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# Adjuvant effects of novel water/oil emulsion formulations on immune responses against infectious bronchitis (IB) vaccine in mice

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ABSTRACT

Vaccines have long made use of adjuvants to boost the immune response of the body and reduce the amount of vaccine needed as well as the expense of producing the vaccine. Many vaccine adjuvants are in development, but their application in veterinary vaccinations is restricted due to their lack of efficacy or undesirable side effects. For this reason, it is essential to develop novel adjuvants. To address the issue that the currently available infectious bronchitis (IB) vaccine often fails to produce sufficient immune responses, Coral Biotechnology tested two of their newly developed water-in-oil (W/O) type emulsion adjuvants (Coralvac RZ 528 and Coralvac RZ 506) in the IB vaccine. These adjuvants were tested in a mouse model to determine whether it worked with an inactive IBV H120 vaccine. Vaccine formulations were prepared by combining a virus concentration of $1 \times 10^6$ $EID_{50}$/0.1 ml with an emulsion of the W/O type in a specific ratio. Once the formulations were ready, it was injected intramuscularly as a single dosage, and the mice were monitored for 21 days afterwards. The results showed that *anti*-IB antibody titer (IgG and IgG1), $CD3^+$ $CD8^+$ T cell responses as well as IFN- γ cytokine production, and splenocyte proliferation were all considerably higher in the IBV H120 with Coralvac RZ 528 and IBV H120 with Coralvac RZ 506 formulation groups than in the viral control group. According to our findings, the humoral and cellular immune responses of mice were significantly enhanced by these novel vaccine adjuvants. Thus, our results provide evidence that the W/O type emulsion adjuvants developed by Coral Biotechnology may be a useful adjuvant in IBV vaccines.

## 1. Introduction

*Coronaviridae* share a large, single-stranded, positive-sense RNA genome with other members of the family. The *Letovirinae* and the Orthocoronavirinae are two sub-families that have been described within this family. Four genera have been recognized in the latter, and the infectious bronchitis virus (IBV) serves as the type species in the *Gammacoronavirus* genus [1]. The extremely contagious IBV is the primary cause of disease in chickens of all ages and kinds. Disease of the urogenital tract is a common complication of IBV infection, despite the fact that the virus often enters the body through the upper respiratory tract. The primary concern in laying and breeding birds is the impact on egg laying performance [2], and there is a wealth of evidence from both field observations and experimental studies [3–5] to support the idea that the virus has an effect on egg production in terms of both quantity and quality.

Vaccination is still the most effective method of preventing IBV infection. Nonetheless, the persistent genetic, antigenic, and tissue tropism alterations of the prevalent IBV have resulted in ongoing instances of vaccination ineffectiveness [6]. Infectious bronchitis (IB) in poultry has been largely managed through the use of live attenuated and inactivated vaccines containing the most epidemiologically significant strains of the IBV. Poor cross-protective immune responses established in the respiratory mucosa, the virus's portal of entry, are attributed to reports of insufficient protection against developing novel variations despite a steady increase in new vaccines [7–9]. High mutation and recombination rates driven by selective pressures exerted by the partial immune status of avian hosts are also responsible for the persistent development of IBV variants, even in vaccinated flocks [10,11].

Adjuvants are described as a heterogeneous group of components

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that enhance the immunogenicity of vaccines through their co-administration with vaccine antigens [12]. Adjuvants encompass a broad spectrum of substances, including synthesized small particles, intricate natural extracts, and particulate materials [13]. The use of adjuvants in vaccines has been extensively employed to enhance the efficacy of immunization. The incorporation of adjuvants in vaccines serves to augment the effectiveness of adaptive immunity through the stimulation of innate immune cells [14]. The basic concept is the function of adjuvants in promoting the generation of antigen-presentation signals (signal 1) and co-stimulatory signals (signal 2) by activating antigen-presenting cells (APCs). The signals for antigen presentation consist of antigen peptides that are bound to major histocompatibility complexes (MHC) and are subsequently displayed on the surface of APCs following the uptake and processing of antigens. These are co-stimulatory signals, which are made up of molecules like CD40, CD80, and CD86 that are found on the surface of APCs. Additionally, secreted inflammatory cytokines, including IL-6, IL-10, IL-12, and TNF-α, also contribute to co-stimulatory signaling. The production of these two signals could greatly increase the activation of naive T cells, leading to a stronger adaptive immune response [15]. Immunostimulants, including pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and chemically manufactured small-molecule agonists of Toll-like receptors (TLRs), have the potential to induce the production of signal 1 and signal 2 by APCs [12,16,17].

Injectable oil emulsion adjuvanted inactivated vaccines against IBV are frequently used in layer and breeder chickens. However, these vaccines are rarely given to broilers [18]. The veterinary field, and the poultry business in particular, have made extensive use of emulsion as an adjuvant for many years. Since the antigen is always found in the aqueous phase, emulsions can be classified as either water-in-oil (W/O) emulsions, in which tiny water droplets are suspended in a continuous oil phase, or oil-in-water (O/W) emulsions, in which tiny oil droplets are suspended in an aqueous phase [19]. As it works, this adjuvant develops a depot that enables the gradual and steady release of antigen [20]. High levels of systemic IBV antibodies are shown during the vaccine response, but T cytotoxic cell activation and local humoral responses are not well elicited, leading to insufficient protection against IBV infection [21,22]. Conversely, inactivated vaccines are thought to be safe because they prevent the virus from replicating, triggering reversion of virulence or gene recombination, or reactivating residual pathogenicity. Thus, oil emulsion adjuvants are necessary, particularly for those aiming to boost immunogenic responses. New adjuvants and/or carrier systems for IBV inactivated vaccines may be useful alternatives to the conventional adjuvants, which are linked to increased toxicity, decreased immunogenicity, and undesirable responses [23,24].

Inactivating viruses makes them non-infectious for downstream use. Before binary ethylenimine (BEI) was developed as a virucide, formaldehyde or formalin was used to inactivate the food and mouth disease virus (FMDV). However, this method often failed or changed how the virus was recognized by the body. Aziridine and its derivatives, acetyl ethylenimine (AEI) and binary ethylenimine (BEI), have been used to inactivate viruses since the 1950s. Due to its stability, improved inactivation kinetics, less residual toxicity, and capacity to preserve FMDV antigenicity and immunogenicity, BEI became the principal inactivation agent for FMDV vaccine formulation. As a virucide, BEI inactivates other viruses. The inactivation benefits of BEI have been thoroughly investigated [25]. The purpose of this study was to assess, for the first time in Turkey, the efficacy of an intramuscular injection of a vaccine containing inactivated IBV H120 combined with Coral Biotechnology's newly developed W/O emulsion adjuvants.

