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Commensal gut microbiota can modulate adaptive immune responses in chickens vaccinated with whole inactivated avian influenza virus subtype H9N2

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ABSTRACT

Variations in the composition of commensal gut microbiota have been reported to be major contributors to differences in responses to vaccination among individuals. In chickens, there is limited information on the role of gut microbiota in responses to vaccination. The current study studied the role of gut microbiota in cell- and antibody-mediated immune responses to vaccination with a whole inactivated avian influenza virus, subtype H9N2. A total of 166 one-day-old specific pathogen free layer chickens (SPF) were randomly assigned to treatments, where a combination of antibiotic depletion, and probiotics (a combination of five *Lactobacillus* species) or fecal microbial transplant (FMT) reconstitution were used to study the dynamics of cell- and antibody-mediated immune responses to primary and secondary vaccinations at days 15 and 29 of age, respectively. Overall, at days 7 and 14 post primary vaccination (p.p.v.), administration of probiotics to non-depleted chickens resulted in significantly higher mean hemagglutination (HI) titre compared to antibiotic treated chickens. Furthermore, at day 21 p.p.v., chickens treated with probiotics or FMT post-antibiotic treatment showed a significantly higher mean HI titre compared to non-depleted chickens treated with probiotics. At day 7 p.p.v., a significantly higher virus specific IgM and IgG titres were observed in non-depleted chickens administered with probiotics compared to antibiotic depleted chickens, and a significantly higher IgG titre was observed in chickens treated with FMT following antibiotic treatment compared to only antibiotic treatment. Analysis of interferon gamma expression in splenocytes to assess cell-mediated immune responses showed a significantly lower expression in antibiotic-treated chickens compared to non-depleted chickens and FMT reconstituted chickens. Taken together, the current study suggests that shifts in the composition of gut microbiota of chickens may result in changes in cell- and antibody-mediated immune responses to vaccination against influenza viruses. Further studies will be needed to highlight the mechanisms involved in this modulation.

1. Introduction

Avian influenza viruses (AIV), which are categorized into high (HPAI) and low (LPAI) pathogenicity viruses pose a significant public health threat [1–3]. Some LPAI viruses such as H9N2 have reached a panzootic proportion [4], and can provide internal genes to HPAI viruses, as in the case of H9N2 that provided internal genes to HPAI viruses of humans such as H5N1, H7N9, H10N8 and H5N6 [5–8]. Although vaccination has been used as a method for controlling the spread of AIV such as H9N2, complementary strategies should also be envisaged to enhance vaccine efficacy [9]. Various natural or synthetic adjuvants mimic molecular structures of commensal microbiota, and they can stimulate cell surface, cytoplasmic and endoplasmic pattern recognition receptors (PRRs) resulting in enhanced vaccine efficacy and reduced viral shedding from chickens [10,11]. Even though there is limited information on the role of commensal microbiota as a vaccine adjuvant, this suggests a potential role for commensal microbiota in vaccine efficacy; understanding the role of commensal gut microbiota of chickens may provide additional insight into the development of new and more effective adjuvants.

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The lumen of the gastrointestinal tract of chickens contains trillions of commensal bacteria, and the microbiome, which is the collection of genes encoded by members of the microbiota that far exceeds the number of genes encoded by the host genome [12,13]. Among the diverse functions of the gut microbiota are the production of energy-rich short chain fatty acids (SCFAs) [14–16], gut morphological development [17,18], nutrient extraction, absorption and utilization [19–21], and development/modulation of the host immune system [22]. The adaptive immune system also plays a critical role in regulating the composition of microbiota and has been suggested to have evolved in part to enable the mutual relationship of the microbiota and the host [23–25]. However, recent research suggests that commensal microbiota may also play an important role in the development and regulation of adaptive immune responses. In human populations, individual variations in response to various vaccines have been attributed to differences in the composition of commensal microbiota, among other factors [26].

Gut microbiota can influence responses induced by influenza virus vaccines in other species, while there is limited information in chickens. Both germ-free and antibiotic-treated mice have been shown to have significantly impaired IgG and IgM responses to a seasonal influenza vaccine, in which Toll-like receptor (TLR)-mediated sensing (mainly TLR5-mediated sensing of flagellin), was necessary for antibody responses [27]. However, other microbial-associated molecular patterns (MAMPs) originating from commensal microbiota can affect vaccine efficacy via other mechanisms involving PRRs such as TLR4 [28] and nucleotide oligomerization domain-2 (NOD2) [29]. Furthermore, commensal microbiota producing metabolites such as SCFAs resulting from fermentation of dietary fiber in the hind gut can regulate B cell gene expression responses for optimal antibody production, and pathogen-specific antibody response against *Citrobacter rodentium* [30]. In chickens, administration of probiotics has been shown to modulate total and antigen-specific antibody responses [31–33]. However, the role of gut microbiota in induction of antibody responses to influenza viruses in chickens is not very well understood. Therefore, the current study was conducted to assess the role of commensal gut microbiota of chickens in induction of cell- and antibody-mediated immune responses following influenza virus subtype H9N2 vaccination using antibiotic-depletion of the microbiota to induce dysbiosis in the gut.

