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Effects of immunosuppression on the efficacy of vaccination against *Mycoplasma gallisepticum* infection in chickens

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Abstract

Immunosuppression can increase the susceptibility of chickens to other disease-causing pathogens and interfere with the efficacy of vaccination against those pathogens. Chicken anaemia virus (CAV) and infectious bursal disease virus (IBDV) are common causes of immunosuppression in chickens. Immunosuppression was induced by experimental infection with either CAV or IBDV to assess the effect of immunosuppression on the efficacy of *Mycoplasma gallisepticum* vaccine strain ts-304 against infection with virulent *M. gallisepticum*, a common bacterial pathogen of chickens worldwide. Birds were experimentally infected with either CAV or IBDV at 1 week of age, before vaccination and challenge with *M. gallisepticum* to examine the effect of immunosuppression at the time of vaccination, or at 6 weeks of age, after vaccination against *M. gallisepticum* but before challenge with virulent *M. gallisepticum*, to investigate the effect of immunosuppression at the time of challenge. All birds were vaccinated with a single dose of the ts-304 vaccine at 3 weeks of age and experimentally challenged with the virulent *M. gallisepticum* strain Ap3AS at 8 weeks of age. In immunosuppressed chickens there was a reduction in protection offered by the ts-304 vaccine at two weeks after challenge, as measured by tracheal mucosal thicknesses, serum antibody levels against *M. gallisepticum*, air sac lesion scores and virulent *M. gallisepticum* load in the trachea. Immunosuppressed birds with detectable serum antibodies against *M. gallisepticum* were less likely to have tracheal lesions. This study has shown that immunosuppression caused by infection with CAV or IBDV can interfere with vaccination against mycoplasmosis in chickens.

Key words: chicken, *Mycoplasma gallisepticum*, vaccine, immunosuppression, chicken anaemia virus, infectious bursal disease virus

1. Introduction

Mycoplasma gallisepticum causes chronic respiratory disease in chickens and infectious sinusitis in turkeys. Infected chickens develop clinical signs of upper and lower respiratory tract disease including coughing, sneezing, râles, and ocular and nasal discharge (Ley et al., 1997; Nascimento et al., 2005). Chronic respiratory disease in chickens can cause significant economic loss by reducing feed conversion efficiency, weight gain and egg production (Ley et al., 1997; Nascimento et al., 2005). Vaccination, antimicrobial therapy, biosecurity, and periodic screening and culling of infected flocks are used to control *M. gallisepticum* in commercial poultry (Kleven, 2008; Levisohn and Kleven, 2000). While several live attenuated *M. gallisepticum* vaccines are available commercially (F strain, strain 6/85, and strain ts-11) (Kleven, 2008), all have some limitations that restrict their universal application in control programs. *M. gallisepticum* ts-304 is a new live attenuated vaccine strain that has been shown to have an enhanced capacity to reduce the incidence and severity of disease caused by infection with virulent *M. gallisepticum* in chickens (Kanci Condello et al., 2020b).

The chronic respiratory disease caused by *M. gallisepticum* in commercial flocks is often multifactorial, with other respiratory pathogens, including Newcastle disease virus (NCDV), infectious bronchitis virus (IBV) and *Escherichia coli* (Nunoya et al., 1995) contributing to disease severity. In addition, immunosuppression can play a key role in the pathogenesis of disease caused by infection with *M. gallisepticum*, exacerbating the severity of the disease and delaying recovery (Bao et al., 2020; Ganapathy and Bradbury, 2003). Infection with immunosuppressive viruses can also reduce the efficacy of vaccination with live-attenuated vaccines by interfering with the development of protective immunity after vaccination (Whithear, 1996). The effect of immunosuppression on vaccinal immunity has been explored for several pathogens of chickens,

including *Pasteurella multocida* (Nathanson et al., 1981), NCDV (Yan et al., 2011), IBV (Winterfield et al., 1978), highly pathogenic avian influenza virus (Spackman et al., 2018), infectious laryngotracheitis virus (ILTV) and fowlpox virus (Cloud et al., 1992). The effects of immunosuppression on the pathogenesis of disease caused by *M. gallisepticum* (Bao et al., 2020; Canter et al., 2019; Ganapathy and Bradbury, 2003) and on the efficacy of antimicrobial treatment against *M. gallisepticum* (Xiao et al., 2016) have been investigated, but its effect on the protective immunity afforded by live attenuated *M. gallisepticum* vaccines has not been examined.

Two different approaches have been used to induce immunosuppression experimentally in chickens – a chemical approach, using immunosuppressive agents such as hydrocortisone, cyclophosphamide or cyclosporine (Bao et al., 2020; Ganapathy and Bradbury, 2003; Nathanson et al., 1981; Xiao et al., 2016), and a biological approach, using infection with immunosuppressive viruses such as chicken anaemia virus (CAV), infectious bursal disease virus (IBDV) or Marek's disease virus (MDV) (Canter et al., 2019; Gallardo et al., 2012; Mohammad Heidari and Dmitry Kireev, 2010; Subler et al., 2006). Chicken anaemia virus predominantly suppresses the cell-mediated immune response of chickens (McConnell et al., 1993), while infectious bursal disease virus mainly suppresses the antibody-mediated immune response (Giambrone et al., 1977). In this study, we aimed to assess the effect of immunosuppression on vaccinal immunity against *M. gallisepticum* in chickens and to explore the role of cell-mediated and humoral immunity in the development and persistence of protective immunity. To achieve this, chickens were infected with either CAV or IBDV before or after vaccination to examine the effect that suppression of cell-mediated immunity or antibody-mediated immunity by these viruses had on the protection afforded by the ts-304 vaccine.

2. Materials and methods

2.1. Preparation of *M. gallisepticum*, CAV and IBDV strains

A freeze-dried culture of *Mycoplasma gallisepticum* ts-304 vaccine was obtained from Bioproperties Pty. Ltd. (Glenorie, NSW, Australia) and diluted to $10^{6.0}$ colour changing units (CCU) per 0.03ml dose in Marek's Disease vaccine sterile diluent (Merial Select Inc., Gainesville, Georgia, USA) as recommended by the manufacturer for vaccination. An ampoule of the virulent *M. gallisepticum* Ap3AS strain (Soeripto et al., 1989) was thawed at 37°C, diluted 1:10 in Mycoplasma growth medium and the culture incubated for 24 h at 37°C. The culture was then diluted to $10^{6.5}$ CCU/ml in Mycoplasma growth medium prior to challenge. Stocks of the CAV strain CAU269/7 (Brown et al., 2000) diluted to 10^4 median tissue culture infectious doses ($TCID_{50}$)/dose and IBDV strain 002/73 (Ignjatovic and Sapats, 2002) diluted to 10^3 $TCID_{50}$/dose were used to induce immunosuppression.

