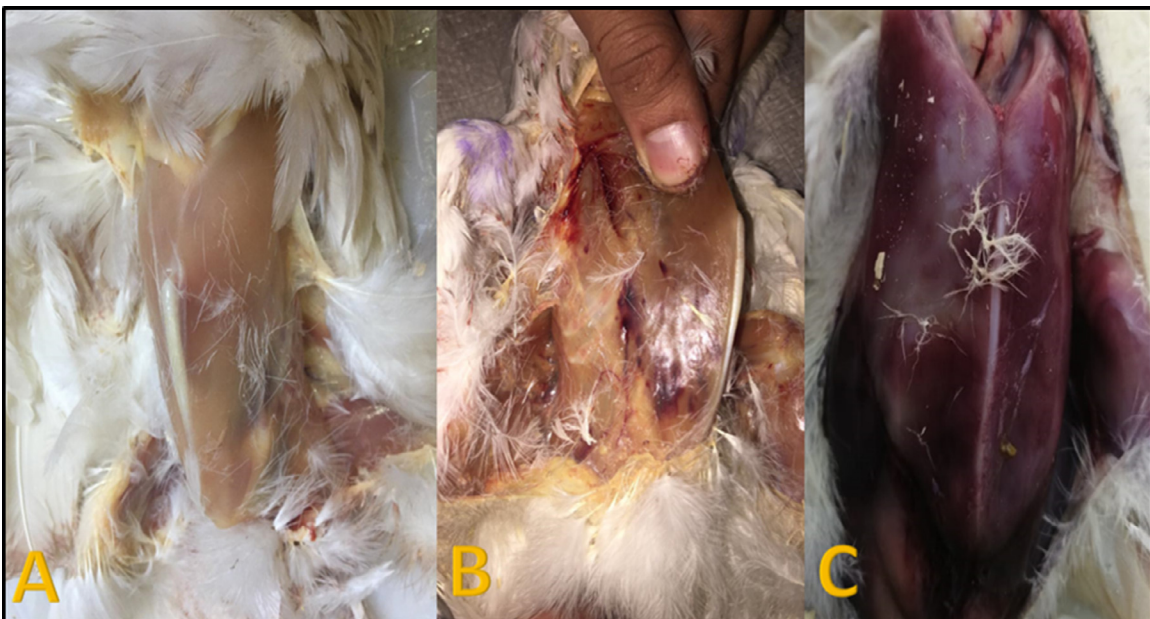

Figure 4. (A) Apparently normal breast muscle appearance in control negative birds. (B) Postmortem in control positive S*almonella enterica* serovar Typhimurium (ST), challenged birds showing congestion and hemorrhages in breast muscle. (C) Postmortem in control positive S*almonella enterica* serovar Enteritidis (SE), challenged birds showing severe congestion of breast muscle with severe septicemic picture.
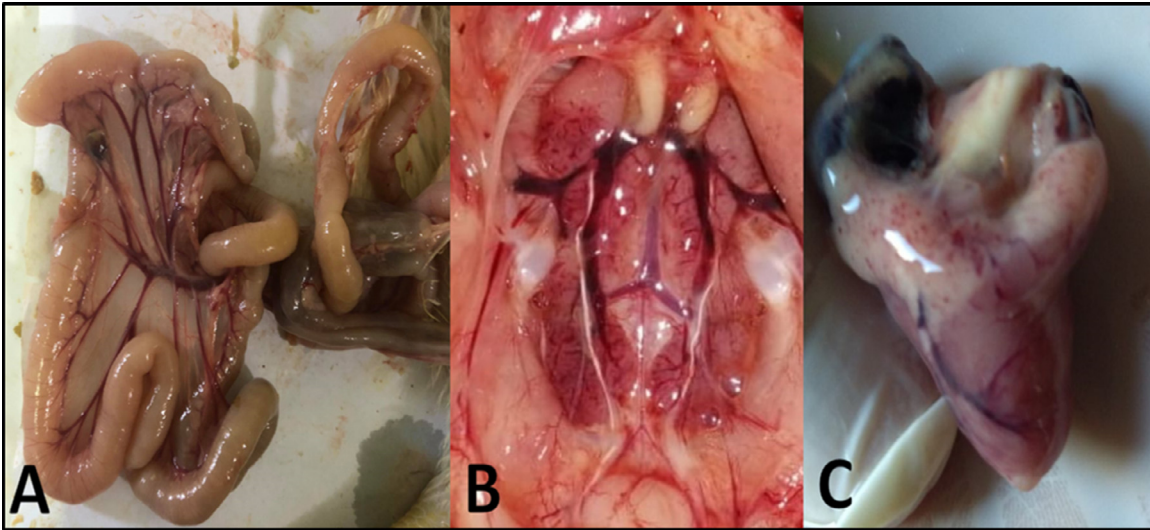
Figure 5. (A) Intestine of *Salmonella enterica* serovar Enteritidis (SE), control positive challenged birds showing enteritis with engorgement of mesenteric blood vessels. (B) Kidney of *Salmonella enterica* serovar Enteritidis (SE), control positive challenged birds showing nephritis and nephrosis with distended ureters. (C) Heart of *Salmonella enterica* serovar Enteritidis (SE), control positive challenged birds showing hemorrhage on coronary fat.