2. Materials and methods

2.1. Experimental design

All experimental procedures were approved by the University of Guelph Animal Care Committee and conducted according to specifications of the Canadian Council on Animal Care. A total of 166 one-day-old specific pathogen free layer chickens (SPF) (CFIA, Ottawa Laboratory, Nepean, ON, Canada) were randomly assigned to seven treatment groups. The following treatments were used. (1) NEG-CON (n = 26); a group where no antibiotic depletion, no probiotics (PROB) or fecal microbial transplant (FMT), and no vaccination were used, (2) POS-CON (n = 10); a group with no antibiotic depletion, no PROB or FMT, but vaccination, (3) PROB (n = 26); a group with no antibiotic depletion but bi-weekly administration of a cocktail of five lactobacilli species (*Lactobacillus salivarius*, *L. johnsonii*, *L. reuteri*, *L. crispatus*, and *L. gasseri*) and vaccination, (4) ABX (n = 26); a group with antibiotic depletion from day 0–12 and vaccination, (5) ABX + PROB (n = 26); a group with ABX and treated with PROB from day 12–15 and vaccination, (6) ABX + FMT (n = 26); a group treated with ABX and FMT administration from day 12–15 and vaccination, and (7) ABX-D35 (n = 26); a group treated with antibiotics from day 0–35. Primary vaccination was performed at day 15 of age and secondary vaccination was performed at day 29 of age. Weekly serum samples were collected for the assessment of influenza-specific IgG and IgM responses until day 43 of age.

2.2. Antibiotics, probiotics and fecal microbial transplant administration

Chickens in groups with antibiotics were gavaged twice daily with a cocktail of antibiotics in 10 mL of water per kg of body weight as described previously [34]. Antibiotics cocktail included 5 mg/ml vancomycin, 10 mg/ml neomycin, 10 mg/ml metronidazole and 0.1 mg/ml amphotericin-B, and 1 g/l of ampicillin continuously provided in drinking water. Lactobacilli bacteria were first individually grown on De Man, Rogosa and Sharpe (MRS) medium overnight and 10^9 CFU/mL of each were mixed, and 1 mL of cocktail was administered daily for four days to each chicken by gavaging to the crop using a 1 mL syringe. Chickens in the FMT group were gavaged with FMT preparation that was prepared according to the method of Li and colleagues [35]; where 8 gm of cecal contents from age matched chickens was homogenized in 10 mL of sterile PBS and centrifuged at 1600*g* for 30 s at 4 °C to pellet the particulate matter. The supernatant was collected and the optical density (OD) determined. An OD of 0.5 represented 10^8 cells and each chicken received 1×10^9 bacterial cells every day for four days post antibiotic treatment. Cecal microbial genomic DNA extraction was performed using QIAamp DNA Stool mini kit (Qiagen, Toronto, Canada) according to the manufacturer's instructions, and DNA concentrations were measured with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The total cecal bacterial population was determined using quantitative real-time polymerase chain reaction (qRT-PCR) using universal primers described in Table 1.

2.3. Virus propagation and vaccine preparation

Propagation of the virus, A/turkey/Wisconsin/1/1966 (H9N2), in embryonated SPF eggs was performed as previously described [34]. Inactivation of the vaccine preparation was performed using beta-propiolactone (BPL) as described previously [36]. Briefly, one part of 0.5 M disodium phosphate was added to 38 parts of virus containing allantoic fluid followed by drop-wise addition of one part 2% BPL solution with continued stirring and incubation for 30 min on ice. The solution was then placed in a 37 °C water bath for 2 h with 15 min mixing intervals. The pH of the mixture was

Table 1
Primer sequences used in qRT-PCR.