2.2. Experimental vaccination and infection of chickens

Eighty White Leghorn chicks were hatched from specific-pathogen-free (SPF) eggs (Australian SPF Services Pty. Ltd., Woodend, Victoria, Australia) and raised to 1 week of age. The chicks were wing-tagged, allocated at random into 8 groups of 10 (Table 1) and each group was housed in a separate HEPA-filtered fibreglass isolator unit under negative pressure, with feed and water provided *ad libitum*. At 1 week of age, the birds in the CAV-vaccinated-challenged group were infected with 10^4 $TCID_{50}$ of CAV strain CAU269/7 by eyedrop inoculation and the birds in IBDV-vaccinated-challenged group were infected with 10^3 $TCID_{50}$ of IBDV strain 002/73 by eye-drop inoculation. At 3 weeks of age, the chickens in the vaccinated groups (Table 1) were inoculated with 30 µl of the ts-304 vaccine containing $10^{6.0}$ CCU by eye drop, while those in the negative control and challenged-only groups were inoculated with 30 µl of sterile Merial diluent (Marek's Disease vaccine sterile diluent) by eye drop. At 6 weeks of age, the chickens in the vaccinated-

CAV-challenged group were infected with 10^4 $TCID_{50}$ of CAV strain CAU269/7 by eye drop inoculation and those in the vaccinated-IBDV-challenged group were infected with 10^3 $TCID_{50}$ of IBDV strain 002/73 by eye-drop inoculation. At 8 weeks of age, all the chickens in the challenged groups (Table 1) were exposed to an aerosol challenge with the *M. gallisepticum* wild-type strain Ap3AS by nebulisation of a culture containing $10^{6.5}$ CCU/ml into a purpose-built infection chamber for 40 minutes using an established protocol (Kanci et al., 2017). Two weeks after challenge, all birds were humanely euthanised and necropsied. In the descriptions of each group of birds throughout this paper, vaccination refers to vaccination with strain ts-304 against *M. gallisepticum*, challenge refers to challenge with the *M. gallisepticum* wild-type strain Ap3AS and infection refers to infection with either CAV or IBDV. All procedures involving animals were reviewed and approved by the University of Melbourne Animal Ethics Committee under approval number 1914907.1.

2.3. Serology

Blood samples were collected from birds before vaccination (at 3 weeks of age) with ts-304, and five (before challenge) and seven (2 weeks after challenge) weeks after vaccination. The rapid serum agglutination (RSA) test was performed to determine the levels of serum antibody against *M. gallisepticum* in each bird using a commercial stained *M. gallisepticum* agglutination antigen (Nisseiken Co. Ltd., Tokyo, Japan). The results were scored on a scale of 0 to 4, as described previously (Whithear, 1993), and a RSA score of ≥ 1 was considered positive. A recombinant protein-based indirect enzyme-linked immunosorbent assay (ID Screen *Mycoplasma gallisepticum* indirect ELISA) was used to detect anti-*Mycoplasma gallisepticum* IgG in chicken sera (ID.Vet, Grabels, France) and an S/P ratio ≥ 0.5 was considered positive. Sera collected from CAV infected birds were tested for CAV-specific antibodies using a commercial competitive ELISA kit (Chicken

Anaemia Test Kit, IDEXX Laboratories Inc., Westbrook, Maine, USA) and an S/P ratio of ≤ 0.6 was considered positive. Specific antibodies against IBDV in the serum of IBDV infected groups were detected using a commercial IBDV antibody test kit (IDEXX) and an S/P ratio of > 0.2 was considered positive.

2.4. Gross examination and scoring of air sac lesions

Air sac lesions were scored grossly for severity as described previously (Nunoya et al., 1987), except lesions were scored on a scale of 0 to 4 (0, no air sac lesions; 1, cloudy appearance or a few yellowish foci; 2, thickening and/or multiple yellowish foci and/or a few small caseous or extensive foamy exudates; 3, diffuse yellowish thickening with large caseous exudate; 4, partial to complete meaty in consistency). A cumulative lesion score was then calculated for each bird by summing the scores for the thoracic and abdominal air sacs (Shil et al., 2011).

2.5. Histopathological examination of the tracheal mucosal thickness

Approximately 2-3 mm thick tracheal cross sections from the upper, middle and lower trachea were collected from each bird and placed in 10% formalin for histopathological processing and examination. The formalin-fixed tracheal cross sections were embedded in paraffin and 3 μm thick sections were stained with haematoxylin and eosin. The mucosal thicknesses of the upper, middle and lower tracheas of each bird were calculated by averaging measurements taken at four points transected by vertical and horizontal lines at 400 × magnification using a light microscope with an eyepiece graticule, as described previously (Shil et al., 2011). The mean mucosal thicknesses of the upper, middle and lower tracheas of each group were calculated and compared to each other within the group. The mean mucosal thicknesses of the upper, middle and lower tracheas were also compared between the groups. The overall average tracheal mucosal thickness of each bird was

also calculated by averaging the mean upper, middle and lower tracheal thicknesses for each bird. The individual birds in the vaccinated/challenged groups were classified as having an increased tracheal mucosal thickness if their overall average tracheal mucosal thickness was more than one standard deviation greater than the mean of the negative control group.

2.6. Detection of *M. gallisepticum* in the trachea

The remainder of each of the tracheas was collected into RNAlater Stabilization Solution (Invitrogen, Carlsbad, CA, USA) and stored at -20°C. DNA was extracted from the tracheas of the individual birds and used as template in PCR assays. Briefly, tracheal cross sections were halved along their longitudinal axes and the mucosa was separated using sterile forceps. The mucosae (20 mg) were disrupted and homogenized in 1 ml of phosphate buffered saline by passage through Discofix 3-way stopcocks using two 5 ml syringes (B. Braun, Melsungen, Germany). Total DNA was extracted from 0.1 ml of each of the tracheal mucosal homogenates using the Applied Biosystems MagMAX CORE Nucleic Acid Purification kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) and the Invitrogen KingFisher Flex Purification System (Thermo Fisher Scientific).

PCR assays were performed as described previously (Lysnyansky et al., 2005). The presence of *M. gallisepticum* DNA was confirmed using the *mgc2* 2F (CGCAATTTGGTCCTAATCCCCAACA) and *mgc2* 2R (TAAACCCACCTCCAGCTTTATTTCC) oligonucleotide primers. Each PCR was performed using a reaction mixture (volume of 50 µl) comprising 0.1 mM of each dNTP (Bioline, Alexandria, NSW, Australia), 2.0 mM $MgCl_2$ (Promega, Auburn, VIC, Australia), 100 mM of each primer, 2.5 U Taq polymerase (5 U/µl) (Promega) and 5 µl of template. The assay was incubated at 94°C for 3 min, then amplified through 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min.

The amplicons were separated in a 2 % (w/v) agarose gel run at 5 V/cm for 60 min and the PCR products were stained with SYBR Safe DNA gel stain (Invitrogen) and visualised using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The expected size of the product was 225 base pairs for the *M. gallisepticum* wild-type strain Ap3AS and 290 base pairs for the ts-304 vaccine strain.