## 2. Materials and methods

### 2.1. Adjuvants

In this study, W/O type emulsion adjuvants (Coralvac RZ 528, Coralvac RZ 506) developed by Coral Biotechnology were used. The adjuvants are shown in the table below (see Table 1).

### 2.2. Viruses

The Avian Infectious Bronchitis Virus (IBV) H120 strain used in the study was supplied by Dr. Fethiye ÇÖVEN from Izmir Bornova Veterinary Control Institute. The production of IBV H120 viruses was carried out in specific pathogen-free embryonated chicken eggs (SPF-ECEs). Since the virus does not have zoonotic properties, it is suitable to work in our laboratory under BSL conditions.

### 2.3. Experimental mice

*In vivo* studies were carried out with the approval of the Ege University Animal Experiments Local Ethics Committee (EÜHADYEK) numbered 2022–013. Swiss albino experimental mice obtained from Izmir Veterinary Control Research were sexually mature, 4–6 weeks old, and weighted between 25 and 34 g.

The animals (20) to be used in the study were quarantined for 7 days at the Ege University Laboratory Animals Application and Research Center (EGEHAYMER) and prepared for the experiment. The animals used in the study were kept in filter cages with individual ventilation. For the experimental environment, laboratory temperatures of 20–24 °C and humidity levels of 45–65 % were provided. A 12-h dark and 12-h light period was applied in the laboratory. The experimental animals were provided with a diet consisting of regular laboratory feed and access to water. The provision of food and water was administered on a daily basis.

### 2.4. Embryonated chicken eggs

Embryonated chicken eggs have been used for the propagation of viruses, which is a practical, inexpensive, and fast method [26]. The SPF-ECEs obtained from Bornova Veterinary Control Research Institute were incubated for 10 days in an incubator at 37 °C. Because embryonic development takes 21 days before hatching and the formation of capillaries continues for up to 11 days, the processes on the eggs take about 9–11 days [27]. The embryo viability of SPF eggs ready for inoculation was examined with the help of a light source. By observing vessel formation and embryo viability, the dead SPF-ECEs were separated and disposed of in accordance with the waste rules. The SPF-ECEs selected for the study were marked with an 'x' form with a pencil about 1–2 mm above the air sac and from the parts that do not contain dense vessels.

### 2.5. Determination of the incubation period for the production of virus strains

The production hour giving the highest titer for the IBV H120 strain was determined by a preliminary trial. For this purpose, 10-day SPF-ECEs were used and divided into 3 different groups, each containing 2 eggs. An incubation period of 24 h was chosen for the first group, 48 h for the second group, and 72 h for the third group. The shells of the 10-day-old SPF ECEs, for which embryo viability controls were made, were sterilized under sterile conditions. The air sac and inoculation points were determined, and a hole was drilled where the eggs would be inoculated. The IBV H120 strain was inoculated with an insulin syringe of 0.1 ml/egg volume at a 45° angle to the chorioallantoic space of all SPF-ECEs.

The holes are sealed with paraffin. With the air sacs on top, SPF-ECEs were incubated in an incubator at 37 °C with 55 % humidity. Daily

**Table 1**
Water-in- oil (W/O) type emulsion adjuvants to be used in the studies.

| Trade name | Emulsion type | Application | Oil type | Immunostimulant | Antigen/Adjuvant (%V/V) |
|---|---|---|---|---|---|
| **Coralvac RZ 528** | W/O | Chicken (IBV) | Non-metabolizable oils and emulsifiers | Not included | 30/70 |
| **Coralvac RZ 506** | W/O | Chicken (IBV) | Non-metabolizable oils and emulsifiers | Not included | 40/60 |

viability checks of SPF-ECEs were made and dead eggs were recorded. At the end of the incubation period, each group was kept at 4 °C overnight. Then, SPF-ECEs were taken into the biosafety cabinet under sterile conditions, and the air sacs were opened, and chorioallantoic fluid (CAF) was collected. The titers of IBV H120 viruses were determined by PCR testing following RNA isolation.

### 2.6. Inoculation, propagation, and harvesting of the IBV virus strain

The SPF-ECEs were incubated in an incubator with approximately 55 % humidity at 37 °C for 9–11 days. At the end of the incubation, the viability of the embryo was checked under light in a dark environment. The air sac and the inoculation point were determined by drawing with a pencil. The shells of SPF-ECEs are sterilized with tincture iodine in the biosafety cabinet. All SPF-ECEs were inoculated with the H120 strain of IBV using an insulin syringe angled at 45° and a volume of 0.1 ml/egg. The holes are sealed with paraffin. With the air sacs on top, SPF-ECEs were incubated in an incubator at 37 °C with 55 % humidity for the optimum incubation time (35 h). The viability of the embryos was checked daily, and the dead eggs were noted.

Following the 35-h incubation period, the SPF-ECEs were stored at 4 °C overnight. Next, the CAF of the SPF-ECEs were collected into sterile 50 ml falcon tubes under sterile conditions. Embryo viability was also observed. One dead egg was identified among the SPF-ECEs. The collected CAF from the whole eggs was centrifuged for 5 min at 4000 rpm in a room temperature condition to separate the unwanted particles and erythrocytes. The supernatant was collected in a sterile 1-L screw cap (schott) bottle. The IBV H120 virus titer for mass production was determined by RT PCR. The remaining CAF was stored at +4 °C for the purification step. As a result of the studies, all egg wastes were disposed of in accordance with the rules.