186.0 before vaccination to 863.1 after the first immunization and 2,220.5 in the third week after the booster administration. The GMT increased to 1,985.7 in the third week after the challenge. In contrast, the GMT against SE increased from 191.0 before vaccination to 867.8 in the third week after the initial immunization and 2,104.3 in the third week after the booster administration. The GMT increased to 2,032.7 in the third week
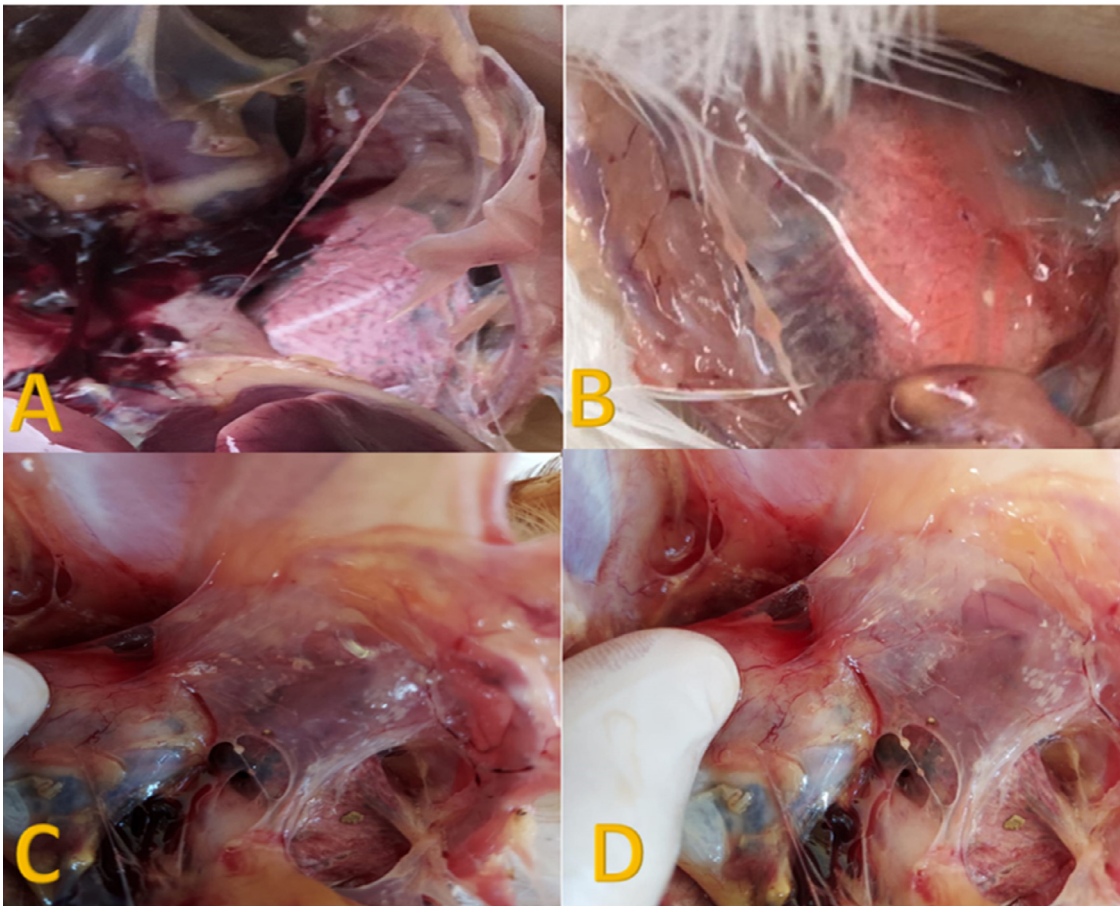
Figure 6. (A) Postmortem examination of sacrificed birds in blank control negative group at the end of the experiment showing normal transparent and clear air sac. (B) Postmortem examination in vaccinated challenged birds with *Mycoplasma gallicepticum* (MG) showing normal air sac appearance. (C and D) Postmortem examination of freshly dead chicken in control positive challenged group with *Mycoplasma gallicepticum* (MG) showing foamy slight turbidity of air sacs.