2.7. Quantification of *M. gallisepticum* wild-type strain Ap3AS in the trachea

The load of *M. gallisepticum* wild-type strain Ap3AS in the trachea was determined using a qPCR assay using F (5'-GTTTGGAGTTGGTGTATAGTTAG-3') and R (5'-TCTTCTTCGAAAACAAAAGG-3') oligonucleotide primers (Ghorashi et al., 2010). A pair of oligonucleotide primers, GAPDH-F (5'-CCCGGGGCCTTCAGTAAATT-3') and GAPDH-R (5'-AGGACAAGCAGTGAGGAACG-3'), which bind to intron regions of the chicken house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were designed using the Geneious Prime sequence analysis software (version 2020.1.2) (https://www.geneious.com). Amplification of each target DNA sequence was performed in 20 µl reaction volumes on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany), with the reaction mixtures containing 3 µl of genomic DNA extract, 25 µM of each primer, 1.5 mM $MgCl_2$, 1250 µM of each dNTP, 5 µM SYTO 9 green fluorescent nucleic acid stain (Invitrogen), 1× Green GoTaq Flexi Reaction Buffer (Promega) and 1 U GoTaq DNA polymerase (Promega). The PCR conditions for *M. gallisepticum* DNA were one cycle of 94°C for 60 s, 40 cycles of 94°C for 10 s, 50°C for 10 s and 72°C for 10 s, and a final cycle of 72°C for 1 min. The PCR conditions for the GAPDH gene were one cycle of 94°C for 5 min, 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final cycle of 72°C for 5 min. The cycle threshold (Ct_{target}) value for *M. gallisepticum* DNA for each tracheal tissue sample was calculated at a threshold fluorescence of 0.05167 and the $Ct_{reference}$ value for the chicken

GAPDH gene for each tracheal tissue sample was calculated at a threshold fluorescence of 0.07399 using the Rotor-Gene Q series software.

Differences in the amount of total DNA in each reaction on the calculations of the *M. gallisepticum* load in each sample were corrected by normalisation against the amount of the chicken GAPDH gene in the same sample. The normalised ($40 - Ct_{target}$) values were calculated using the equation

$$normalised\ (40 - Ct_{target}) = (40 - Ct_{target}) + (Ct_{reference} - mean\ Ct_{reference})$$

2.8. Statistical analyses

Median RSA scores and air sac gross lesion scores were compared using the Kruskal-Wallis test and Dunn's multiple comparison test. The comparisons of the mean S/P ratios in the ELISAs to detect antibodies against *M. gallisepticum*, CAV or IBDV between the groups at each time point, or between the time points in each group, were performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests or Student's *t*-tests. The comparisons of the mean mucosal thicknesses of the upper, middle and lower trachea between the groups and between the levels of the trachea within each group were conducted using a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. The differences in the median normalised ($40 - Ct_{target}$) values for *M. gallisepticum* loads in the CAV and IBDV immunosuppressed groups were compared between the two different time points of infection using Mann–Whitney U tests. A *P*-value ≤ 0.05 was considered significant. All statistical analyses were conducted using GraphPad Prism version 8.3.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com)

3. Results

3.1. *Mycoplasma gallisepticum* specific antibodies

3.1.1. RSA scores

No antibodies against *M. gallisepticum* were detected in the serum of any bird before vaccination at 3 weeks of age. All chickens in the negative control group were seronegative for antibodies against *M. gallisepticum* throughout the experiment, while the challenged-only group had no detectable antibodies at the time of vaccination or before challenge. At 5 weeks after vaccination (before challenge), the vaccinated-only group had the highest proportion (6/10 birds) of seropositive birds, followed by the vaccinated-and-challenged group (5/10 birds), the vaccinated-CAV-challenged group (5/10 birds), the vaccinated-IBDV-challenged group (5/10 birds), the CAV-vaccinated-challenged group (4/10 birds) and the IBDV-vaccinated-challenged group (3/10 birds). At 7 weeks after vaccination (2 weeks after challenge), all birds in the vaccinated and/or challenged groups were seropositive, except for the vaccinated-IBDV-challenged group, in which 9/10 birds were seropositive.

Before challenge, the median RSA scores of the vaccinated-only, the vaccinated-and-challenged, the vaccinated-CAV-challenged and the vaccinated-IBDV-challenged groups were significantly higher than those of the two control groups (the negative control and challenged-only groups), while there was no significant difference between the median RSA scores of the CAV-vaccinated-challenged and IBDV-vaccinated-challenged groups and those of any of the other groups. After challenge, all the groups vaccinated against *M. gallisepticum* and/or challenged with *M. gallisepticum* except the vaccinated-IBDV-challenged group had significantly higher median RSA scores than the negative control group. The RSA score results are summarised in Table 2.

3.1.2. ELISA results

None of the birds had antibodies against *M. gallisepticum* detectable by ELISA before vaccination (3 weeks of age), and all the birds in the negative control group were seronegative throughout the experiment. Prior to challenge, the mean S/P ratios did not differ significantly between the six groups of birds vaccinated with the ts-304 strain (Figure 1A). The proportion of ELISA positive birds was highest in the vaccinated-only and vaccinated-and-challenged groups (6/10 birds in each group), while 4/10 birds were seropositive in the CAV-vaccinated-challenged group, the vaccinated-CAV-challenged group and the vaccinated-IBDV-challenged group. Only one bird was seropositive in the IBDV-vaccinated-challenged group before challenge. At two weeks after challenge, the vaccinated-and-challenged group had the highest proportion of seropositive birds (9/10 birds) and the mean S/P ratio of this group was significantly higher than that of the negative control group (Figure 1B). The proportions of seropositive birds in the CAV and IBDV infected groups were less than the proportion of seropositive birds in the vaccinated-only group (6/10 birds), but higher than the proportion of seropositive birds in the challenged-only group (2/10 birds) (Figure 1B).

3.2. Anti-CAV and anti-IBDV antibodies

No birds in the CAV-vaccinated-challenged group had detectable antibodies against CAV at 2 weeks after infection with CAV (3 weeks of age), but all the birds were seropositive at 7 and 9 weeks after infection (8 and 10 weeks of age) and the mean S/P ratios were significantly lower than at 3 weeks of age (Figure 2A). In the vaccinated-CAV-challenged group, no birds had detectable antibodies against CAV at 2 weeks after infection with CAV (8 weeks of age), but all birds were seropositive at 4 weeks after infection (10 weeks of age), and the mean S/P ratio was significantly lower than at 8 weeks of age (Figure 2B).

No birds in the IBDV-vaccinated-challenged group had detectable antibodies against IBDV at 2 weeks after infection with IBDV (3 weeks of age), but all the birds were seropositive at 7 and 9 weeks after infection (8 and 10 weeks of age) and the mean S/P ratios were significantly higher than at 3 weeks of age (Figure 2C). In the vaccinated-IBDV-challenged group, all the birds had detectable anti-IBDV antibodies at 2 and 4 weeks after infection with IBDV (8 and 10 weeks of age) and there was no significant difference in the mean S/P ratios at the different time points (Figure 2D).

3.3. Gross air sac lesions

Gross air sac lesions were most prevalent in the challenged-only (5/10 birds) and vaccinated-CAV-challenged groups (5/10 birds), followed by the vaccinated-IBDV-challenged group (3/10 birds). There was no significant difference between any of the groups in the median air sac lesion scores. The air sac lesion scores are summarised in Table 3.