Gene	Sequences	Annealing temp. (°C)	Reference
β-actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	60	[47]
IFN-γ	F ACACTGACAAGTCAAAGCCGACA R AGTCGTTCATCGGGAGCTTGGC	60	[40]
Universal	F: AAACTCAAAKGAATTGACGG R: CTCACRRCACGAGCTGAC	61	[48]
Firmicutes	F: TGAAACTYAAAGGAATTGACG R: ACCATGCACCACCTGTC	61	[48]
Bacteroidetes	F: CRAACAGGATTAGATACCCT R: GGTAAGGTTCCTCGCGTAT	61	[48]
α-Proteobacteria	F: CIAGTGTAGAGGTGAAATT R: CCCCGTCAATTCCTTTGAGTT	61	[48]
γ-Proteobacter	F: TCGTCAGCTCGTGTYGTGA R: CGTAAGGGCCATGATG	61	[48]
Lactobacillus group	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	58	[49]

adjusted to 7.3–7.4 using 7% sodium bicarbonate solution, and stored at 4 °C until confirmation of inactivation, which was confirmed using both embryonated eggs and Madin-Darby canine kidney (MDCK). Chickens were vaccinated intramuscularly with 15 μg of the whole inactivated virus, corresponding to approximately 960 HA units.

2.4. Hemagglutination inhibition assay

A two-fold serial dilution was performed with 50 μL serum samples followed by 30 min of incubation with 50 μL (8 HA units) of H9N2 virus at room temperature in 96-well V-bottom plates (Corning Inc., Corning, New York, USA). Chicken red blood cells (RBCs) at 0.5% were then added and plates were further incubated for 30 min. Hemagglutination inhibition (HI) titres were determined as the reciprocal of the greatest sample dilution resulting in complete inhibition of the RBCs.

2.5. Measurement of anti-H9N2 IgG and IgM by ELISA

Virus-specific IgG and IgM titres in serum were determined by indirect ELISA as described previously [34]. Briefly, 96-well ELISA plates (Nunc MaxiSorp™, Thermo Fisher Scientific Inc., Mississauga, Ontario, Canada) were coated overnight at 4 °C with 100 μL per well of whole inactivated H9N2 virus (800 ng/100 μL of carbonate-bicarbonate buffer, pH 9.6). After washing the plates three times with 300 μL PBS containing 0.05% Tween 20 (PBS-T), 100 μL of blocking solution (PBS-T containing 0.25% fish gelatin) was added and plates were incubated for 1 h at 37 °C. After washing the plates three times, 100 μL two-fold diluted serum samples starting at 1:800 dilution (1:800 to 1:409,600) were added and the plates were incubated at room temperature for 1 h. After washing three times, 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-chicken IgY was added into each well followed by incubation of the plates at 37 °C for 1 h. After further washing, 100 μL of an HRP substrate solution (ABTS peroxidase substrate system) (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) was added to each well, followed by an incubation period of 30 min in the dark at room temperature and reading of the optical density (OD) at 405 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, Vermont USA). The end-point titre calculation was performed as previously described [37].

2.6. Virus neutralization assay

Virus neutralization (VN) was performed for D7 post-secondary vaccination samples (Day 36 of age prior to infection) as described previously [38] with some modifications. Briefly, a 2-fold serial dilution of serum was incubated with 400 $TCID_{50}$/mL of H9N2 virus for 1 h at 37 °C. One hundred microliters of the mixture were then added to MDCK cells that were cultured for 24 h at 37 °C followed by further incubation for 72 h at 37 °C. The reciprocal of the highest serum dilution to inhibit cytopathic effect was used to calculate the VN titre.

2.7. Cell-mediated immune response

At 5 days post-secondary vaccination, spleen mononuclear cells were prepared from 5 chickens per treatment as described previously [39] and seeded in 48-well flat bottom plates at a density of 5×10^6 cells/well and incubated at 41 °C and 5% CO_2. Mononuclear cells were then stimulated with 1 μg/mL of BPL inactivated H9N2 virus. At 24 h post-stimulation, cells were collected and RNA extraction, cDNA synthesis and qRT-PCR for the quantification of interferon (IFN)-γ were performed as described previously [34]. Primers for IFN-γ quantification using qRT-PCR are presented in Table 1. At 48 h and 72 h post-stimulation, supernatant was collected for the assessment of IFN-γ production using a chicken IFN-γ sandwich ELISA kit as per manufacturer's recommendations (Invitrogen™).

2.8. Calculations and statistical analysis

Relative expression of IFN-γ with β-actin as a housekeeping gene, and quantification of cecal bacterial populations relative to total microbial population was conducted as described previously [40]. Comparison of multiple treatments for all data (including cecal bacterial populations, HI titre, IgG and IgM titres in serum, VN, relative expression of IFN-γ, and serum IFN-γ) were performed with one-way ANOVA when data was normally distributed, and Kruskal-Wallis test was used when data was not normally distributed. Differences were considered significant at $P < 0.05$. Data for HI titre, IgM and IgG are described in post-primary vaccination time points (p.p.v.).

