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Efficacy of Combined Vaccine against Salmonellosis and Infectious Coryza in Poultry

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ABSTRACT

In the present study, efficacy of two prepared combined vaccines against salmonellosis and infectious coryza in poultry has been studied. Two vaccines were prepared using *Salmonella* Typhimurium and Enteritidis combined with *Avibacterium paragallinarum* serovars A, B, and C. one vaccine was adjuvanated with aluminium hydroxide gel and the other adjuvanated with montanide ISA71. The two vaccines were assayed in six weeks old Specific Pathogen Free (SPF) white Lohman layer chickens by injecting two doses of each vaccine 3 weeks apart. These chickens were challenged with either *Salmonella* virulent strains or *Avibacterium paragallinarum* different serovars 3 weeks post second dose. Antibody titers in sera of chickens against different antigens were higher in groups vaccinated with montanide oil vaccine than those vaccinated with aluminium hydroxide gel vaccine as detected by different serological tests; ELISA, micro-agglutination test and haem-agglutination inhibition test. Protection rate against challenge test were 80% and 85% for *Salmonella* and (80%; 90%, and 70%) and (90%; 100%, and 90%) to *Avibacterium paragallinarum* serovars A, B, and C respectively for combined vaccine adjuvanated by aluminum hydroxide gel and montanide ISA71. The protection rate was 15% against *Salmonella* Typhimurium and Enteritidis and 0% against infectious coryza among the unvaccinated chicken group.it could be concluded that producing a vaccine from locally isolated *Salmonella* and Avibacterium (Haemophilus) paragallinarum strains adjuvanated with montanide ISA71 is recommended to aid in controlling avian salmonellosis and Infectious coryza at the same time.

Key words: Aluminum hydroxide gel, Chicken, Infectious coryza, Salmonellosis, Vaccine.

INTRODUCTION

Salmonella is a persistent pathogen in the environment, able to easily survive and proliferate. The most commonly isolated serovars worldwide from various animal sources continue to be *Salmonella* Enteritidis and *Salmonella* Typhimurium which, besides producing gastroenteritis, are found in asymptomatic carriers in a wide variety of animal species. Of these, *Salmonella* Enteritidis is the most prevalent one followed by *Salmonella* Typhimurium (52.3% and 23.3% of the cases, respectively) (López-Martín et al., 2016). *Salmonella* has remained to be one of the most frequently detected causative agents in the food-borne outbreaks reported (26.6% of outbreaks). Eggs and egg products are frequently associated with *Salmonella* outbreaks. *Salmonella* Enteritidis and to a lesser extent, *Salmonella* Typhimurium are associated with egg-related outbreaks (EFSA, 2004).

Avian Infectious Coryza is a serious respiratory tract infection of domestic fowls caused by an opportunistic pathogen *Avibacterium paragallinarum* having an economic implication on the poultry industry and ornamental bird's population (Priya et al., 2012).

Infectious Coryza is a contagious bacterial disease of poultry; it is a common bacterial disease in the commercial poultry (Gayatri et al., 2010). It mainly affects the upper respiratory tract of chickens. The meat of the affected chicken is condemned if it is infected with *A. paragallinarum* (Blackall et al., 2005).

Combined vaccines have the advantage of protection against more than one disease at the same time, besides, reducing vaccination expenses, decreasing the stress of vaccination for different vaccines, number of vaccination performed and saving time. So this study evaluates the efficacy of a prepared combined vaccine against salmonellosis and infectious coryza using two different adjuvants; aluminium hydroxide gel and montanide ISA 71.

MATERIALS AND METHODS

Bacterial strains

***Salmonella* Typhimurium and *Salmonella* Enteritidis**

These two strains are local field isolates kindly obtained from Department of Bacterial Sera and Antigens, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. These strains were used for preparation of vaccines under test.

Avibacterium paragallinarum

The reference strains *Avibacterium paragallinarum* strain W (serovar A-1) and Modesto strain (serovar C-2) were obtained from MSD Animal Health/Intervet International bv., Boxmeer, The Netherlands; and reference strain 0222 (serovar B-1) was obtained from Dr. R.B. Rimler, USDA National. Animal Disease Center, Ames, Iowa, USA. Local field strain (A) has been originally isolated by Anaerobic Vaccines Research Department, VSVRI from an outbreak of Infectious Coryza in a laying flock in Egypt, confirmed using species level and serotype using serological tests with standard antisera against reference serovars.