3.4. Tracheal mucosal thickness and lesions

The mean mucosal thickness of the upper trachea of the challenged-only group was significantly greater than those of the negative control, vaccinated-only and vaccinated-and-challenged groups. The mean mucosal thickness of the middle trachea of the challenged-only group was significantly greater than those of all other groups except those of the vaccinated-CAV-challenged and vaccinated-IBDV-challenged groups. The mean mucosal thickness of the lower trachea of the challenged-only group was significantly greater than those of all other groups except that of the vaccinated-IBDV-and-challenged group. The mean mucosal thicknesses of the upper, middle and lower tracheas of each group are summarised in Table 3. There were no significant differences between the mean mucosal thicknesses of upper, middle and lower tracheas within each of the

groups. There were 4/10 birds in the vaccinated-only group, 5/10 birds in the vaccinated-and-challenged group (Table 4), 4/10 birds in the CAV-vaccinated-challenged group, 6/10 birds in the vaccinated-CAV-challenged group (Table 5) and 5/10 birds each in the IBDV-vaccinated-challenged and vaccinated-IBDV-challenged groups (Table 6) that had an average tracheal mucosal thickness greater than the mean + one standard deviation of the negative control group.

The tracheal mucosae of the birds in the challenged-only group had diffuse intraepithelial inflammatory cell infiltration, with a disintegrated pseudostratified columnar epithelium, loss of cilia, and reduction or absence of the intraepithelial mucous glands and goblet cells (Figure 3A). Approximately half of the birds in each of the CAV or IBDV infected groups had diffuse intraepithelial inflammatory cell infiltration (Figure 3E-H) similar to that seen in the challenged-only group, while the remainder of the birds in these groups had no detectable lesions, similar to the birds in the negative control (Figure 3B), vaccinated-only (Figure 3C) and vaccinated-and-challenged (Figure 3D) groups, with an intact pseudostratified ciliated columnar epithelium and abundant simple intraepithelial mucous glands containing several mucus-secreting cells.

3.5. Detection and quantification of *M. gallisepticum* in the tracheal mucosa

The Ap3AS challenge strain was more commonly detected in the challenged-only group (9/10 birds) than in any of the other challenged groups (results not shown). The vaccinated-and-challenged group had the lowest proportion of birds with detectable Ap3AS in the trachea (2/10 birds) (Table 4), followed by the CAV-vaccinated-challenged group (6/10 birds) (Table 5), the vaccinated-CAV-challenged group (6/10 birds) (Table 5), the IBDV-vaccinated-challenged group (6/10 birds) and the vaccinated-IBDV-challenged group (7/10 birds) (Table 6). No *M. gallisepticum* vaccine strain ts-304 was detected in any of the vaccinated birds and no challenge strain was detected in either the negative control group or the vaccinated-only group (Table 4).

315 The amount of *M. gallisepticum* wild-type strain Ap3AS colonising the trachea was quantified
316 using qPCR and normalised (40 - Ct_{target}) values were calculated (Table 4, 5 and 6). Only two birds
317 in the vaccinated-and-challenged group had detectable strain Ap3AS DNA in the trachea and they
318 had a normalised (40 - Ct_{target}) value of > 8 (Table 4). The six birds in the CAV-vaccinated-
319 challenged group with detectable strain Ap3AS DNA in the trachea had normalised (40 - Ct_{target})
320 values ranging from 3.79 to 6.31, while the six birds in the vaccinated-CAV-challenged group with
321 detectable strain Ap3AS DNA in the trachea had normalised (40 - Ct_{target}) values ranging from
322 0.81 to 9.46 (Table 5). There were six birds in the IBDV-vaccinated-challenged group with
323 detectable strain Ap3AS DNA in the trachea and their normalised (40 - Ct_{target}) values ranged from
324 1.74 to 8.32, while the seven birds in the vaccinated-IBDV-challenged group with detectable strain
325 Ap3AS DNA in the trachea had normalised (40 - Ct_{target}) values ranging from 3.78 to 9.10 (Table
326 6). There was no significant difference between the median normalised (40 – Ct_{target}) values for the
327 groups infected with CAV or IBDV at different time points.

328 **4. Discussion**

329 This study investigated the effect of immunosuppression on vaccinal immunity against chronic *M.*
330 *gallisepticum* infection in chickens. Immunosuppressed birds showed increased susceptibility to
331 *M. gallisepticum* infection, exhibiting more severe tracheal and air sac lesions, and had lower
332 concentrations of serum antibodies against *M. gallisepticum* compared to normal vaccinated
333 chickens.

334 A few studies have investigated the effect of coinfection with *M. gallisepticum* and respiratory
335 viruses, such as infectious bronchitis virus and low pathogenicity avian influenza virus, and have
336 observed more severe pathology in coinfected birds (Canter et al., 2019; Chu and Uppal, 1975;
337 Stipkovits et al., 2012), possibly as a result of indirect immunosuppression by the viral pathogen.

However, only two studies have investigated the direct effect of immunosuppression on the pathogenesis of disease caused by infection with *M. gallisepticum* (Bao et al., 2020; Ganapathy and Bradbury, 2003) and both of these used chemical immunosuppression. The study described here used biological immunosuppression, using two different viruses that predominantly attack different arms of the immune system, and investigated the effect of this immunosuppression on the efficacy of the live attenuated ts-304 vaccine against *M. gallisepticum*. Both CAV and IBDV are endemic in chicken flocks throughout the world and are frequently detected during the early stages of life in co-infections with other bacterial and viral pathogens (Giambrone et al., 1977; McConnell et al., 1993). Therefore, in this study we used both CAV and IBDV to induce immunosuppression in chickens that might be encountered in typical field situations (Bao et al., 2020), and to explore which components of the immune response might have a greater impact on the efficacy of the protection provided by the ts-304 vaccine.

All chickens infected with CAV had detectable serum antibodies against CAV at 4 weeks after infection, while all those infected with IBDV had detectable serum antibodies against IBDV at 2 weeks after infection, confirming that experimental infection with each of the viruses had been successfully established (Mahgoub, 2014; Singh et al., 2010). Previous studies investigating the clinical aspects of immunosuppression induced by CAV and IBDV in chickens have found that the chickens exposed to IBDV or CAV recover from the immunosuppression and return to near normal function within 6 weeks from the time of infection (Kim et al., 1999; McConnell et al., 1993). The experiments described here used two different time points for infection with CAV and IBDV: 1) two weeks before vaccination, but more than 6 weeks before challenge, to examine whether immunosuppression at the time of vaccination with *M. gallisepticum* ts-304 vaccine has an effect on the development of immunity after vaccination, and 2) three weeks after vaccination,

but two weeks before challenge, to explore the effect immunosuppression might have on the protective effect of the immunity induced by vaccination.