3. Results

3.1. Microbial composition

Analysis of microbial composition at 15 days of age showed that treatment of chickens with antibiotics resulted in significantly lower phyla Firmicutes and Bacteriodetes, and class Alphaproteobacteria ($P < 0.05$), while treatment with either probiotics or FMT after antibiotics resulted in significantly higher phyla Firmicutes, Bacteriodetes, and class Alphaproteobacteria compared to antibiotic-treated chickens ($P < 0.05$). Treatment with probiotics and FMT to antibiotic treated chickens also resulted in a significantly higher Lactobacillus group ($P < 0.05$). At 35 days of age, chickens treated with antibiotics for 35 days showed a significantly reduced phyla Firmicutes, and Bacteroidetes, class Alphaproteobacteria and Lactobacilli group ($P < 0.05$). For this time point, chickens treated with antibiotics for 15 days did not show a significant difference in phylum Bacteroidetes compared to the control and PROB groups ($P > 0.05$), while a significantly higher class Alphaproteobacteria was observed compared to control, ABX + PROB and ABX + FMT treatments ($P < 0.05$) (Fig. 1).

3.2. Hemagglutination inhibition and virus neutralization antibody titers

There was no detectable agglutination in the NEG-CON treatment at all time points. At D7 p.p.v., a significantly higher mean HI titre was observed in the PROB treatment compared to all treatments ($P < 0.05$) except for POS-CON ($P > 0.05$). At D14 p.p.v., a significantly higher mean HI titre was observed in the PROB treatment compared to all treatments ($P < 0.05$) except for POS-CON and ABX treatments ($P > 0.05$). There was no significant difference among treatments in mean HI titre at D21 p.p.v., while at D28 p.p.v., a significantly higher mean HI titre was observed in ABX + PROB and ABX + FMT treatments compared to PROB treatment ($P < 0.05$), while no significant difference was observed among all other treatment comparisons ($P > 0.05$) (Fig. 2). Virus neutralization measured at D7 post-secondary vaccination (D21 p.p.v.) showed a significantly higher neutralizing antibody titres in the ABX + PROB, ABX + FMT and ABX-D35 treatments compared to all other treatments ($P < 0.05$) (Fig. 3).