### 2.7. Preliminary study for determination of inactivation concentration

The application of BEI was employed as a means of viral inactivation [28]. A volume of 5 ml of freshly prepared BEI was utilized. In order to make a 5 ml solution of BEI, a quantity of 35 mg (0.175 M) of NaOH was dissolved in ultrapure water. The resulting solution was then maintained at a temperature of 37 °C. A 1 % concentration of beta-naphthol violet, a pH indicator, was prepared and thereafter added into the solution with a volume of 2.5 μl. Subsequently, a quantity of 0.1025 g (0.1 M) of 2-bromoethylammonium bromide was added to the solution. The anticipated transition in color from purple to orange was observed. Following the observation of a change in color, the inactivation investigation employed the use of BEI. In order to evaluate the effectiveness of the BEI formulation, various concentrations of BEI (1 %, 3 %, and 5 %) were prepared and examined for their ability to inactivate the H120 virus. Following a sequential addition of each BEI formulation to 5 ml of IBV H120 virus in sterile vials, the vials were subjected to overnight incubation at room temperature on a shaker operating at 150 rpm. In order to ensure successful inactivation, each experimental group was inoculated into two separate SPF-ECEs with a volume of 0.1 ml, and incubated for a duration of 72 h. The collection of CAF fluid from SPF-ECEs was carried out following inoculation, and the efficacy of inactivation in the samples was assessed using RT PCR analysis.

### 2.8. Large-scale virus production and virus inactivation in SPF-ECEs

Large-scale virus production was carried out using 55 SPF-ECEs. A 3 % BEI concentration (14.53 ml) was then calculated for CAF containing 470 ml of IBV H120. The required volume of freshly prepared BEI was then added to the virus suspension. It was incubated overnight with stirring at 250 rpm at room temperature. The inactivated virus was inoculated into 2 SPF-ECEs and incubated for 72 h for inactivation control. This process was continued in three passages in the SPF-ECEs to demonstrate the success of inactivation. The PCR analysis was performed on CAF samples collected from SPF-ECEs after the end of the passages.

### 2.9. Determination of median $LD_{50}$ for IBV H120

The median $LD_{50}$ value of IBV H120 was calculated according to the Reed and Muench method [29]. The virus was diluted in PBS by a 10-fold serial dilution between $10^{-1}$ and $10^{-13}$. Each dilution was inoculated into the chorioallantoic fluid of 3 eggs in a volume of 100 μl. After 35 h of incubation, the viability of the embryos was checked and calculated using the Reed and Muench method.

### 2.10. Determination of median $EID_{50}$ for IBV H120

The median $EID_{50}$ value of IBV H120 was calculated according to the Reed and Muench method [29]. The virus was diluted in PBS by a 10-fold serial dilution between $10^{-1}$ and $10^{-13}$, and each dilution was inoculated into the chorioallantoic fluid of 3 eggs. After 35 h of incubation, CAF was collected, and PCR analysis was performed for each egg sample using the High Pure Viral Nucleic Acid Kit (Roche, USA).

### 2.11. Purification of IBV virus

After inactivation, CAF was first clarified by passing through 80 μm and 45 μm membrane filters at room temperature. A commercial ultrafiltration system (Sartocon Slice 200 Holder) and polyethersulfone (PES) cassettes with a 100 kDa cut-off value were used for purification. Sterile washing solutions (0.5 M NaOH) were passed through the cassettes before use. A peristaltic pump was used to provide the required (approximately 2 bar) membrane pressure. The virus suspension was concentrated approximately 9 times. The sample was stored at +4 °C.

### 2.12. Immunological studies

The immunization processes of the mice were carried out at the "Ege University Laboratory Animals Application and Research Center". Immunization studies were started on July 26, 2022, ended on August 16, 2022, and thus the 21-day immunization process was followed. During immunization studies, prepared virus and adjuvant formulations were administered to male/female Swiss albino mice aged 4–6 weeks and 25–34 g in weight as an intramuscular injection of 1 dose. In order to conduct the experiment, 20 Swiss albino mice were separated into groups, and each group received an injection of a different vaccine formulation. All injection procedures were performed with sterile insulin needles. In the experimental group, 0.1 ml intramuscular injection was made into the hind leg muscle of Swiss albino mice. Mice were administered intramuscular injections of vaccine on day 0, and on the 21st day, the mice were sacrificed and their blood samples and spleens were collected. Blood samples were collected in EDTA blood collection tubes, and the experimental groups were labeled. Spleen samples collected from mice were collected in falcon tubes containing 10 % sterile PBS (Phosphate Buffered Salt Solution) and stored at −20 °C after

labeling the experimental groups. The groups are shown in the table below (see Table 2).

### 2.13. Preparation of adjuvant based inactive IBV H120 vaccine formulations

In parallel with the studies in the literature, each dose of the vaccine is prepared with virus at a concentration of $1 \times 10^6$ $EID_{50}$/0.1 ml for IBV and adjuvant (W/O) type emulsion [30,31]. After calculating the virus in the experimental groups containing the oil-based emulsion as adjuvant, the volumes were calculated as 30:70 ratio for (Coralvac RZ 528) adjuvant and 40:60 ratio for (Coralvac RZ 506) adjuvant, according to IBV H120 $1 \times 10^6$ $EID_{50}$ was prepared in a volume of 1.5 ml. For Coralvac RZ 528 adjuvant, 15 ml formulation was prepared with 10.5 ml of emulsion content and 4.5 ml of virus content (1.5 ml of virus stocks and 3 ml of 0.9 % sterile physiological saline (NaCl). For Coralvac RZ 506 adjuvant, 15 ml of formulation was prepared with 9 ml of emulsion content and 6 ml of virus content (1.5 ml of virus stocks and 4.5 ml of sterile 0.9 % NaCl). Two adjuvants were passed through a sterile 0.22 μm filter before use. The prepared formulations were homogenized on ice in a homogenizer (IKA, Germany) at 15,000 rpm for 10 min and stored in a refrigerator at +4 °C for 24 h.

For the IBV positive control group formulation, the volume required to be withdrawn from the virus stocks tasked to $1 \times 10^6$ $EID_{50}$/ml was determined as 100 μl. For injection to 5 Swiss albino mice used as the IBV virus control group, 2000 μl formulation was prepared with 200 μl virus content and 1800 μl sterile 0.9 % NaCl. For the 0.9 % NaCl control group, which was prepared as the negative control group, 2000 μl of sterile physiological saline solution was used.

### 2.14. Physical stability test

To evaluate the stability of W/O emulsion formulations, all antigen-adjuvant formulations produced for use in IBV vaccines were incubated at two different storage temperatures, 25 °C and +4 °C, respectively. This experimental setup is designed so that formulations can be tested at any temperature. Each antigen-adjuvant formulation prepared in our laboratory was labeled, transferred to a 1 ml sterile Eppendorf tube, and incubated at 25 °C. The remainder of the antigen-adjuvant formulations were incubated in a refrigerator at +4 °C. These procedures were carried out in a class 2 biosafety cabinet. It was subsequently examined for stability at 7 days, two months, and six months. As a function of storage time and stability parameters, all formulations were visually evaluated for phase separation and agglomeration.