In our previous studies we have observed a correlation between the serological response and the protection induced by the ts-304 vaccine. However, some chickens in the vaccinated groups have not had detectable levels of serum antibodies, yet were still protected against the development of tracheal and air sac lesions (Kanci Condello et al., 2020a; Kanci Condello et al., 2020b; Shil et al., 2011). Similarly, a small proportion of vaccinated immunocompetent chickens in the study described here did not have a detectable serological response or tracheal/air sac lesions, while another small proportion of birds had a detectable serological response and had mild tracheal lesions. These findings suggest that mucosal humoral and cell mediated immune responses are probably the key contributors to the protection offered by the ts-304 vaccine, with serum antibody concentrations usually, but not always, reflective of these mucosal responses, as has been seen after vaccination with another attenuated *M. gallisepticum* strain (Javed et al., 2005). Nevertheless, the reduced serological response in the birds in the four immunosuppressed groups, compared to those in the vaccinated-and-challenged group, reflected the adverse effect of immunosuppression with either IBDV or CAV on the immune response induced by vaccination with strain ts-304, as has been seen in previous studies investigating the effect of immunosuppression on protection provided by vaccination against other pathogens in chickens (Cloud et al., 1992; Nathanson et al., 1981; Spackman et al., 2018; Winterfield et al., 1978; Yan et al., 2011). The reduction in the serological response was more apparent in the two groups infected with IBDV than the two groups infected with CAV, suggesting, as might be expected, that IBDV had a greater suppressive effect on the humoral response to vaccination with ts-304 and to subsequent infection with wild-type *M. gallisepticum*. Similar observations have been made

about the serological response in chickens infected with either CAV or IBDV before vaccination against Newcastle disease virus (Cloud et al., 1992).

The intermediate severity of the lesions observed in the upper tracheas of the four immunosuppressed groups, between that seen in the challenged-only group and in the vaccinated-and-challenged group clearly showed that immunosuppression had interfered with the protection against the development of lesions in the trachea afforded by vaccination with ts-304. Similar changes were seen in the middle trachea of birds in the vaccinated-CAV-challenged and vaccinated-IBDV-challenged groups, and in the lower trachea of birds in the vaccinated-IBDV-challenged group. Thus, the birds in the vaccinated-IBDV-challenged group had severe pathological changes throughout the length of the trachea, more so than the birds in the other three immunosuppressed groups. However, the greatest effect of immunosuppression, based on the number of individual birds with increased tracheal mucosal thicknesses compared to the negative control group, was seen in the vaccinated-CAV-challenged group. These findings suggest that immunosuppression by infection with either CAV or IBDV at the time of challenge had more adverse effect on pathological changes in the trachea than immunosuppression at the time of vaccination. The birds immunosuppressed at the time of challenge also developed more air sac lesions. These findings are consistent with previous studies that have examined the effect of the immunosuppression caused by infection with these viruses on the exacerbation of clinical signs and lesions associated with disease caused by other pathogens in vaccinated but immunosuppressed chickens (Nathanson et al., 1981; Spackman et al., 2018; Winterfield et al., 1978; Yan et al., 2011).

In contrast to the vaccinated-and-challenged group, the majority of the individual chickens in the immunosuppressed groups had detectable persistence of virulent *M. gallisepticum* in the trachea

and they were less likely to have detectable serum antibodies against *M. gallisepticum.* In addition, the development of serum antibodies against *M. gallisepticum* was inversely correlated with the *M. gallisepticum* load in the tracheas of these birds. Interestingly, no differences were detected between the two CAV-infected groups with respect to the detection of *M. gallisepticum* in the trachea and the highest rates of persistence of virulent *M. gallisepticum* were detected in the tracheas of vaccinated-IBDV-challenged group. These findings suggest that the suppression of humoral immunity at the time of challenge with *M. gallisepticum* may have favoured colonisation of the trachea by virulent *M. gallisepticum*. Therefore, it is highly likely that antibodies may play a role in the protection induced by the ts-304 vaccine against colonisation of the trachea by virulent *M. gallisepticum*, rather than competitive exclusion of the virulent strain by the vaccine strain, as suggested previously (Kanci Condello et al., 2020a; Kanci Condello et al., 2020b).

In the study described here, suppression of humoral immunity or cell-mediated immunity at the time of vaccination or at the time of challenge had an impact on the level of serum antibodies against *M. gallisepticum*. However, suppression of humoral immunity or cell-mediated immunity at the time of challenge with virulent *M. gallisepticum* had a greater effect on development of lesions in the trachea and air sacs and colonisation of the trachea by virulent *M. gallisepticum* than immunosuppression at the time of vaccination. Our study thus provides evidence for the involvement of both cell-mediated and humoral immunity in the development of protective immunity against pathological changes caused by mycoplasmas including *M. gallisepticum* as suggested by previous studies (Erb and Bredt, 1979; Jones et al., 2002), and in immunological effector responses in vaccinated birds. Further investigations to identify the specific cells involved in these responses and to determine differences in cytokine gene expression levels in the trachea

will be useful to further define the specific components of the cell-mediated immune response that have been affected by immunosuppression.

The most common strategy used to control CAV and IBDV infections in chickens is immunisation of parent breeder flocks (Steer and Noormohammadi, 2011) to provide passive protection against early infections. Although the passive immunity plays a key role in delaying the immunosuppression induced by CAV and IBDV, maternal antibodies deplete after hatching and are lost completely by approximately 3 to 4 weeks of age (Otaki et al., 1992; Sharma et al., 2000). Subsequent infection with CAV and/or IBDV can increase the economic losses associated with other pathogens, including *M. gallisepticum*, by reducing the efficacy of vaccinal protection, as highlighted by the findings in this study.

Conclusion

In conclusion, the study described here clearly demonstrated the adverse effect of immunosuppression on the protection afforded by vaccination with the ts-304 live attenuated vaccine against chronic infection with *M. gallisepticum* in chickens, providing further insights into the factors that can reduce the efficacy of vaccines in the field.

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Table 1. Treatments administered to each group of birds in this experiment.

Group	Vaccination at 3 weeks of age	Infection at:		Challenge at 8 weeks of age
		1 week of age	6 weeks of age	
Negative control	-	-	-	-
Vaccinated-only	ts-304	-	-	-
Challenged-only	-	-	-	Ap3AS
Vaccinated-and-challenged	ts-304	-	-	Ap3AS
CAV-vaccinated-challenged	ts-304	CAV	-	Ap3AS
Vaccinated-CAV-challenged	ts-304	-	CAV	Ap3AS
IBDV-vaccinated-challenged	ts-304	IBDV	-	Ap3AS
Vaccinated-IBDV-challenged	ts-304	-	IBDV	Ap3AS

CAV, chicken anaemia virus; IBDV, infectious bursal disease virus; ts-304, *M. gallisepticum* live attenuated vaccine strain ts-304; Ap3AS, *M. gallisepticum* wild-type strain Ap3AS. Vaccination refers to vaccination with ts-304 against *M. gallisepticum*, infection refers to infection with either CAV or IBDV, and challenge refers to challenge with *M. gallisepticum* wild-type strain Ap3AS.

Table 2. Serum antibodies against *M. gallisepticum* as detected using the Rapid Serum Agglutination test (RSA) at the time of challenge (5 weeks after vaccination) and 2 weeks after challenge with *M gallisepticum* wild-type strain Ap3AS.