Table 2. *Salmonella* shedding rates.

Weeks for shedding of *Salmonella*	*Salmonella enterica* serovar Typhimurium (ST)		*Salmonella enterica* serovar Enteritidis (SE)		*Salmonella enterica* serovar Kentucky (SK)	
	Pentavalent vaccinated group	Control positive challenged group	Pentavalent vaccinated group	Control positive challenged group	Pentavalent vaccinated group	Control positive challenged group
1st wk	30	100	30	100	30	100
2nd wk	20	100	20	100	10	100
3rd wk	0	100	0	100	0	100

after the challenge. In contrast, the serum ELISA antibody titer in the unvaccinated chicks was 187.0.

Furthermore, for ST, a sudden increase in antibody titers was noted in the third week after exposure, with antibody titers of 176.8, 191.1, and 1,128.0. For SK, the value was 187.0. For SE, the increase in antibody titers was also noted in the third week after exposure, with antibody titers of 176.8, 191.1, and 1,200.7, and finally a figure of 187.0. Lastly, the increase in antibody titers was also noted in the third week after exposure, with antibody titers of 176.8, 191.1, and 1,150.4, as shown in Figures 7−11.

Body Weight Gain of the Experimental Birds

The statistical analysis showed no detectable difference between the pentavalent vaccinated group and the negative control group. Despite this result, there remained a difference between the positive and negative control groups, as shown in Figure 12.

Protective Efficacy of the Prepared Vaccine

According to mortality after challenge, the manufactured vaccine had a 100% protection rate as the survival rate of the vaccinated birds were 100% postchallenge, the prepared vaccine diminished the severity of the clinical signs in vaccinated birds, reduced bacterial shedding, provided protective antibody titers, and did not affect body weight gain in the vaccinated birds in comparison with control group.