Experimental birds

SPF one day old chicks. Forty chicks were used for safety testing of the prepared vaccines.

SPF white Lohman layer chickens. A total number of 150, six weeks old SPF white Lohman layer chickens were obtained from SPF Farm at Koom Osheem Fayuom province, Egypt. They were housed in batteries with the network floor. All birds were ascertained first to be free from *Salmonella* and coryza (organism and antibodies). They were fed on free balanced rations, and used for evaluation of prepared vaccines.

Vaccine preparation

Two combined vaccines were prepared according to Blackall et al. (1992) and Charles et al. (1994). Briefly, ST and SE were cultured on specific media. Equal volumes of each culture (adjusted to contain 1×10^8 CFU/ml) were mixed together and inactivated by adding 0.5% Formalin. Also cultures of *Avibacterium paragallinarum* serovars A, B and C were prepared (adjusted to contain 1×10^6 CFU/ml) and equal volume of each serotype were mixed and inactivated by adding 0.5% Formalin and 0.01% (w/v) of thimerosal was added as a preservative agent. Then the above cultures were combined together and divided into 2 parts; one part adjuvanated with 20% (v/v) aluminum hydroxide gel and the other part with Montanide ISA-71 (30:70 v/v).

Experimental design

A total of 150, six weeks old SPF white Lohman layer chickens were divided into three groups 50 chicks per each. Group 1 contained fifty chickens were vaccinated with the prepared combined aluminium hydroxide gel vaccine in a dose of 0.5 ml S/C. Group 2 contained fifty chickens were vaccinated with the prepared combined montanide ISA71 vaccine with dose of 0.5 ml S/C. Group 3 contained fifty chickens injected 0.5 ml S/C with normal saline, left as a control group.

Birds in group (1) and group (2) were boostered with the same vaccine (by the same route and dose) 3 weeks after first immunization. Serum samples were obtained regularly before immunization, weekly for 3 weeks after the 1st vaccination and every 2 weeks post boostering for 22 weeks. Then pooled and stored at -20 °C till used for following up the induced antibodies.

Quality control testing of the prepared experimental vaccines

Sterility test. The prepared vaccines were tested to be free from any external contaminant (aerobic and anaerobic bacteria, fungus and mycoplasma) according to OIE (2016).

Safety test. Safety of the prepared vaccines was monitored through the injection of a double field dose (1 ml) of the vaccine subcutaneously in each of 20 one day old SPF chicks. The chicks were observed daily for two weeks for any signs of local reactions, clinical signs or deaths.

Determination of immune response to the prepared vaccines Serological evaluation of humeral immune response of the vaccinated chickens against *Salmonella* Typhimurium and *Salmonella* Enteritidis

Micro-agglutination test (MAT)

Antibody titer in vaccinated and unvaccinated chickens was followed up on regular intervals post vaccination applying Micro-agglutination test (MAT) using sonicated antigen, according to the method described by Thaxton et al. (1970) and Brown et al. (1981).

ELISA

The developed humoral immune response against ST and SE in the vaccinated chickens was measured by ELISA in the sera using *Salmonella* antibody test kit (BioChek Poultry Immunoassays cat # CK117 for *S. enteritidis* and CK118 for *S. typhimurium*) according to Haider et al. (2007). Calculation of the antibody titers in ELISA were performed in relation to S/P ratio according to the following formulae:

$$\text{S/P ratio} = \frac{\text{Sample mean} - \text{Negative control}}{\text{Positive control} - \text{Negative control}}$$

Calculation of Antibody Titer Log_{10} Titer=1.13(Log $_{S/P}$) +3.156.

Antibody titer = AntiLog

Serological evaluation of humeral immune response of the vaccinated chickens against Avibacterium paragallinarum serovars A, B, and C

Haemagglutination inhibition test

Antibody response in vaccinated and unvaccinated chickens was followed up on regular intervals post vaccination applying Haemagglutination Inhibition (HI) test using sonicated antigen, according to the method described by Blackall et al. (1990).