Group	Before challenge		After challenge	
	Proportion RSA positive	Median RSA score (range)	Proportion RSA positive	Median RSA score (range)
Negative control*	0/10	0 (0, 0)[a]	0/10	0 (0, 0)[a]
Vaccinated-only*	6/10	1 (0, 2)[b]	10/10	1 (1, 2)[b]
Challenged-only	0/10	0 (0, 0)[a]	10/10	2 (2, 2)[b]
Vaccinated-and-challenged	5/10	0.5 (0, 2)[b]	10/10	1 (2, 2)[b]
CAV-vaccinated-challenged	4/10	0 (0, 2)[a, b]	10/10	1 (2, 2)[b]
Vaccinated-CAV-challenged	5/10	0.5 (0, 2)[b]	10/10	1 (1, 2)[b]
IBDV-vaccinated-challenged	3/10	0 (0, 2)[a, b]	10/10	1 (1, 2)[b]
Vaccinated-IBDV-challenged	5/10	0.5 (0, 2)[b]	9/10	1 (0, 2)[a, b]

* Unchallenged. All the groups were negative for anti-*M. gallisepticum* antibodies by RSA before vaccination. Values marked with the same superscript letter in the same column are not significantly different ($P > 0.05$).

591 **Table 3**. Air sac lesion scores and tracheal mucosal thicknesses in birds at 2 weeks after challenge

592 with *M. gallisepticum* wild-type strain Ap3AS.

Group	Proportion with air sac lesions	Median air sac lesion score (range)	Mean tracheal mucosal thickness (μm) ± SD		
			Upper	Middle	Lower
Negative control*	0/10	0 (0, 0)[a]	79 ± 9[a]	55 ± 10[a]	58 ± 13[a]
Vaccinated-only*	1/10	0 (0, 0.5)[a]	78 ± 14[a]	61 ± 16[a]	73 ± 14[a]
Challenged-only	5/10	0.25 (0, 5)[a]	188 ± 73[b]	187 ± 125[b]	184 ± 49[b]
Vaccinated-and-challenged	1/10	0 (0, 0.5)[a]	86 ± 26[a]	71 ± 26[a]	66 ± 15[a]
CAV-vaccinated-challenged	1/10	0 (0, 0.5)[a]	121 ± 75[a, b]	70 ± 33[a]	86 ± 80[a]
Vaccinated-CAV-challenged	5/10	0.25 (0, 7)[a]	117 ± 70[a, b]	91 ± 76[a, b]	90 ± 62[a]
IBDV-vaccinated-challenged	0/10	0 (0, 0)[a]	111 ± 47[a, b]	81 ± 68[a]	84 ± 68[a]
Vaccinated-IBDV-challenged	3/10	0 (0, 10.5)[a]	121 ± 84[a, b]	102 ± 89[a, b]	120 ± 86[a, b]

593 CAV, chicken anaemia virus; IBDV, infectious bronchitis virus; SD, standard deviation. *Unchallenged. Values with

594 the same superscript letter in the same column are not significantly different ($P > 0.05$).

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606 **Table 4**. Tracheal mucosal thicknesses, *M. gallisepticum* wild-type strain Ap3AS loads in the trachea, gross air sac lesions and serum

607 antibodies against *M. gallisepticum* in individual birds in the vaccinated-only and vaccinated-and-challenged groups.

Group	Bird ID	Increased tracheal mucosal thickness	MG Ap3AS detection	MG load in the trachea (40 - Ct)	Air sac lesions	Anti-MG Abs	
						Before challenge	After challenge
Vaccinated-only*	566	+					
	613	+				+	+
	614	+				+	+
	632	+				+	+
	572				+	+	+
	584					+	+
	593					+	+
	624						
	627						
	636						
Vaccinated-and-challenged	630	+					+
	578	+	+	8.92		+	+
	597	+				+	+
	610	+				+	+
	638	+			+	+	+
	569		+	8.11		+	+
	561						+
	573						+
	606						
	607					+	+

608 MG Ap3AS, *M. gallisepticum* wild-type strain Ap3AS; *Unchallenged; Abs, antibodies. An individual bird was regarded as having increased tracheal mucosal

609 thickness if its average tracheal mucosal thickness (across the upper, middle and lower trachea) was greater than the mean + one standard deviation of the average

610 tracheal mucosal thickness for the negative control group.

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612 **Table 5**. Tracheal mucosal thicknesses, *M. gallisepticum* wild-type strain Ap3AS loads in the trachea, gross air sac lesions and serum

613 antibodies against CAV and *M. gallisepticum* in individual birds in the groups infected with CAV.

Group	Bird ID	Increased tracheal mucosal thickness	MG Ap3AS detection	MG load in the trachea (40 - Ct)	Air sac lesions	Anti-CAV Abs		Anti-MG Abs	
						Before challenge	After challenge	Before challenge	After challenge
CAV-vaccinated-challenged	602	+	+	6.31		+	+		
	617	+	+	5.36		+	+		
	621	+	+	5.81		+	+		
	623	+	+	3.79		+	+		
	571		+	4.45	+	+	+		
	616		+	4.75		+	+		
	633					+	+	+	+
	577					+	+	+	+
	615					+	+	+	+
	628					+	+	+	+
Vaccinated-CAV-challenged	570	+	+	9.12	+		+		
	582	+	+	6.73	+		+		
	620	+			+		+		
	564	+	+	9.46	+		+		
	574	+					+		
	631	+	+	6.69			+		+
	589		+	6.09	+		+	+	+
	559		+	0.81			+	+	+
	563						+	+	+
	567						+	+	+

614 CAV, chicken anaemia virus; MG Ap3AS, *M. gallisepticum* wild-type strain Ap3AS; Abs, antibodies. An individual bird was regarded as having increased tracheal

615 mucosal thickness if its average tracheal mucosal thickness (across the upper, middle and lower trachea) was greater than the mean + one standard deviation of

616 the average tracheal mucosal thickness for the negative control group.

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Table 6. Tracheal mucosal thicknesses, *M. gallisepticum* wild-type strain Ap3AS loads in the trachea, gross air sac lesions and serum antibodies against IBDV and *M. gallisepticum* in individual birds in the two groups infected with IBDV.

Group	Bird ID	Increased tracheal mucosal thickness	MG Ap3AS detection	MG load in the trachea (40 - Ct)	Air sac lesions	Anti-IBDV Abs		Anti-MG Abs	
						Before challenge	After challenge	Before challenge	After challenge
IBDV-vaccinated-challenged	611	+	+	8.02		+	+		
	625	+	+	4.48		+	+		
	629	+	+	8.32		+	+		
	562	+				+	+		
	591		+	5.58		+	+		
	595		+	6.97		+	+		
	608					+	+	+	+
	626	+	+	1.74		+	+		+
	585					+	+		+
	599					+	+		
Vaccinated-IBDV-challenged	618	+	+	6.8	+	+	+		
	622	+	+	8.84	+	+	+		
	596	+			+	+	+		
	587	+	+	9.10		+	+		
	635	+	+	7.61		+	+	+	
	581		+	3.78		+	+	+	+
	594		+	4.48		+	+	+	+
	637		+	4.23		+	+	+	+
	592					+	+		
	619					+	+		

IBDV, infectious bursal disease virus; MG Ap3AS, *M. gallisepticum* wild-type strain Ap3AS; Abs, antibodies. An individual bird was regarded as having increased tracheal mucosal thickness if its average tracheal mucosal thickness (across the upper, middle and lower trachea) was greater than the mean + one standard deviation of the average tracheal mucosal thickness for the negative control group.