DISCUSSION

There has been a rapid increase in the world's principal poultry products (meat and eggs) output (Abd El-Hack et al., 2022a). In turn, this reflects customer need and acceptance for those high-quality items and the comparatively low price due to the efficiency of the manufacturing process (Abd El-Hack et al., 2022b). Between 1995 and 2005, worldwide consumption and production have grown (Scanes, 2007). In the chicken industry, respiratory and enteric infection due to bacterial, viral, parasitic, or managemental causes are common devastating problems that results in severe economic losses in the poultry industry (Setta et al., 2018; Abd El Hamid et al., 2019; Salem and Attia, 2021; Salem et al., 2021). When it comes to poultry, *Mycoplasma* and *Salmonella* are responsible for considerable economic losses. They are connected to respiratory and enteric diseases, poor performance in the field, decreased
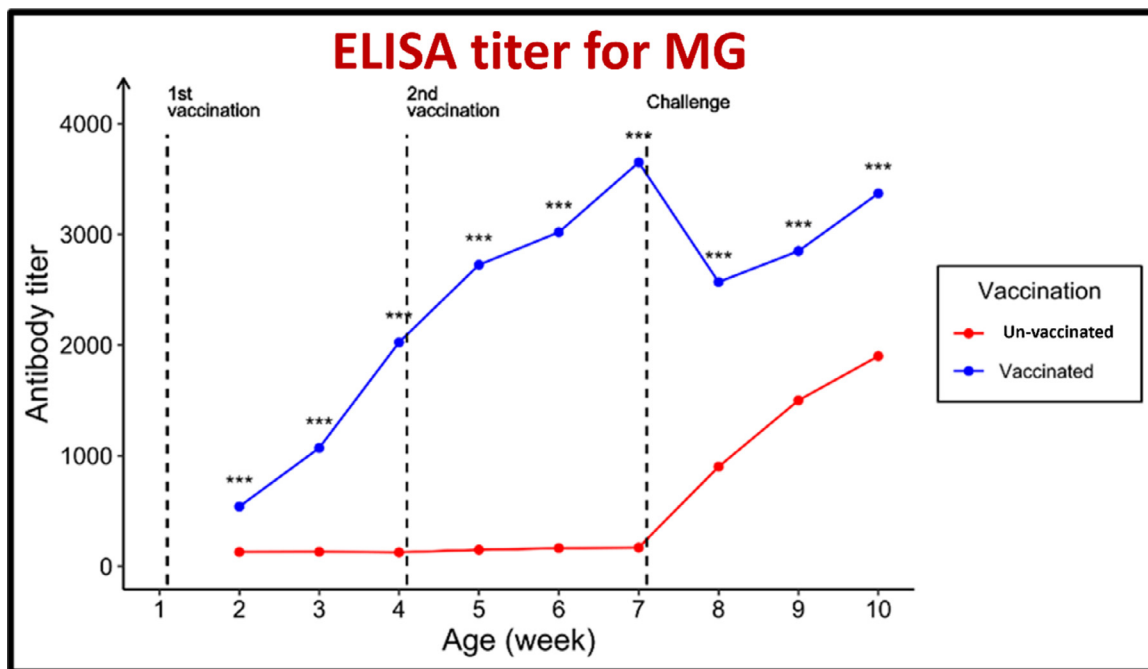
Figure 7. Effect of vaccination and challenge with *Mycoplasma gallicepticum* (MG) on the ELISA titer MG antibodies. Significance between titers in the same age is expressed as *** (*P*-value < 0.001) (n = 5).
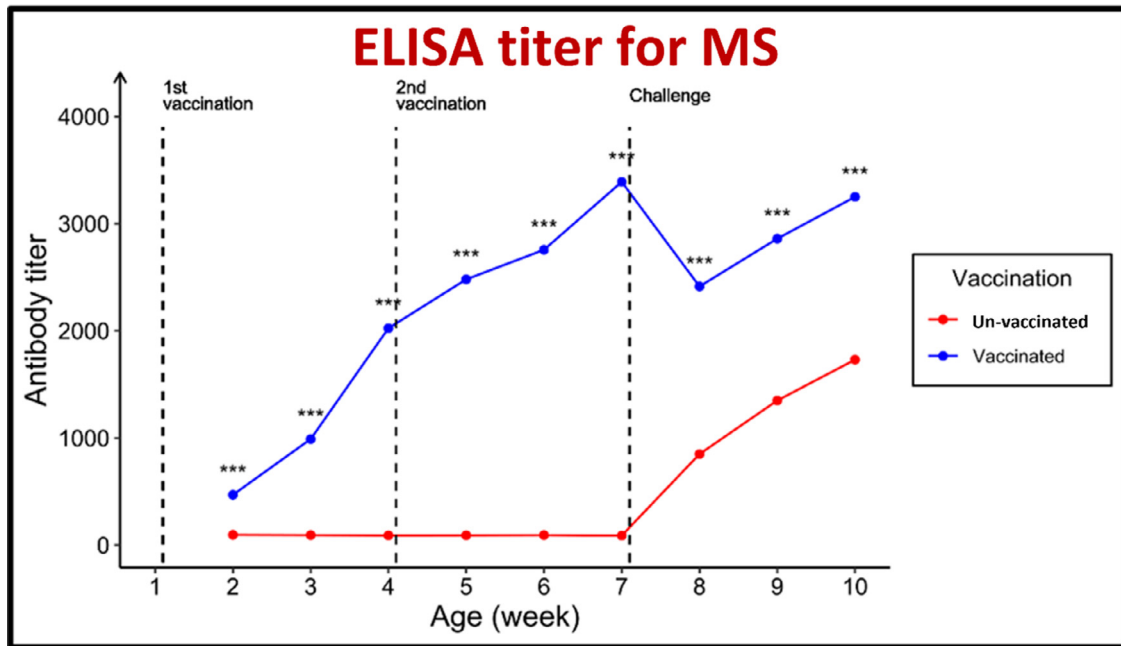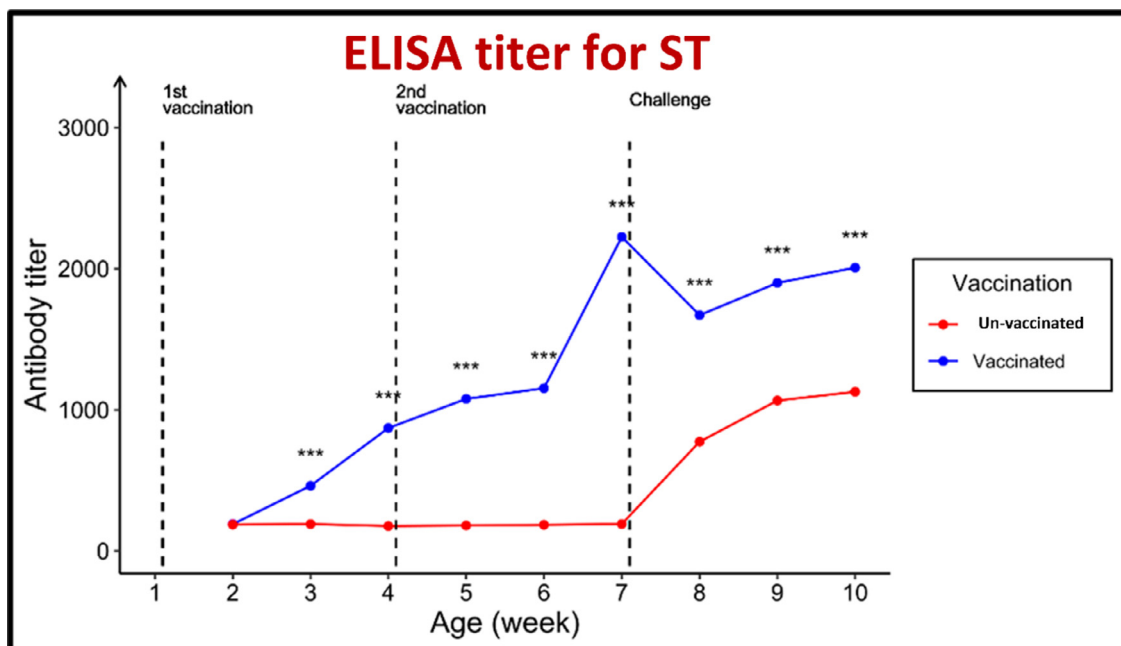
Figure 8. Effect of vaccination and challenge with *Mycoplasma synoviae* (MS) on the ELISA titer MS antibodies. Significance between titers in the same age is expressed as *** (P-value < 0.001) (n = 5).