### 2.15. Detection of IBV (H120) specific antibody response by indirect ELISA

The level of IBV-specific antibodies (IgG, IgG1, and IgG2a) in mouse serum was determined using an indirect ELISA established in our laboratory. There is cross-reactivity between different serotypes in IBV infection, so only the coated 96-well plate from the IBV ELISA CK119 kit (BioChek, UK) was used and labeled as part of the study. After that, diluted serum (1:500) at an amount of 100 μl/well was added to the wells, and all was incubated for 30 min at 22–27 °C. After 30 min, the plate taken from the incubator was washed 4 times with washing solutions (eBioscience) and discarded. Then, to the appropriate areas of the plate, 100 μl/well of HRP-conjugated goat anti-mouse antibodies (IgG, IgG1, and IgG2a) were added at a dilution of 1:8000 (Santa Cruz Biotechnology, USA) and incubated for 30 min at 22–27 °C. After 30 min, the plate was washed 4 times. After washing, a 100 μl/well solution of 3,3,5,5′-tetramethylbenzidine substrate (TMB) was employed to induce a color shift (Elabscience, USA). Color development was carried out in the dark at 22–27 °C for 15–30 min. Finally, 50 μl of stopping solution (2 M $H_2SO_4$) was added to terminate the reaction (eBioscience TM, USA). The 450 nm optical density values were measured with a Multiskan Sky spectrophotometer, and the findings were reported in SkanIt Software 6.0.2.

**Table 2**
Vaccination groups.

| Number of groups | Number of mice | Vaccinated and unvaccinated control groups |
|---|---|---|
| **1** | 5 | Unvaccinated control (−) |
| **2** | 5 | Inactivated IBV virus control (+) |
| **3** | 5 | Inactivated IBV H120 vaccine + Coralvac RZ 528 adjuvant |
| **4** | 5 | Inactivated IBV H120 vaccine + Coralvac RZ 506 adjuvant |

### 2.16. Analysis of cytokines using ELISA

For the aim of cytokine analysis, serum samples were collected from each group and quantified using an ELISA kit according to the manufacturer's instructions to measure levels of interferon-γ (IFN-γ) (Finetest, China, Catalogue No.: EM0093), interleukin-4 (IL-4) (Finetest, China, Catalogue No.: EM0119), and interleukin 1-β (IL 1-β) (Finetest, China, Catalogue No.: EM0109).

### 2.17. Splenocyte proliferation test

To perform the splenocyte proliferation test, a concentration of $5 \times 10^7$ cells/ml was seeded into the 96 well-plates at a volume of 100 μl/well [32,33]. In order to stimulate cells, LPS (final concentration: 10 μg/ml) for the positive control [34], and IBV H120 at $1 \times 10^3$ $EID_{50}$/ml concentration for the tested groups were added and incubated at 37 °C in a humidified 5 % $CO_2$ incubator. After 72-h incubation, the MTT test [35] was performed and absorbance at 570 nm was measured by a spectrophotometer (Multiskan Sky, USA). All wells were examined by inverted microscope at 0 and 72-h.

The following formula was used to calculate the stimulation index (SI).

$$Stimulation\ Index = \frac{OD\ of\ stimulated\ cells - OD\ of\ unstimulated\ cells}{OD\ of\ unstimulated\ cells}$$

### 2.18. Flow cytometric analysis of the immune system

The spleens of groups of mice sacrificed by cervical dislocation under ketamine (100 μl/mouse) anesthesia were collected in a pool. Spleens suspended with RPMI-1640 medium (Gibco, USA) in a single cell were cultivated at a $5 \times 10^5$ cells/ml initial concentration in 6-well microplates. An additional $1 \times 10^3$ $EID_{50}$/ml IBV (H120) was applied to the wells and incubated for 72 h at +37 °C in an incubator containing 5 % $CO_2$.

After incubation, the cells were collected in tubes and treated with 50 μl anti-mouse CD3, 50 μl anti-mouse CD8, 100 μl fixation/permeabilization solution and 50 μl IFN-γ antibodies (BD Biosciences, USA), respectively and incubated for 30 min at +4 °C separately. Between each step, cells were washed twice by adding 250 μl of PBS containing 3 % FBS and centrifuged at 2000 rpm for 5 min. After the final wash, the supernatant was removed and pellets resuspended by adding 300 μl of PBS (containing 3 % FBS) before being transported to the flow cytometer (BD Biosciences, USA) for analysis. The data obtained were analyzed using the BD Accuri C6 software.

### 2.19. Statistical evaluation

Statistical analyses in the study were conducted using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). A statistical significance control was conducted at a significance level of $p < 0.05$, comparing the control group to the treatment groups. The groups were

compared using one-way analysis of variance (ANOVA) and t-tests. The statistical significance levels were denoted as follows: n. s., indicating not significant ($P > 0.05$); *, representing a significance level of $P < 0.05$; **, indicating a significance level of $P < 0.01$; ***, representing a significance level of $P < 0.001$; and ****, indicating a significance level of $P < 0.0001$.

## 3. Results

### 3.1. Results of RNA isolation after determination of incubation time for IBV virus production

A preliminary trial was conducted to find the production hour resulting in maximum titer for strain IBV H120. The titers of IBV H120 viruses after chorioallantoic fluid collection were determined by RT PCR testing following RNA isolation (see Table 3). As a result of PCR, it was determined that the best incubation period for IBV H120 production was around 35 h.

### 3.2. RNA isolation results after inactivation concentration determined in preliminary study

As a result of inoculation, CAF fluid from SPF ECEs was collected, and the inactivation success of the samples was evaluated by PCR test. Table 4 shows the RT PCR results for different BEI concentrations (see Table 4). As a result of PCR, it was determined that inactivation was successful at all concentrations. The concentration containing 3 % BEI, which is also commercially applied, was chosen for the inactivation of the H120 virus.

### 3.3. Control of virus inactivation after large-scale IBV virus production in SPF-ECEs

For inactivation control of the inactivated IBV virus, CAF was harvested from SPF-ECEs and submitted through a RT PCR test. Table 5 shows the PCR results for the 3 % BEI concentration (see Table 5).