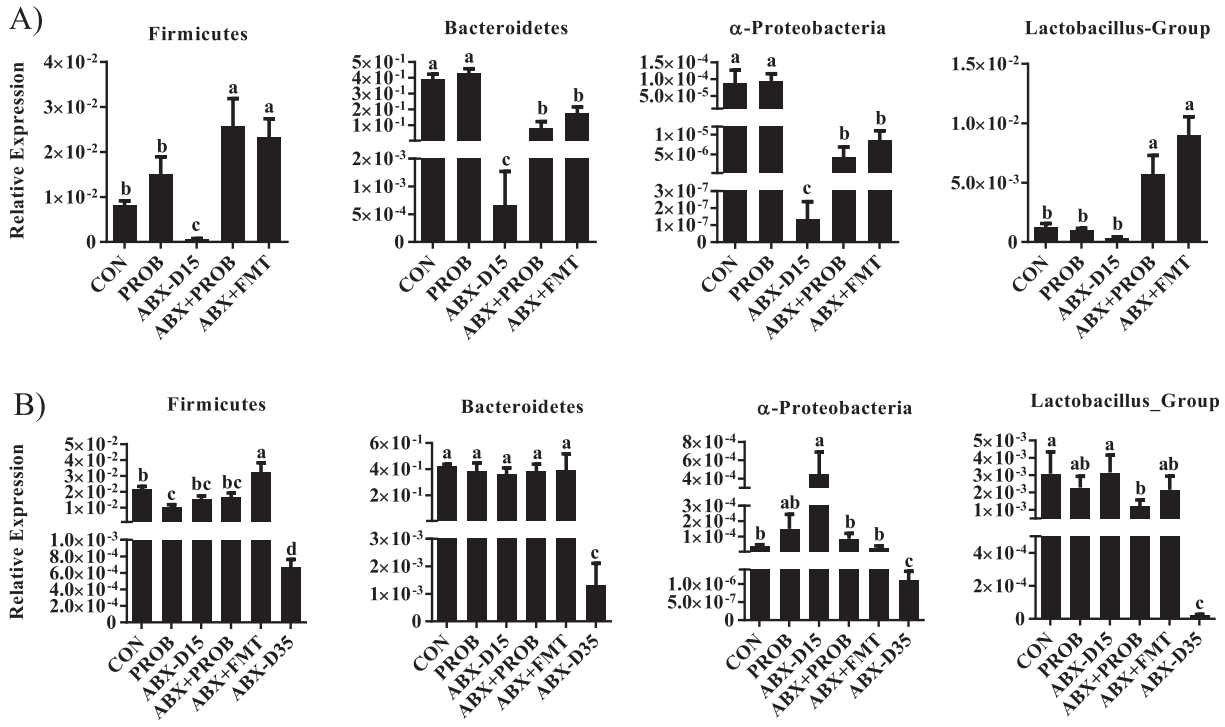

Fig. 1. Bacterial populations in chickens were assessed at the end of antibiotic treatment (A) Day 15 and (B) Day 35 of age using quantitative real-time PCR. Relative expressions were calculated relative to total bacteria. ABX-D15 represents a treatment where chickens received with a cocktail of antibiotics twice daily for 15 days. ABX + PROB and ABX + FMT represent treatments where chickens were administered with a cocktail of antibiotics for 12 days and reconstituted with a cocktail of five *Lactobacillus* and a fecal microbial transplant (FMT), respectively. ABX-D35 represents chickens treated with a cocktail of antibiotics twice daily for 35 days. Bars with different symbols differ significantly from each other (n = 10/group; $P < 0.05$). Bars not sharing a common letter are significantly different from each other.

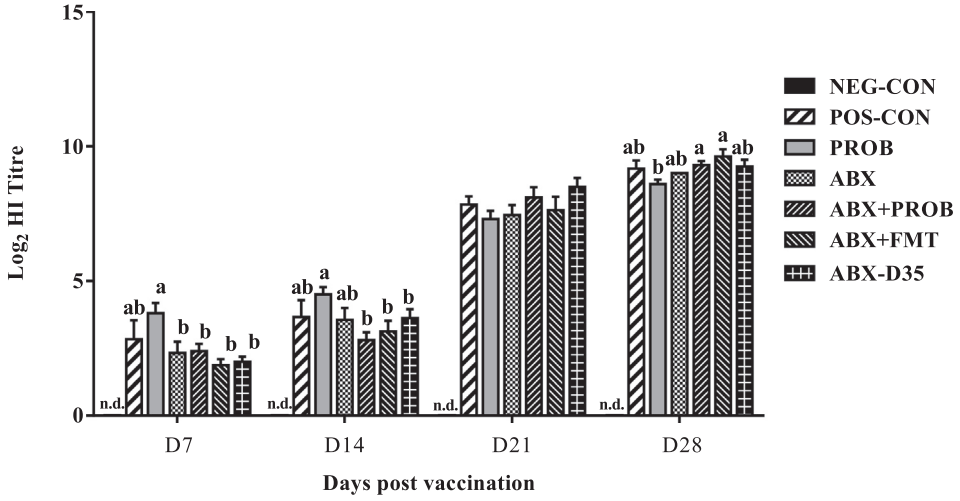

Fig. 2. Hemagglutination inhibition (HI) antibody titers in chickens belonging to various groups (n = 10/group) at days 7, 14, 21, and 28 post primary vaccination. Bars with different symbols in a time point differ significantly from each other ($P < 0.05$). **n.d.** = not detected. Bars not sharing a common letter are significantly different from each other.

3.3. H9N2 virus-specific IgM and IgG responses

At D7 p.p.v., a significantly higher mean IgM titre was observed in the PROB treatment compared to ABX and ABX + FMT treatments ($P < 0.05$), while no significant differences were observed among other treatment comparisons ($P > 0.05$). No significant difference in mean IgM titre was observed among treatments at D14 p.p.v. ($P > 0.05$). At D21 p.p.v., a significantly higher mean IgM titre was observed in ABX + FMT and ABX-D35 treatments compared to PROB treatment ($P < 0.05$), while no significant difference was observed among all other treatment comparisons ($P > 0.05$). There was no significant difference among treatments at D28 p.p.v. ($P > 0.05$).

At D7 p.p.v., a significantly higher IgG titre was observed in the POS-CON and PROB treatments compared to ABX + FMT and ABX-D35 ($P < 0.05$), while no significant difference was observed among all other treatment comparisons ($P > 0.05$). At D14 p.p.v., a significantly higher mean IgG titre was observed in the ABX + FMT compared to ABX, ABX + PROB and ABX-D35. There was no significant difference in mean IgG titre among treatments at D21 p.p.v., and