egg production, decreased fertility, and increased embryonic mortalities worldwide (Murugkar et al., 2005; Marouf et al., 2020, 2022).

In the current study, the chicks immunized by the prepared pentavalent vaccine showed a protective titer against MG, MS, ST, SE, and SK after challenge with 100% protection percentage. This protection percentage indicated the promising efficacy of using the prepared vaccine as mentioned by Heddleston (1975) and Knight-Jones et al. (2014). These studies confirmed that if the

produced vaccine gave more than 80% protection value, it is considered acceptable for the usage.

Three weeks post challenge, the vaccinated group of hens had fecal shedding of 8.33%, whereas the unvaccinated control group had fecal shedding of 25%. In the vaccinated group, no shedding was identified in the fourth week after the challenge. While the unvaccinated control group shed 16.66%, the vaccinated group shed 16.66%. Sayed (2010) and Ibrahim et al. (2018) showed similar fecal shedding rates.

Figure 9. Effect of vaccination and challenge with *Salmonella enterica* serovar Typhimurium (ST), on the ELISA titer ST antibodies. Significance between titers in the same age is expressed as *** (P-value < 0.001) (n = 5).
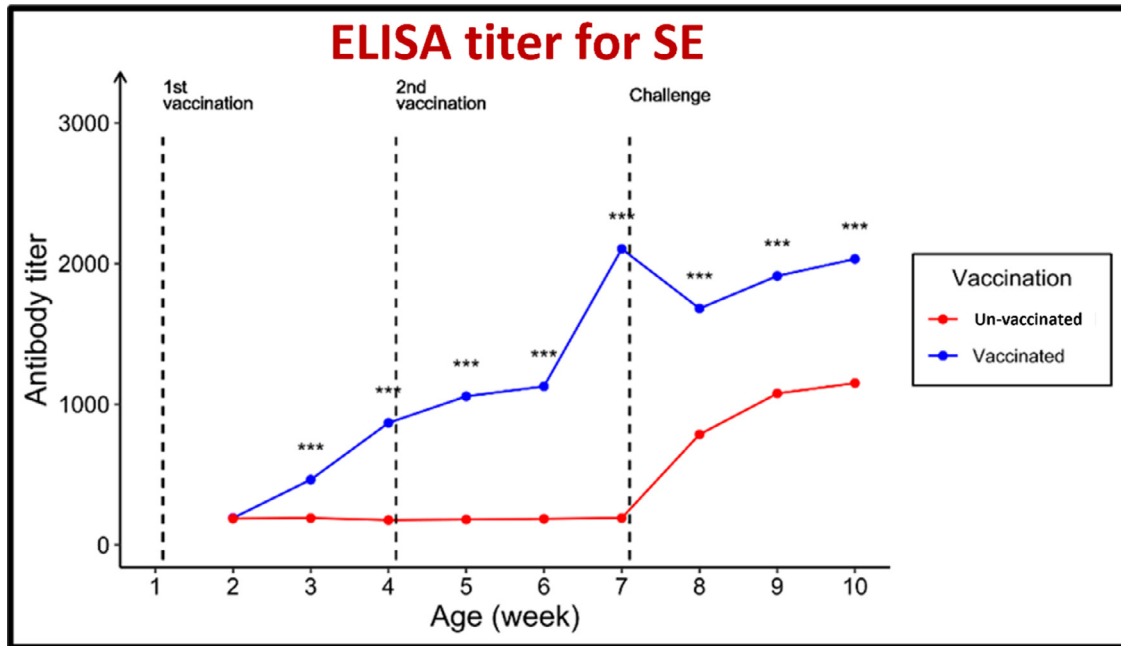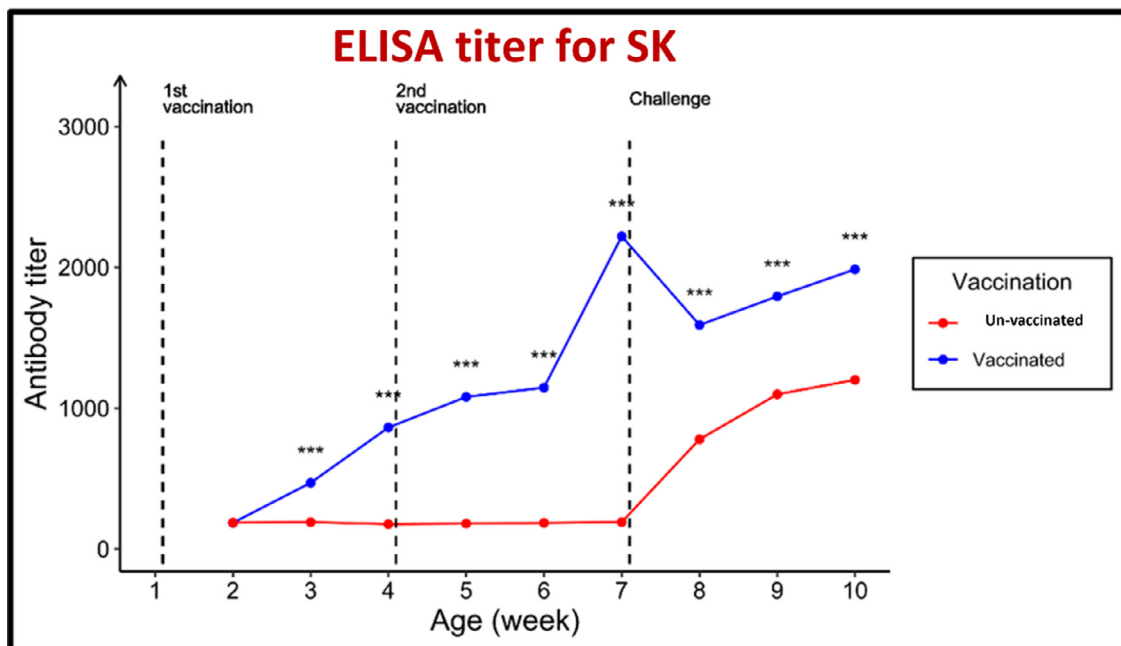
Figure 10. Effect of vaccination and challenge with *Salmonella enterica* serovar Enteritidis (SE), on the ELISA titer SE antibodies. Significance between titers in the same age is expressed as *** (*P*-value < 0.001) (n = 5).