### 3.4. Determination of median $LD_{50}$ for IBV H120

The virus was diluted in PBS by a 10-fold serial dilution between $10^{-1}$ and $10^{-13}$. Each dilution was inoculated into the chorioallantoic fluid of 3 eggs in a volume of 100 μl. After 35 h of incubation, the viability of the embryos was checked and calculated using the Reed and Muench method (Table S1). As a result of the calculation made according to the Reed and Muench method, the $LD_{50}$ value of IBV H120 was found to be $10^{5.75}$ $LD_{50}$/ml.

### 3.5. Determination of median $EID_{50}$ for IBV H120

As in $LD_{50}$, Reed and Muench method was used to obtain IBV titer. The embryo viabilities were observed (Fig. S1) and the samples determined as positive and negative according to the PCR results were recorded to calculate $EID_{50}$ titer (Table S2). As a result of the calculation made according to the Reed and Muench method, the $EID_{50}$ value of IBV H120 was found to be $10^{7.25}$ $EID_{50}$/ml.

**Table 3**
PCR results of optimum incubation time studies for IBV H120 production.

| Sample | Cycle Threshold (CT) |
|---|---|
| IBV H120 24 h | 33.44 |
| IBV H120 35 h | 18.08 |
| IBV H120 36 h | 21.29 |
| IBV H120 48 h | 33.93 |
| IBV H120 72 h | 37.00 |
| IBV H120 Positive Control | 29.04 |
| IBV H120 Negative Control | 0 |

**Table 4**
PCR results for IBV H120 inactivation against BEI concentrations.

| Sample | Cycle Threshold (CT) |
|---|---|
| BEI 1%- IBV H120 | 0 |
| BEI 3%- IBV H120 | 0 |
| BEI 5%- IBV H120 | 0 |
| IBV H120 Positive Control | 34.37 |
| IBV H120 Negative Control | 0 |

**Table 5**
PCR results for inactivation of large-scale production of IBV-H120.

| Sample | Cycle Threshold (CT) |
|---|---|
| BEI 3%- IBV H120 | 0 |
| IBV H120 Positive Control | 32.54 |
| IBV H120 Negative Control | 0 |

### 3.6. Purification of IBV virus

At the end of the purification step, the retentant (concentrated virus suspension) and permeat were collected to confirm the purification success using the RT PCR test. Table 6 shows the PCR results for the purification step (see Table 6). The titer of IBV in the retentant was higher than that in the permeat suspension because the retentant's cycle threshold value (18.21) was lower than that of the permeat suspension (31.44).

### 3.7. Stability of prepared oil emulsion adjuvant-antigen combinations

All the antigen-adjuvant formulations intended for use in vaccines (inactive IBV H120 + Coralvac RZ 528 adjuvant and inactive IBV H120 + Coralvac RZ 506 adjuvant) were incubated at two different temperatures for analysis of W/O emulsion stability. These temperatures are predetermined at 25 °C and +4 °C, respectively (Figs. S2 and S3).

No evidence of a change in physical state (phase separation) or agglomeration was detected in any of the formulations incubated at 25 °C and then visually examined, the only exception was the formulation of the inactive IBV H120 + Coralvac RZ 506 adjuvant which showed separation after 7 days of incubation. Visual inspection revealed no changes to any of the formulations after 2 months of incubation, with the exception of the IBV H120+ Coralvac RZ 506 adjuvant formulation, which showed a new change (increased separation) after 2 months of incubation. Two formulations were observed to have changed (phase separation) after 6 months of incubation at 25 °C (Fig. S2). Even after 1 week and 2 months of storage at 4 °C, it showed no signs of separation in any of the IBV formulations. When kept at +4 °C for 6 months, visual inspection revealed that all formulations had changed (phase separation) (Fig. S3). The results showed that +4 °C was the optimal storage temperature for at least two months to store two formulations.

### 3.8. Detection of specific antibody levels by indirect ELISA

To test the effect of water/oil (W/O) emulsion adjuvants on systemic humoral immune responses, total IgG, IgG1 and IgG2a antibodies against IBV were determined by indirect ELISA in serum samples taken 21 days after vaccination. Mice receiving adjuvanted inactive IBV H120 exhibited an immune response, as evidenced by higher absorbance

**Table 6**
PCR results after IBV H120 purification.

| Sample | Cycle Threshold (CT) |
|---|---|
| IBV H120 Before purification | 18.08 |
| IBV H120 Retentant (concentrated virus suspension) | 18.21 |
| IBV H120 Permeat | 31.44 |

values compared to the virus control group (see Fig. 1). A Th2-dominant immune response has been linked to IgG1 antibody. The experimental group treated with inactivated virus + Coralvac 528 adjuvant formulations had higher levels of IgG ($P < 0.001$) and IgG1 ($P < 0.0001$) immunoglobulin titers compared to the experimental control group treated with inactivated IBV H120 virus control alone. The combination of inactive virus + Coralvac 506 adjuvant resulted in a statistically significant ($P < 0.05$) increase in IgG1 immunoglobulin titers compared to the inactive IBV H120 virus control group.

### 3.9. Evaluation of cytokine responses in mice immunized with IBV inactivated vaccines

Serum levels of various cytokines (IFN-γ, IL-4, and IL-1β) were measured using ELISA to examine whether or not IBV H120 formulation vaccines influenced cytokine production in mice. IFN-γ is a representative Th1 cytokine that is also expressed by cytotoxic T lymphocytes, IL-4 is a Th2 cytokine, whereas IL 1-β is a macrophage cytokine. The levels of IFN-γ significantly increased in mice immunized with IBV + CV RZ 528 ($P < 0.05$), and CV RZ 506 ($P < 0.05$) vaccine groups when compared to mice vaccinated with the IBV viral control group (see Fig. 2), while the levels of IL-4, and IL 1-β were not significant in all adjuvanted vaccine groups compared to mice vaccinated with the IBV viral control group (see Figs. 3 and 4). These results suggest that newly developed oil emulsion adjuvants (Coralvac RZ 528 and Coralvac RZ 506) could promote the expression of IFN-γ cytokines in Th1 and cytotoxic T lymphocytes in mice immunized with newly manufactured IBV vaccines.