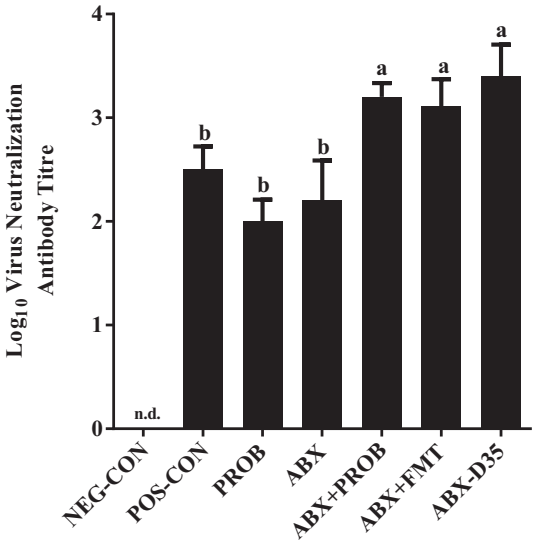

Fig. 3. Virus neutralization (VN) assay of antibody responses in chickens (n = 10/group) at day 21 post primary vaccination. Bars with different symbols in a time point differ significantly from each other ($P < 0.05$). **n.d.** = not detected. Bars not sharing a common letter are significantly different from each other.

at D28 p.p.v., a significantly lower mean IgG titre was observed in the PROB treatment compared to all treatments ($P < 0.05$), except for POS-CON ($P > 0.05$) (Fig. 4).

3.4. Cell-mediated responses

Analysis of IFN-γ expression in splenocytes showed a significantly higher expression of IFN-γ in both Pos-CON, PROB, ABX + FMT and ABX-D35 treatments compared to both Neg-CON and ABX treatments ($P < 0.05$). Analysis of IFN-γ protein using ELISA at 48 h post-stimulation showed a significantly higher IFN-γ protein in the ABX + FMT and ABX-D35 treatments compared to Neg-CON, ABX, and ABX + PROB treatments ($P < 0.05$), while at 72 h post-stimulation, significantly higher level of IFN-γ protein was observed in the ABX-D35 compared to Neg-CON and ABX treatments ($P < 0.05$) (Fig. 5).

4. Discussion

Influenza viruses, mainly LPAI viruses, infect the gastrointestinal tract (GIT) in addition to the upper respiratory tract, both of which are inhabited by diverse microbiota. The GIT of chickens is inhabited by trillions of microbes, which benefit the host in different ways including the development and modulation of the immune system [22]. The role of commensal gut microbiota in vaccine-induced responses to influenza virus and other pathogens is poorly understood. Recent studies have shown that microbiota can influence vaccine-induced responses to influenza virus. Germ-free or antibiotic-treated mice have significantly impaired IgG and IgM responses to seasonal influenza vaccines [27]. Therefore, the current study was designed to assess the role of commensal gut microbiota of chickens in cell- and antibody-mediated immune responses to a whole inactivated influenza virus vaccine using a model of antibiotic-depletion of the gut microbiota, with modulation of microbial composition using either a combination of five probiotics or FMT.

In the current study, administration of five *Lactobacillus* species to non-depleted chickens resulted in higher HI titre at days 7 and 14 p.p.v. compared to chickens that were treated with antibiotics to induce dysbiosis of the gut microbiota regardless of reconstitution of the microbiota with either probiotics or FMT. In a human study examining the efficacy of *Lactobacillus rhamnosus* GG as an adjuvant to influenza vaccine, administration of 1.0×10^{10} CFU of *L. rhamnosus* GG twice daily induced a significantly higher HI titre at 28 days post-vaccination compared to those receiving placebo [41]. A strong correlation between Toll-like receptor 5, which recognizes flagellin, and the magnitude of HI titers 4 weeks post-vaccination was observed [42], suggesting that commensal gut microbiota play an important role in vaccine efficacy. This may be the mechanism by which shifts in the microbial composition post-administration of the lactobacillus cocktail in the current study resulted in higher HI titres, even though other mechanisms involving other PRRs cannot be ruled out. Furthermore, the same study showed macrophages as a critical antigen presenting cell mediating TLR5 dependent modulation of antibody responses to a trivalent inactivated influenza vaccine (TIV) [42]. Despite the lack of significant differences in HI titres in the present study at day 21 p.p.v. among treatments, VN results indicated that at this time point, a significantly higher VN antibody titre was present in groups that were reconstituted with either the combination of lactobacilli or FMT after treatment with antibiotics. We have previously showed that reconstitution of antibiotic treated chickens with either probiotics or FMT resulted in recovery of innate response mechanisms including some key cytokines and tissue remodeling processes of the GIT [43]. Another study using West Nile virus in mice had demonstrated PRRs of the innate immune system to be key modulators of virus-neutralizing antibody responses; mice with compromised innate response signaling had reduced virus neutralization capacity compared to wild type mice [44]. Therefore, administration of probiotics or FMT may have enhanced the innate signaling mechanisms that in turn resulted in enhanced virus neutralization capacity in the current study.