It is well known that *Mycoplasma* infections are widespread prevalent in commercial layer and broiler hens and backyard chickens in rural regions (Marouf et al., 2022). Birds with *Mycoplasma* infection generally exhibited nasal discharge, synovitis, and dyspnea with mouth breathing, as well as gross postmortem lesions of a congested lung, hyperemic mucoid trachea, and swollen air sacs after death (Talha, 2003). Increased mortality, carcass condemnation, lowered egg production, and hatchability are all expenses associated with MG-related diseases where depopulation of animals is not practicable on large commercial multiage layer farms (Ley,

2008). So, the most practical approach to disease management is vaccination (Bermudez and Kalbac, 1988; Branton et al., 2002).

From our findings, the pentavalent prepared inactivated Montanide ISA70-based *Salmonella* and *Mycoplasma* vaccine showed accepted antibodies levels when tested in chickens using the ELISA technique. The tested vaccine protected challenged birds and neither showed mortalities nor clinical signs. The results from the current study are supported by Ferguson-Noel et al. (2012), who found that *Mycoplasma* inactivated vaccines can reduce vertical transmission, lessen the

Figure 11. Effect of vaccination and challenge with *Salmonella enterica* serovar Kentucky (SK) on the ELISA titer SK antibodies. Significance between titers in the same age is expressed as *** (*P*-value < 0.001) (n = 5).
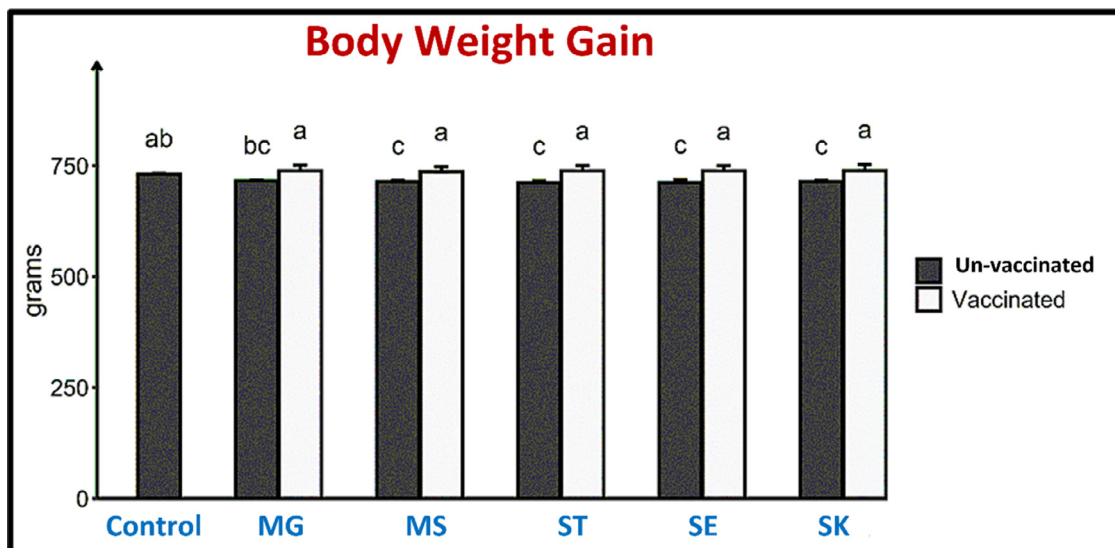
Figure 12. The performance of the birds in the different experimental groups; effect of vaccination on the body weight gain in different pathogenic challenges. *Mycoplasma gallicepticum* (MG), *Mycoplasma synoviae* (MS), *Salmonella enterica* serovar Typhimurium (ST), *Salmonella enterica* serovar Enteritidis (SE), and *Salmonella enterica* serovar Kentucky (SK). Data are expressed as mean ± SEM. Bars without shared letters indicate a significant difference between them (P-value < 0.05) (n = 5).

severity of illness, and provide significant protection for the respiratory system and reproductive system. Also, El-Naggar et al. (2022) concluded that bivalent MG and MS inactivated vaccines adjuvanted with Montanide ISA70 revealed a protective antibodies titer and gave 80% protection against MG and 90% against MS in challenged birds. In the same way, Ibrahim et al. (2018) found that the inactivated trivalent *Salmonella* vaccine revealed protection and provoked protective antibodies titer in vaccinated chickens. Feberwee et al. (2000) also found that the inactivated SE vaccine resulted in the reduction of SE reinfection in broiler breeder flocks.

On the other hand, Evans and Hafez (1992) noticed that SPF layer hens whose were given an inactivated MG vaccination at 30 wk and subsequently challenged with a virulent R strain of MG revealed fair protection. However, the usage of inactivated *Mycoplasma* vaccinations is somewhat effective, and can reduce the spread of MG germs, but it cannot prevent horizontal transmission of the infection (El-Naggar et al., 2022). Also, Jacob et al. (2014) concluded that in commercial layer production facilities with diverse age groups, some feel bacterins have a limited impact in protecting against MG infection. Reviewing the available literature, no available data about pentavalent *Salmonella* and *Mycoplasma* in poultry were found; thus, from our finding, we recommend this vaccine to be applied to protect poultry flocks in Egypt or elsewhere.

CONCLUSIONS

The Egyptian locally prepared pentavalent vaccine against *Mycoplasma* and *Salmonella* infections is both safe and effective and provide 100% protection, reduce the clinical signs, produce protective antibody titers, lower the bacterial shedding, and did not affect the body

weight gain with 100% survival rate in the vaccinated birds. Further studies are recommended to evaluate the efficacy of the prepared vaccine under field condition, especially those with layer or breeder flocks, to combat these major problems that threaten poultry industry in Egypt.

DISCLOSURES

The authors declare no conflict of interests.

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