Enzyme-Linked Immunosorbent Assay (ELISA)

It was done according to Ryuichi et al. *(*2012) for *Avibacterium paragallinarum* serovars (A, B, and C). Optical Density (OD) was measured at 490 nm by using a micro plate reader (DYANA Tech., USA). The S/P ratio was calculated and expressed as ELISA titer.

$$\text{S/P ratio} = \frac{\text{Sample mean} - \text{Negative control}}{\text{Positive control} - \text{Negative control}}$$

Efficacy test (Challenge)

Challenge by Avibacterium paragallinarum serovars A, B and C

All challenge was done by intra sinus inoculation with 0.1 ml overnight broth culture of *Avibacterium paragallinarum* serovars A, B and C challenge dose containing $1x10^6$ CFU/ml. Clinical signs of Infectious Coryza were recorded from day-1 to day-7 after inoculation. The presences of any nasal discharge and facial edema in challenged chickens were recorded. A protected chicken was defined as a chicken that had shown no clinical signs.

***Challenge by Salmonella* Typhimurium and *Salmonella* Enteritidis strains**

Via administrating the vaccinated chickens 3 weeks post boostering dose by a dose of 1 ml virulent ST and SE broth culture containing $1x10^8$ CFU /ml (OIE, 2016).

Fecal shedding

Shedding of *Salmonella* was detected in the fecal samples collected from challenged vaccinated and non-vaccinated chicks up to 4 weeks post challenge.

Statistical analysis

The level of protection present in the vaccinated groups were analyzed and compared with parametrical correlation using Student's T test (significant difference at $P < 0.05$) (Snedecor and Cochran, 1980).

Ethical approval

All animal procedures were approved by the Animal Ethics Committee at Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

RESULTS

Safety and sterility of prepared vaccines

Both of two vaccines were found to be safe and sterile.

Humeral immune response of the vaccinated chickens against *Salmonella* Typhimurium and *Salmonella* Enteritidis

Table 1 and 2 illustrated results of MAT and ELISA which are parallel to each other as the antibody titers started rising 2 weeks post first vaccination and reached peak sixth week post boostering. It was clear that MAT and ELISA titer for combined montanide ISA 71 vaccine was higher or double the titer of combined aluminium hydroxide gel vaccine for both antigens. The obtained results shown in tables 1 and 2 were analyzed statistically using Student's T test and it was found that there is a significant difference at $P \leq 0.05$ between group 2 (vaccinated with combined montanide ISA71 vaccine) and group 1 (vaccinated with combined aluminium hydroxide gel vaccine).

Humeral immune response of the vaccinated chickens against *Avibacterium paragallinarum* serovars A, B, and C

Results of Haem-agglutination Inhibition (HI) text and ELISA as shown in table 3 and 4 were in accordance to those of *Salmonella* organisms of both vaccines. As antibody titers start raising two weeks post first vaccination and reached peak six weeks post boostering. The obtained results in tables (3 and 4) were analyzed statistically using Student's T test and it was found that

there is a significant difference at P $\geq$ 0.05 between group 2 (vaccinated with *combined* montanide ISA71 vaccine) and group 1(vaccinated with combined aluminium hydroxide gel vaccine).

Concerning ELISA titers for *Avibacterium paragallinarum* serovars (A and C) in both vaccines as shown in table 4, wee paralleled with that of HI, also there was a statistically significant difference in ELISA titer between both vaccines.

Table 1. Measurement of antibody against *Salmonella* Typhimurium and Enteritidis in sera of vaccinated and unvaccinated layer chickens using microagglutination test.

Weeks post vaccination	Group (1)		Group (2)*		Control
	Serovar Typhimurium	Serovar Enteritidis	Serovar Typhimurium	Serovar Enteritidis	Serovar Typhimurium and Enteritidis
0	0	0	0	0	0
2WPV	40	40	40	40	0
3WPV	40	40	80	80	0
Boostering					
2 WPB	80	80	160	80	0
4WPB	160	160	320	320	0
6WPB	320	320	640	640	0
8WPC	320	320	320	320	0
10WPC	320	320	320	320	0
12WPC	160	160	320	320	0
14WPC	160	160	160	160	0
16WPC	80	80	160	160	0
18WPC	80	80	80	80	0
20WPC	40	40	40	80	0
22WPC	20	20	20	40	0