### 3.10. Splenocyte proliferation test in immunized mice

A splenocyte proliferation assay was used to evaluate the stimulating effects of different groups of vaccine formulations on immune cells. Mice were given ketamine intraperitoneally prior to euthanasia, and then their spleen cells were stimulated with viral and LPS mitogens, with proliferation level determined as a stimulation index (SI). Mouse splenocyte-seeded plates were examined under an inverted microscope zero hours after virus, medium, and LPS were added to assess cell proliferation after 72 h (Fig. S4). After 72 h of incubation, spleen cells were observed under the microscope at 10× magnification to determine if proliferation had occurred. Evidence for the presence and viability of cells was provided by the control group. Since the control group did not undergo antigen activation, it served as a negative control. Cells stimulated with LPS were used as standard for comparison (positive control), since LPS is also a mitogen for T cells [36]. After 72 h, clusters of T cells were seen under the microscope (Figs. S5, S6, S7, and S8). According to the findings of the study, IBV H120 groups given IBV + CV RZ 506 ($P < 0.0001$) and IBV + CV RZ 528 ($P < 0.0001$) formulations had the highest SI values. Compared with the virus control IBV groups, these two groups show significantly better proliferation (see Fig. 5). Based on these findings, formulations of (W/O) emulsion adjuvants (Coralvac RZ 528 and Coralvac RZ 506) developed by Coral Biotechnology have been proven to provide better immunization and have a greater potential for use against IBV infection.

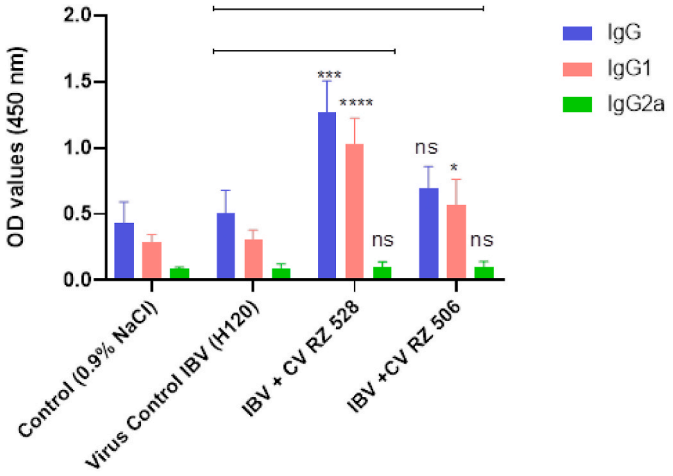


**Fig. 1.** Based on the mean absorbance values of the IBV H120 antibody responses measured at 450 nm, a bar graph identifies the various vaccination groups with positive standard errors.

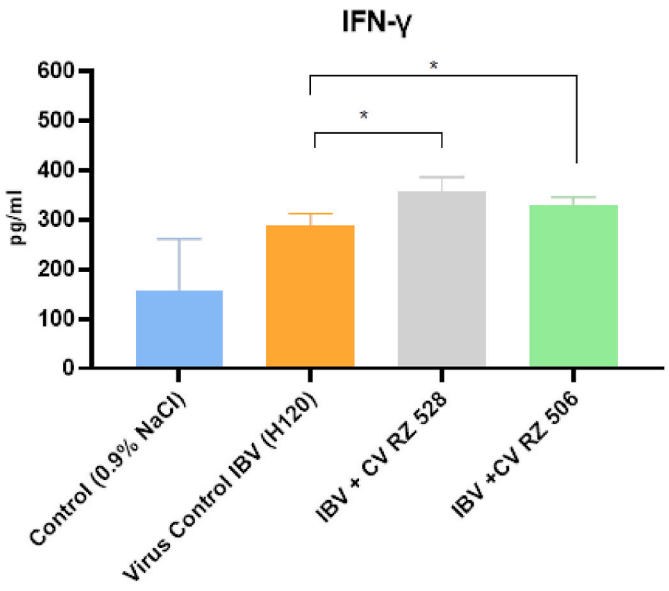


**Fig. 2.** The effects of an immunization on the levels of the IFN-γ cytokine in the serum of mice. Following a period of 21 days, serum samples from mice in each group were taken, and a quantitative ELISA kit was utilized to determine the levels of IFN-γ. The data reflect the mean ± SD of five mice from each group.

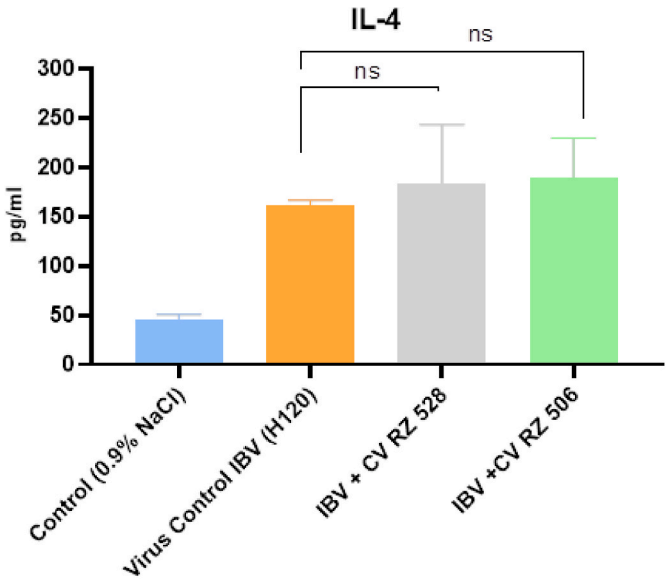


**Fig. 3.** The effects of an immunization on the levels of the IL-4 cytokine in the serum of mice. Following a period of 21 days, serum samples from mice in each group were taken, and a quantitative ELISA kit was utilized to determine the levels of IL-4. The data reflect the mean ± SD of five mice from each group.

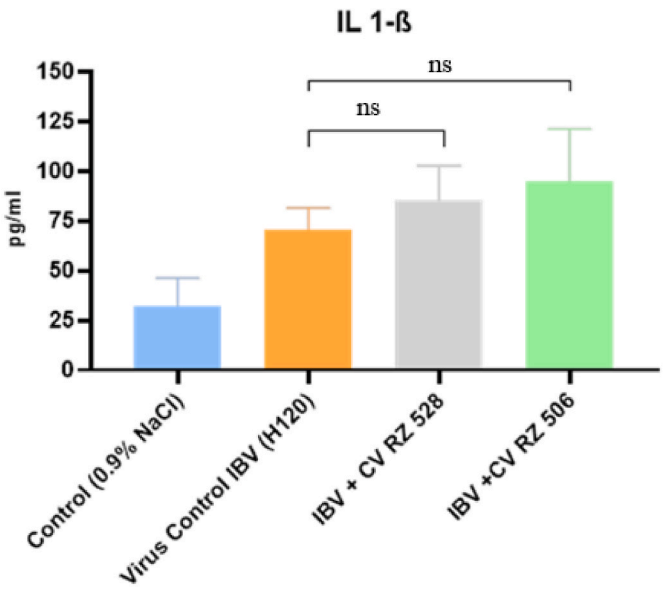


**Fig. 4.** The effects of an immunization on the levels of the IL 1-β cytokine in the serum of mice. Following a period of 21 days, serum samples from mice in each group were taken, and a quantitative ELISA kit was utilized to determine the levels of IL 1-β. The data reflect the mean ± SD of five mice from each group.