Administration of a cocktail of lactobacilli to non-depleted chickens also resulted in a significantly higher mean IgM antibody titres compared to antibiotic-treated chickens (that did or did not receive probiotics). Furthermore, depletion of gut microbiota and administration of FMT as well as administration of antibiotics for 35 days resulted in a significantly lower mean IgG antibody titers compared to non-depleted chickens with or without administration of probiotics in the current study. In a human clinical trial, after influenza vaccination, administration of the probiotic bacterium *L. fermentum* CECT571 after influenza vaccination resulted in a significantly higher total IgM compared to subjects receiving placebo, while no significant difference was observed in influenza specific IgM antibodies [45]. In a separate study, volunteers receiving *Bifidobacterium animalis* ssp. *lactis* and *Lactobacillus paracasei* subsp. *paracasei* developed significantly higher influenza specific IgG titers compared to those receiving a placebo [46]. Similar to our findings in the current study, Oh et al. [27] observed that treatment of SPF mice with a broad-spectrum antibiotic cocktail resulted in substantial reduction in immune responses to vaccination with trivalent inactivated influenza vaccine (TIV) at day 7 post-vaccination. Responses steadily increased to levels comparable to untreated mice by day 28 post vaccination. These findings suggest that modulation of the gut microbiota of chickens, either using probiotics or antibiotics can significantly impact antibody-mediated immune response to influenza virus vaccination.

Expression of IFN-γ by splenocytes isolated from immunized chickens and re-stimulated in vitro with vaccine antigen, showed that treatment of chickens with antibiotics resulted in impaired expression of IFN-γ compared to non-depleted chickens. However, the administration of probiotics and FMT after antibiotic treatment restored the expression of IFN-γ to that of the control group. This suggests that the gut microbiota may play an important role in cell-mediated immune responses. A study in mice showed that the gut microbiota in association with TLR5