Group (1): SPF layer chickens vaccinated with combined aluminium hydroxide gel vaccine; Group (2): SPF layer chickens vaccinated with combined montanide ISA71 vaccine; Control: Unvaccinated group; WPV: Weeks post vaccination; WPB: Weeks post boostering; WPC: weeks post challenge; * Significant at P < 0.05; The antibody titer in MAT was expressed as Geometric Mean Titer (GMT)

Table 2. Measurement of antibody against *Salmonella* Typhimurium and Enteritidis in sera of vaccinated and unvaccinated layer chickens using ELISA

Weeks post vaccination	Group (1)		Group (2)*		Control
	Serovar Typhimurium	Serovar Enteritidis	Serovar Typhimurium	Serovar Enteritidis	Serovar Typhimurium and Enteritidis
0	93	100	93	100	100
2WPV	975	850	1530	1443	112
3WPV	1453	1413	2553	2721	111
Boostering					
2WPB	2189	2189	3517	3617	128
4WPB	2344	2544	3782	3982	123
6WPB	2763	2791	4543	4484	130
8WPV	2675	2547	3925	3855	143
10WPC	2320	2250	3845	3745	135
12WPC	2230	2130	3667	3686	156
14WPC	1970	1940	3253	3354	122
16WPC	1515	1465	2180	2370	129
18WPC	1325	1298	2020	2120	125
20WPC	1250	1110	1890	1970	123
22WPC	1140	1020	1680	1730	128

Group (1): SPF layer chickens vaccinated with combined aluminium hydroxide gel vaccine; Group (2): SPF layer chickens vaccinated with combined montanide ISA71 vaccine; Control: Unvaccinated group;WPV: Weeks post vaccination; WPB: Weeks post boostering; WPC: weeks post challenge; * Significant at P < 0.05

Table 3. Geometric mean of Haem-agglutinating Titer against *Avibacterium paragallinarum* serovars A and C in sera of vaccinated layer chickens.

Weeks post vaccination	Group (1)		Group (2)*		Control
	Serovar A	Serovar C	Serovar A	Serovar C	Serovar A and C
0	0	0	0	0	0
2WPV	40.32	40.31	28.50	32	0
3 WPV	40.23	40.31	35.78	43.11	0
Boostering					
2WPB	40.8	57.01	57.01	80.63	0
4WPB	50.79	57.01	71.83	90.50	0
6WPB	57.01	71.83	101.59	114.04	0
8WPB	57.01	71.83	101.59	114.04	0
10WPB	57.01	71.83	101.59	114.04	0
12WPB	50.79	57.01	90.50	101.59	0
14WPB	40.34	57.01	90.50	101.59	0
16WPB	40.87	50.79	90.5	101.59	0
18WPB	35.91	50.79	80.63	90.50	0
20WPB	28.50	40.31	80.63	90.50	0
22WPB	28.50	40.31	80.63	90.50	0

Group (1): SPF layer chickens vaccinated with combined aluminium hydroxide gel vaccine; Group (2): SPF layer chickens vaccinated with combined montanide ISA71 vaccine; Control: Unvaccinated group; WPV: Weeks Post Vaccination; WPB: Weeks Post Boostering; *Significant at $P < 0.05$

Table 4. ELISA results (S/P ratio) of vaccinated and unvaccinated layer chickens against *Avibacterium paragallinarum* serovars A and C.

Weeks post vaccination	Group (1)		Group (2)*		Control
	Serovar A	Serovar C	Serovar A	Serovar C	Serovar A and C
0	0.031	0.023	0.044	0.021	0.002
2WPV	1.304	1.474	1.292	1.344	0.011
3 WPV	1.388	1.476	1.549	1.598	0.233
Boostering					
2WPB	1.454	1.455	1.936	1.942	0.043
4WPB	1.474	1.519	1.975	1.936	0.022
6WPB	2.190	2.274	2.095	2.011	0.056
8WPB	2.130	2.235	2.164	2.274	0.044
10WPB	2.091	2.064	2.278	2.274	0.070
12WPB	1.782	1.940	2.087	2.164	0.033
14WPB	1.566	1.885	2.011	2.036	0.056
16WPB	1.431	1.850	1.975	2.011	0.099
18WPB	1.519	1.770	1.907	1.942	0.043
20WPB	1.472	1.549	1.869	1.936	0.065
22WPB	1.199	1.454	1.848	1.907	0.023