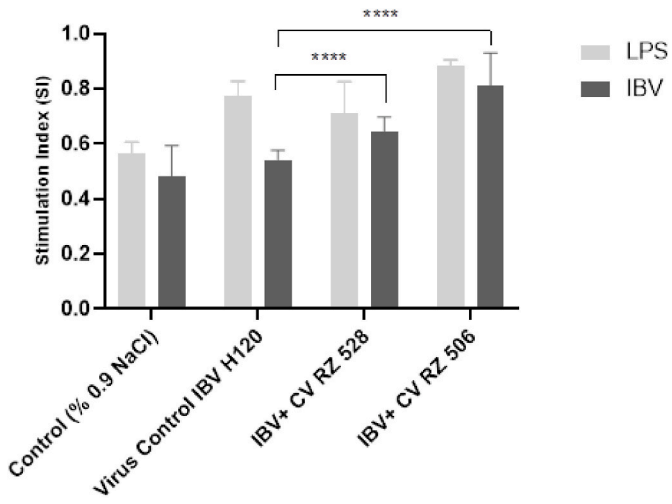


**Fig. 5.** Result of splenocyte proliferation of vaccination groups induced by LPS and IBV (H120).

### *3.11. Analysis of the immune system using flow cytometry*

Spleen cells from different vaccine formulations and control groups were stimulated with IBV H120 antigens for 72 h. This led to an increase in the $CD8^+$ T cell population (see Fig. 6a and Fig. S9), the percentage of IFN-γ secreting $CD8^+$ T cells (Fig. 6b and Fig. S10) and the total percentage of IFN-γ secreting CD3+T cells in all adjuvant formulation groups (see Fig. 6c and Fig. S11). The IBV + CV RZ 528 vaccine formulation and IBV + CV RZ 506 vaccine formulation groups had a significantly higher CD8 immunological response compared to the virus control group ($p < 0.0001$). These two formulations of vaccine preparations have a CD3/CD8 ratio of roughly 9 % (see Fig. 6a and Fig. S9). Evaluation of $CD8^+$ T cells that secrete IFN-γ was performed using CD8/IFN-γ analysis. In IBV H120 containing groups, IBV + CV RZ 528 (5.3 %), a formulation vaccine, was found to be most effective. Compared with the virus control IBV group, the same formulation group showed significant results ($P < 0.0001$). The second-best result in the study was found in the IBV + CV RZ 506 group (5.1 %), with a statistically significant difference ($P < 0.001$) compared to the virus control IBV group (see Fig. 6b and Fig. S10). The CD3/IFN-γ ratio and the T cell population that secretes the most effective IFN-γ cytokine were also investigated in this study. The IBV + CV RZ 506 group reached 7.6 %, which showed a significant increase compared to the virus control group ($p < 0.0001$). In terms of the percentage of IFN-γ secreting T cells in the total population, IBV + CV RZ 528 was shown to be the second most effective group of vaccines (6.8 %). There was a statistically significant difference between this vaccine group and the virus control IBV group ($p < 0.001$) (see Fig. 6c and Fig. S11).

## 4. Discussion

Immunity induced by vaccination is currently the backbone of IBV infection prevention and control [3,22]. Since IBV variations can be found all over the world, most currently available immunizations generally offer poor cross-protection [37–40]. Vaccines targeting the Mass genotype, for instance, provide only partial protection against genotypes not belonging to that strain [11,22]. Diseases like IB, which spread rapidly, cost the chicken business a lot of money. Today, this disease in commercial chickens is typically managed and prevented with the help of live-attenuated and inactivated vaccines. However, there are safety and immunogenicity issues with existing commercial vaccines [41]. Although live-attenuated vaccines have the potential to introduce new variants into the population through mutation and recombination, they are not efficient at eliciting humoral immune responses [42,43]. Low levels of cellular and mucosal immune responses are generated by inactivated vaccines, although they can still be used to promote humoral immunity [44,45]. Multiple serotypes of IBV have coexisted, however there are now dominant serotypes of IBV strains in the field [46,47]. In the case of IBV, the predominant serotypes of field strains do not match the targets of currently available vaccines. Therefore, standard immunizations do not adequately protect against endemic strains. A new vaccination that targets the most common serotypes in order to counteract the immunological deviation seen with older vaccines is, thus, an essential necessity. Previous research has highlighted the significance of comprehending the epidemiology of coronaviruses capable of infecting a diverse range of hosts, including rodents that are considered problems in the context of chicken production [48]. The goal of this study was to develop an inactive IB vaccine candidate in Turkey using newly developed oil emulsion adjuvants (Coralvac RZ 528 and Coralvac RZ 506).

In the present study, the inactive IBV H120 + water-in-oil (W/O) emulsion type adjuvant vaccines demonstrated significantly improved thermostability at various storage temperatures when inspected visually. Figs. 2 and 3 show that these vaccines were stable for up to 7 days at 25 °C and for up to or more than 2 months at +4 °C without separating, as shown by the release of water or oil.