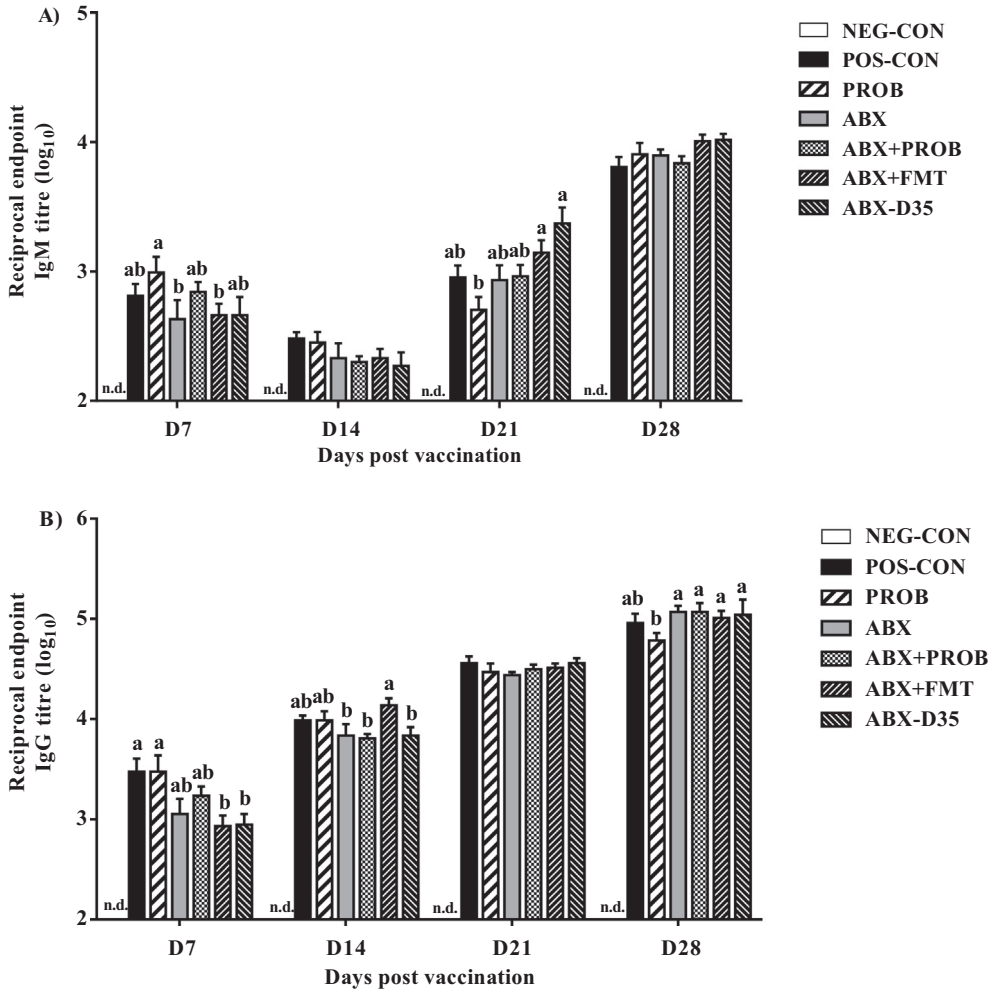

Fig. 4. Serum IgM (A) and IgG (B) antibodies against H9N2 virus. Serum samples were collected at days 7, 14, 21, and 28 post primary vaccination (day 15 of age) from chickens (n = 10) vaccinated with a whole inactivated influenza virus subtype H9N2. Bars with different symbols in a time point differ significantly from each other ($P < 0.05$). **n.d.** = not detected. Bars not sharing a common letter are significantly different from each other.

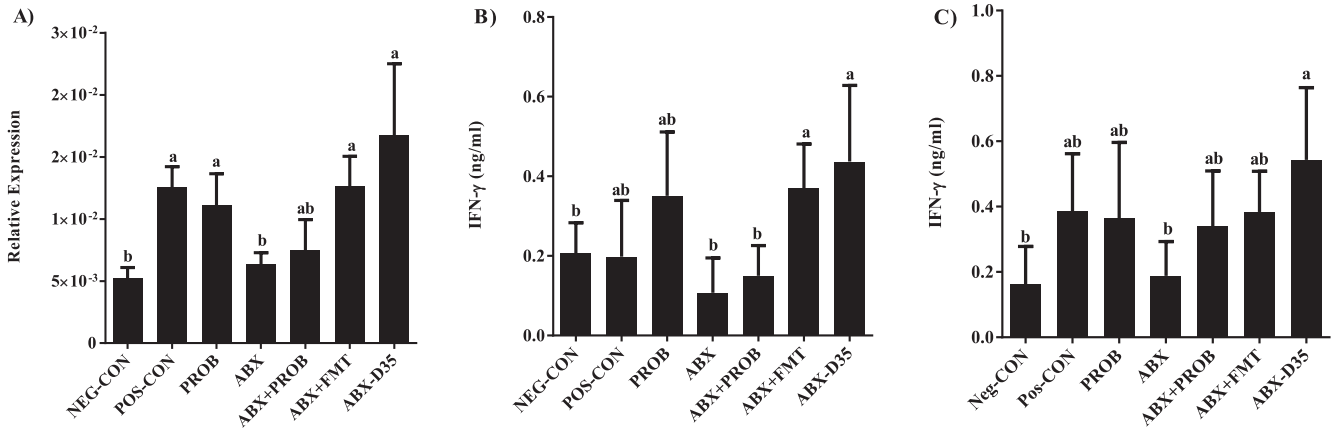

Fig. 5. IFN-γ production in splenocytes stimulated with whole inactivated influenza virus subtype H9N2. Splenocytes were isolated from 5 chickens per treatments at 5 days post-secondary vaccination (Day 26 post primary vaccination). (A) Relative IFN-γ mRNA expression of splenocytes relative to β-actin was calculated using quantitative real-time PCR. Concentration of IFN-γ, measured in triplicates, in the supernatant of splenocytes at 48 h (B) and 72 h (C) post-stimulation. Bars with different symbols in a time point differ significantly from each other ($P < 0.05$). Bars not sharing a common letter are significantly different from each other.

signaling plays an important role in differentiation or function of memory B cells following vaccination with TIV [27]. In agreement with the current study, re-stimulation of splenocytes from antibiotic-treated mice vaccinated intranasally vaccinated with cholera toxin showed a significantly reduced IFN-γ production compared to conventional mice [29], suggesting that intact gut microbiota may be necessary for the generation of long term immune memory.

In conclusion, commensal gut microbiota of chickens may play an important role in induction of adaptive immune responses to influenza virus vaccination both in magnitude and functionality of antibodies produced and possibly in cell-mediated immune responses. However more studies investigating the mechanisms that link innate and adaptive responses are necessary to further understand how modulation of the gut microbiota can be used to enhance protective responses to influenza virus vaccines. Furthermore, this study used an empirical approach by using antibiotic depletion, and probiotics and FMT reconstitution. In order to understand the role of gut microbiota in influenza vaccine immunogenicity, a more targeted approach may be needed to understand the specific link between influenza vaccine and selected members of the gut microbiota. This finding can then be utilized in developing adjuvants either from probiotics, their cell wall, cytoplasmic components, and metabolites, alone, or in combination.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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