Group (1): SPF layer chickens vaccinated with combined aluminium hydroxide gel vaccine; Group (2): SPF layer chickens vaccinated with combined montanide ISA71 vaccine; Control: Unvaccinated group; WPV: Weeks Post Vaccination; WPB: Weeks Post Boostering; *Significant at $P < 0.05$

Results of Challenge test

As shown in tables 5 and 6, the protection rates in chickens vaccinated either with combined aluminium hydroxide gel vaccine or combined montanide ISA71 vaccine were 80% and 85% for *Salmonella* organisms. On the other hand it was (80%, 90% and 70%) for combined aluminium hydroxide gel vaccine and (90%, 100% and 90%) for combined montanide ISA71 vaccine against *Avibacterium paragallinarum* serovars A, B, and C. Meanwhile, the protection rate was 15% against *Salmonella* Typhimurium and *Salmonella* Enteritidis and 0% against infectious coryza among the unvaccinated chicken group.

Fecal shedding of *Salmonella* Typhimurium and *Salmonella* Enteritidis from challenged chickens

Fecal shedding of *Salmonella* Typhimurium and *Salmonella* Enteritidis as shown in table (7), from chickens vaccinated with either combined aluminium hydroxide gel vaccine or combined montanide ISA71 vaccine in the 1st, 2nd and 3rd weeks post challenge were (25%, 12.5% and 12.5%) and (22.22%, 11.11% and 0%) respectively while in the 4th week the fecal shedding disappeared. Regarding the control unvaccinated birds the fecal shedding were 66.66%, 66.66%, 33.33% and 33.33% in the 1st, 2nd, 3rd and 4th weeks post challenge respectively.

Table 5. Protective Efficacy of combined vaccine against salmonellosis in SPF chickens challenged with virulent strains

VACCINE	Serovar	No. of inoculated chickens#	Survived chickens	Protection %
Group (1)	Typhimurium	10	8	80
	Enteritidis	10	8	80
Group (2)	Typhimurium	10	8	80
	Enteritidis	10	9	90
Control	Typhimurium	10	1	10
	Enteritidis	10	2	20

*Protection % = (Survival birds/ total number of birds) x 100; Group (1): SPF layer chickens vaccinated with combined aluminium hydroxide gel vaccine; Group (2): SPF layer chickens vaccinated with combined montanide ISA71 vaccine;# Challenge with virulent *Salmonella* Typhimurium and *Salmonella* Enteritidis; Control: Unvaccinated group.

Table 6. Protective Efficacy of combined vaccine against infectious coryza in SPF chickens challenged by *Avibacterium paragallinarum* serovars A, B, and C

VACCINE	serovar	No. of inoculated chickens#	Survived chickens	Protection %
Group (1)	A	10	8	80
	B	10	9	90
	C	10	7	70
Group (2)	A	10	9	90
	B	10	10	100
	C	10	9	90
Control	A	10	0	0
	B	10	0	0
	C	10	0	0

Group (1): SPF layer chickens vaccinated with combined aluminium hydroxide gel vaccine; Group (2): SPF layer chickens vaccinated with combined montanide ISA71 vaccine; # Challenge with virulent *Avibacterium paragallinarum* serovars A, B, and C; Control: Unvaccinated group.

Table 7. Results of fecal shedding of *Salmonella* Typhimurium and *Salmonella* Enteritidis from layer chickens after challenge

Chicken groups	No. of birds positive for isolation / total No. of living birds			
	1st week	2nd week	3rd week	4th week
Group (1)	2/8 (25%)	1/8 (12.5%)	1/8 (12.5%)	0/8 (0%)
Group (2)	2/9 (22.22%)	1/9 (11.11%)	0/9 (0%)	0/9 (0%)
Control	2/3 (66.66%)	2/3 (66.66%)	1/3 (33.33%)	1/3 (33.33%)

Group (1): SPF layer chickens vaccinated with combined aluminium hydroxide gel vaccine; Group (2): SPF layer chickens vaccinated with combined montanide ISA71 vaccine; Control: Unvaccinated group.

DISCUSSION

Avian salmonellosis is a large group of acute and chronic diseases of poultry caused by any one or more member of genus *Salmonella*. However, particular *Salmonella* Enteritidis is the most prevalent one followed by *Salmonella* Typhimurium (Capita et al., 2003).