Figures

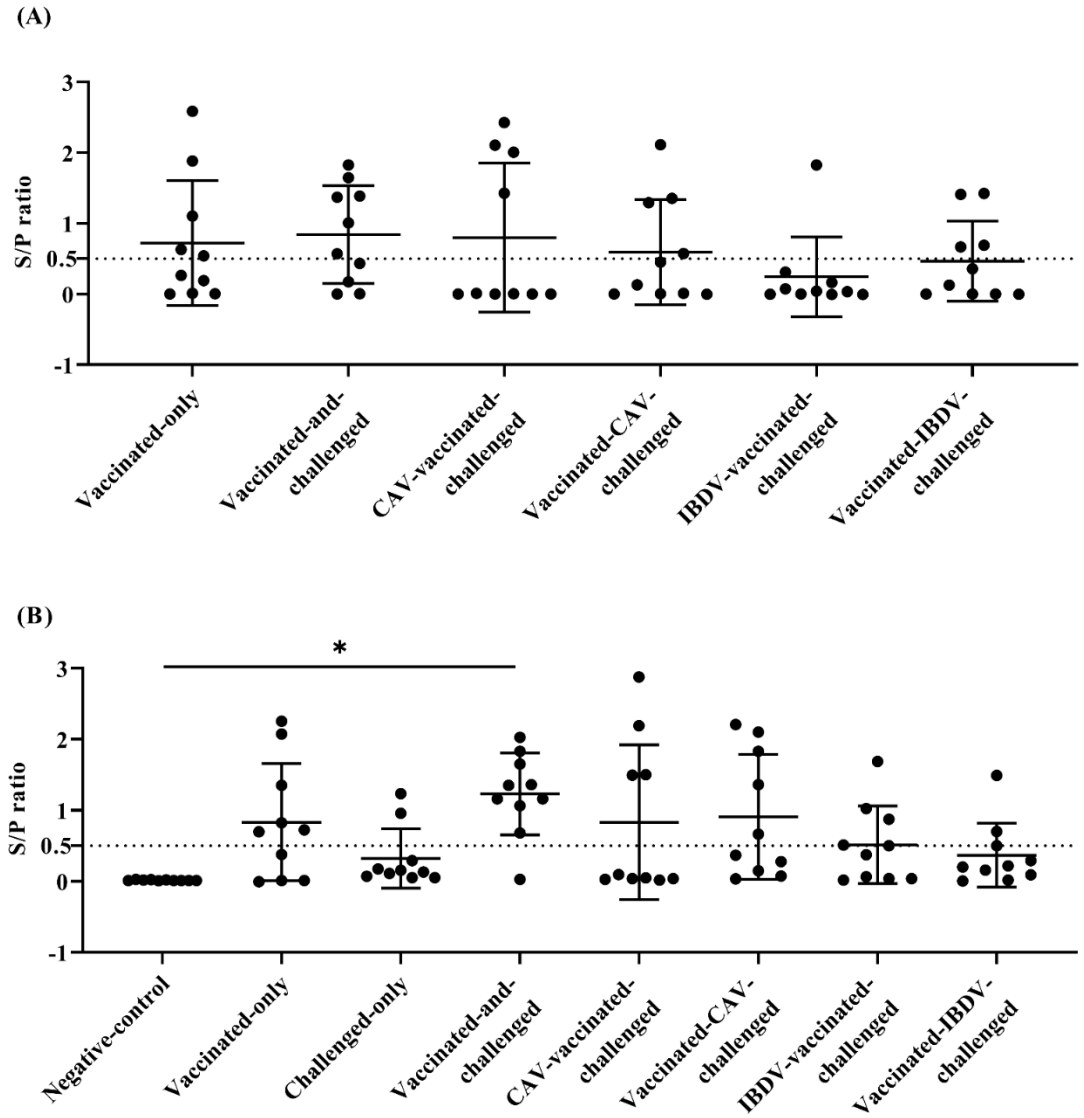
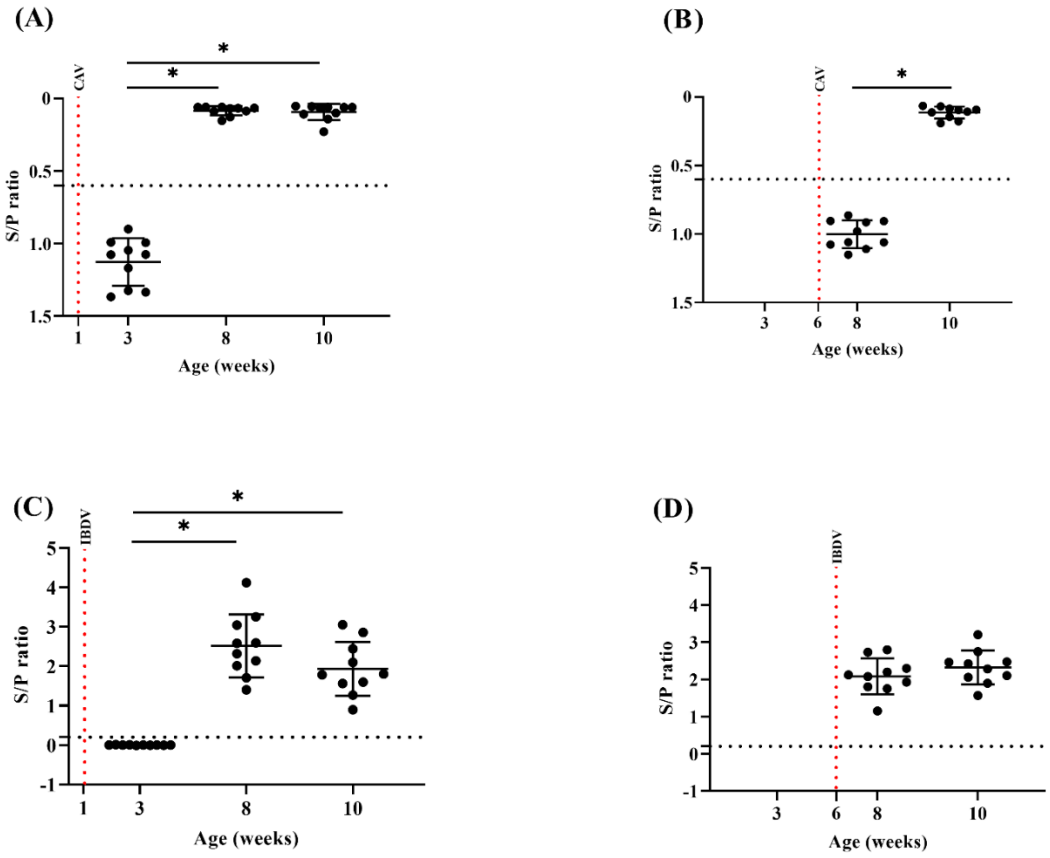


Figure 1. Serum antibodies against *M. gallisepticum*, as detected by ELISA, for individual birds in each group at **(A)** 5 weeks after vaccination (before challenge) and **(B)** 7 weeks after vaccination (2 weeks after challenge)**.** A S/P ratio of ≥ 0.5 (horizontal dotted line) was considered positive, and * indicates a significant difference ($P < 0.05$) between groups. Horizontal bars indicate the mean S/P ratio and the standard deviation for each group. All birds were negative by ELISA for antibodies against *M. gallisepticum* before vaccination (results not shown).