In our previous study (unpublished data), the adjuvant was tested alone, and the results showed that it did not induce any immune response or induce an immune response against itself. Antibody concentration is a useful measure of humoral immunity in the struggle against IBV infection. It is widely accepted that the humoral response is an important part of the protective immune response against IBV. Reports have linked immunity to IBV infection with elevated levels of protective antibodies [8]. In general, Th2 cytokines, which encourage humoral immune responses, are reflected in the formation of IgG1 isotype antibodies, whereas IgG2a isotype antibodies reflect the participation of Th1-type cytokines, which encourage the generation of cellular immunological responses. The nature of the immunological response to a specific antigen can be understood from the relative abundance of these two isotypes [49,50]. More than 60 % of all IgG is IgG1, making it the most common subtype [51]. Mouse serum *anti*-IBV antibody titers (IgG and IgG1) were observed to be higher in the IBV H120 + Coralvac RZ 528 and IBV H120 + Coralvac RZ 506 formulation vaccine groups compared to the viral control IBV group. Therefore, the stimulation of

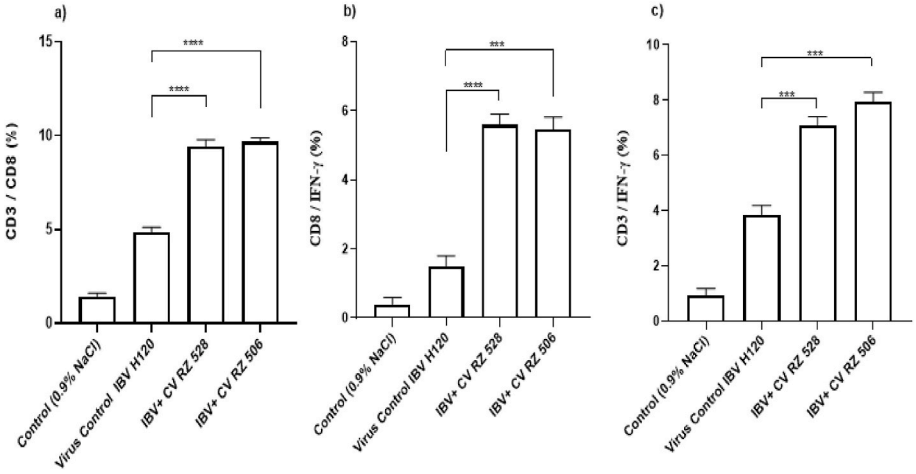


**Fig. 6.** Effect of different IBV vaccine groups on spleen $CD3^+$, $CD8^+$ T cell response and IFN-γ cytokine.

Th2 cells, which will have a substantial effect in providing strong protection against IBV infection, is indicated by the generation of IBV H120-specific IgG1 by oil adjuvant formulations with inactive IBV virus in mice after a single dose intramuscular injection. This result agrees with a previous study that mice were vaccinated to show that the spike protein generated by the recombinant vaccinia virus was immunogenic. Sera collected from groups of mice injected with recombinant vSP19-1 produced antibodies that bound to pure IBV Beaudette in ELISA [52].

An excessive inflammatory response, as a vital immune response of the organism, can cause a dramatic increase in cytokines in the organism, causing a disruption of the immune system and ultimately irreversible damage to the host. It has been reported that coronaviruses have developed strategies to evade the host innate mechanism by increasing the expression of proinflammatory cytokines and decreasing the production of type 1 interferons [53,54]. Furthermore, it has been established that proinflammatory cytokines play a critical role in IBV infection [55]. In the current study, in response to the heightened proinflammatory cytokine storm in IBV-infected mice, newly developed oil emulsion adjuvants significantly enhanced the anti-inflammatory cytokine IFN-γ production when compared with the control group. IFN-γ is the only type II IFN found in birds and mammals, and it acts as a bridge between innate and adaptive immunity. IFN-γ regulates the maturation and differentiation of a variety of immune cells and activates T helper 1-type immune responses. Some studies have distinctly defined the antiviral action of IFN-γ against viruses of various genetic natures, including Newcastle disease virus (NDV) and Marek's disease virus (MDV) [56,57]. However, extensive future research is needed to underpin the mechanisms of IFN-γ induction in the antiviral state. Th2 cells secrete IL-6 and IL-4, and IL-6 is closely associated with the inflammatory response. The present study showed that the inactive IBV H120 with newly developed oil emulsion adjuvants blend could promote IFN-γ expression in the serum of administered mice. These results suggested that oil emulsion adjuvants could improve the cell-mediated immune response.

Another part of the adaptive immune response, the cell-mediated immune system, contributes to the management of acute IBV infection [44]. In the present study, alterations in $CD8^+$ T cell populations, IFN-γ production in IBV-specific $CD8^+$ T lymphocytes and IFN-γ production in total IBV-specific T lymphocytes in mouse splenocytes were examined after administration of oil adjuvant formulations with inactive IBV virus. One of the most important factors in preventing IBV infection is the $CD8^+$ CTL [58]. In comparison to mice given IBV H120 virus control alone, mice immunized with IBV + CV RZ 528 and IBV + CV RZ 506 demonstrated high percentages of $CD3^+CD8^+$ T lymphocytes, IFN-γ production in $CD8^+$ T lymphocytes and IFN-γ production in total $CD3^+$ T lymphocytes. The increased production of T cells after vaccination with oil adjuvant formulation vaccines is evidence of successful cellular immunization.

The splenocyte proliferation assay is widely used to assess cell-mediated immunity by measuring the specific and nonspecific T cell responses of activated splenocytes with various antigen-adjuvant preparations [59]. The spleen is an important immunological organ located in the body's periphery that helps get rid of foreign substances by boosting lymphocyte proliferation and cytokine production [60]. Mice vaccinated with the recently developed oil emulsion adjuvant formulation vaccine showed statistically substantial increases in splenocyte proliferation compared to the IBV H120 virus control group. Liu et al. [61] demonstrated that the vaccine-like particles containing membrane and spike proteins from IBV could generate much stronger cellular immune responses than inactivated IBV vaccine in mice, and they could also elicit effective humoral immune responses in mice and chickens [61]. The current study showed conclusively that the inactive H120 strain of IBV combined with the recently developed oil emulsion adjuvants successfully stimulated humoral and cellular immune responses against IBV in mice. If the findings of this study are confirmed in clinical trials (chicken), the promising outcomes may pave the way for the beginning of clinical investigations with vaccine formulations incorporating oil-based emulsions. The data presented above shows that the same success seen in mice may be replicated in chickens.

## 5. Conclusions

Despite spending huge amounts of money to control IB, outbreaks involving classical and newly emerging virus serotypes are constantly reported. The increasing emergence of IBV genotypes and the lack of cross-protective immunity have augmented the pace of interest in the development of novel IBV vaccines. In conclusion, this study represents the first investigation to evaluate inactivated IBV H120 combined with Coral Biotechnology's newly developed water-in-oil (W/O) emulsion adjuvant vaccines on immune responses in mice. This study has demonstrated that these vaccines induce significantly higher humoral and cellular immune responses.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biologicals.2023.101736.

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