Infectious coryza is an acute respiratory disease of chickens caused by the bacterium *Avibacterium paragallinarum.* The greatest economic losses associated with infectious coryza results from poor growth performance in growing birds and marked reduction (10-40%) in egg production in layers (Blackall and Matsumoto, 2003).

Charoenvisal et al. (2017) examined efficacy of four commercial Infectious Coryza vaccines available in Thailand for protection rate against Thai field isolates serovar A, B, and C. The study revealed that the protection rate of Infectious Coryza vaccines depended on the strains isolated from each country**.**

So in this study combined vaccines have the advantage of protection against more than one disease at the same time, beside, reducing vaccination expenses, number of vaccination performed and saving time. The efficacy of a prepared combined vaccine against salmonellosis and infectious coryza using two different adjuvants; aluminium hydroxide gel and montanide ISA 71 was monitored in sera of vaccinated chickens using HI, MAT and ELISA. It was clear that antibody titers in sera of chickens for all tests were paralleled to each other in starting and increasing titer and also after boostering as illustrated in tables 1, 2, 3, 4 and 5 which agree with that obtained by Akeila et al. (2014). Who evaluated a combined vaccine against *A. paragallinarum* and *S.* Enteritidis and found that antibody titers reached the maximum levels at the 6th WPV in the vaccinated groups.

With serovar B vaccines, a HI test was not done as it is based on a hyaluronidase-treated antigen and formaldehyde-treated RBC and gave only very low HI titers following vaccination (as compared with serovar A or C vaccines) but the vaccinated birds were significantly protected against homologous challenge, These results correlate with other studies done by Yamaguchi et al. (1991).

The protection rates against *Salmonella* Typhimurium and Enteritidis as measured by challenge test were 80% and 85% in chickens vaccinated with combined aluminium hydroxide gel vaccine and combined montanide ISA71 vaccine are respectively, as shown in table 5.

Also the protection rates against *Avibacterium paragallinarum* serovars A, B, and C were 80%, 90% and 70% in chickens vaccinated with combined aluminium hydroxide gel vaccine and were 90%, 100% and 90% of the montanide ISA71 vaccine respectively (Table 6).

Meanwhile, the protection rate was 15% against *Salmonella* Typhimurium and Enteritidis and 0% against infectious coryza among the unvaccinated chicken group and these results agreed with by Akeila et al. (2014) who reported 73.3% and 93.3% protection rate against *S.* Enteritidis and *A. paragallinarum*, respectively in a combined vaccine containing both organisms.

The fecal shedding of *Salmonella* Typhimurium and Enteritidis in the 1st, 2nd and 3rd weeks post challenge in chickens vaccinated with combined aluminium hydroxide gel vaccine was 25%, 12.5% and 12.5% , while it was 22.22%, 11.11% and 0% for those vaccinated only with montanide ISA71 vaccine, respectively. The fecal shedding disappeared in the 4th week.

Regarding the control unvaccinated birds the fecal shedding were 66.66%, 66.66%, 33.33% and 33.33% in the 1st, 2nd, 3rd and 4th weeks post challenge and these result agreed with Nourhan et al. (2015) who found that fecal shedding of *Salmonella* organisms in vaccinated group of chickens with *S.* Kentucky reached 8.33% while the unvaccinated control group at 3 week post challenge revealed fecal shedding of 25 %. No shedding was detected at the fourth week post challenge in the vaccinated group, while there was 16.6% shedding in control unvaccinated group.

So, the SPF layer chickens vaccinated with combined vaccine against salmonellosis and infectious coryza adjuvanted with montanide ISA71 gave high immune response and protection which is capable of improving vaccine efficacy via the induction of a strong and long lasting immunity. Also it is an excellent adjuvant stimulating humoral and cellular responses. This product is recommended for producing a potent vaccine able to protect layer chickens against salmonellosis and infectious coryza.

CONCLUSION

From the above results it could be concluded that producing a vaccine from locally isolated *Salmonella* and *Avibacterium* (Haemophilus) *paragallinarum* strains adjuvanted with montanide ISA71 is recommended to aid in controlling avian salmonellosis and infectious coryza at the same time.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors participated in making the design, performing the experiment, analyses of the data, and writing the paper.

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