631 **Figure 2**. Serum antibodies against CAV, as detected by ELISA, for individual birds in (**A)** the
632 CAV-vaccinated-challenged group and (**B**) the vaccinated-CAV-challenged group, and serum
633 antibodies against IBDV, as detected by ELISA, for individual birds in (**C**) the IBDV-vaccinated-
634 challenged group and (**D**) the vaccinated-IBDV-challenged group. A S/P ratio of ≤ 0.6 (horizontal
635 dotted line) was considered positive in the anti-CAV ELISA (**A** and **B**) and a S/P ratio of > 0.2
636 (horizontal dotted line) was considered positive in the anti-IBDV ELSA (**C** and **D**)**.** The chickens
637 in (**A**) the CAV-vaccinated-challenged group and (**C**) the IBDV-vaccinated-challenged group
638 were infected with CAV or IBDV, respectively, at 1 week of age (red vertical dotted line), while
639 those in (**B**) the vaccinated-CAV-challenged group and **(D)** the vaccinated-IBDV-challenged
640 group were infected with CAV or IBDV, respectively, at 6 weeks of age (red vertical dotted line).

All the chickens were vaccinated with ts-304 at 3 weeks of age, challenged with virulent *M. gallisepticum* strain AP3AS at 8 weeks of age, and euthanised at 10 weeks of age. Bars indicate the mean S/P ratio and the standard deviations for each group at each age and * indicates a significant difference between groups ($P < 0.05$). ELISAs to detect antibodies against CAV and IBDV were not performed on **(B)** the vaccinated-CAV-challenged group or **(D)** the vaccinated-IBDV-challenged group at 3 weeks of age.

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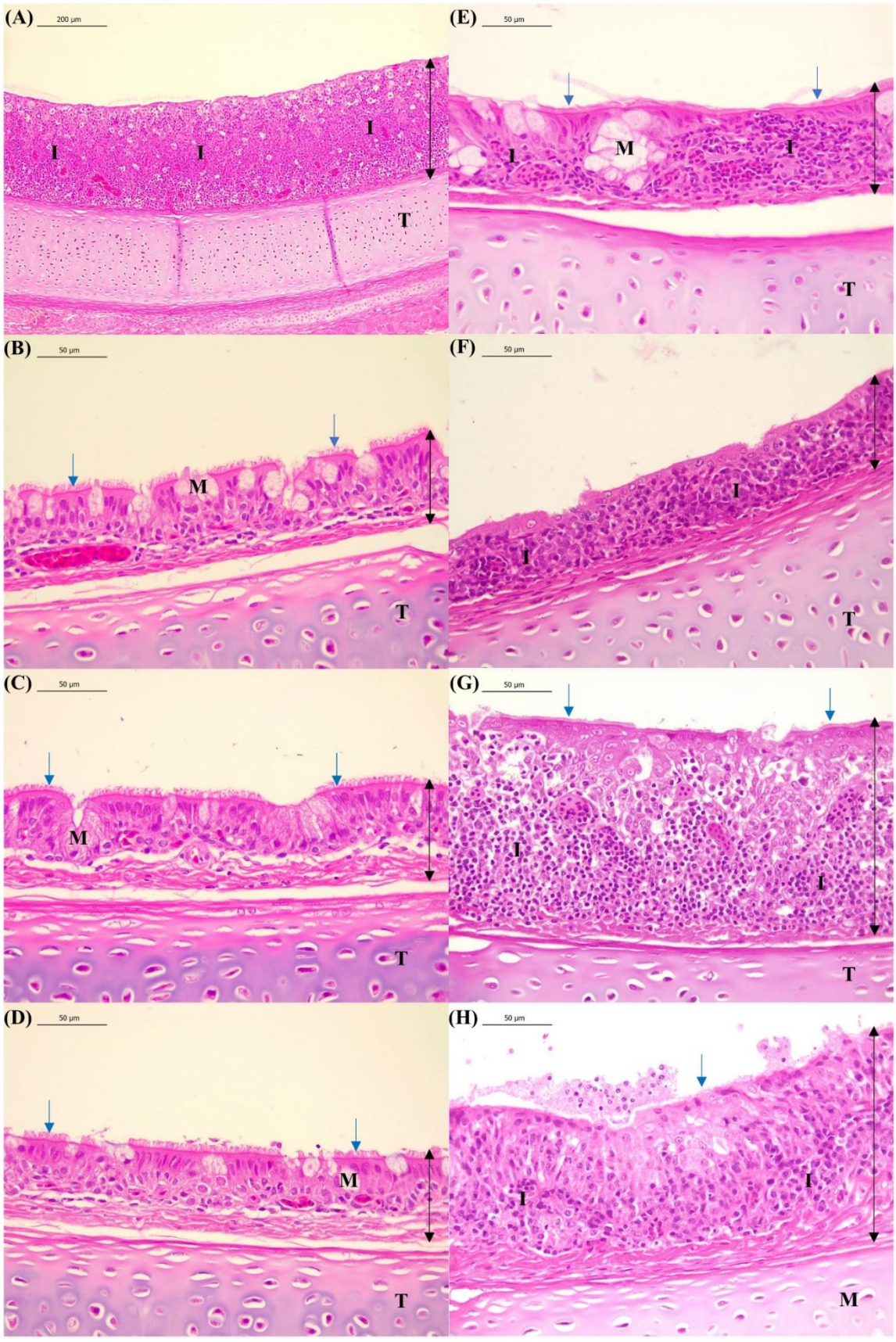
(A) 200 µm
I
I
I
T
(E) 50 µm
I
M
I
T
(B) 50 µm
M
T
(F) 50 µm
I
I
T
(C) 50 µm
M
T
(G) 50 µm
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I
T
(D) 50 µm
M
T
(H) 50 µm
I
I
M

Figure 3. Photomicrographs of upper tracheal cross sections from representative birds in the (**A**) challenged-only, (**B**) negative control, (**C**) vaccinated-only, (**D**) vaccinated-and-challenged, (**E**) CAV-vaccinated-challenged, (**F**) vaccinated-CAV-challenged, (**G**) IBDV-vaccinated-challenged and (**H**) vaccinated-IBDV-challenged groups after staining with H & E. Diffuse inflammatory cell infiltration, increased mucosal thickness, loss of cilia and disintegrated epithelia are visible in **A**, **E**, **F**, **G** & **H,** while pseudostratified ciliated columnar epithelia, intact cilia (**blue arrow**) with numerous mucous glands (**M**) are visible in **B**, **C** & **D**. **T**, cartilage; **I**, Inflammatory cell infiltration; **double headed arrow,** thickness of the mucosa. Bar in **A** = 200 µm. Bar in **B**-**H** = 50 µm.