

# Protection against avian pathogenic *Escherichia coli* and *Salmonella* Kentucky exhibited in chickens given both probiotics and live *Salmonella* vaccine

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**ABSTRACT** Commercial poultry farms are increasingly threatened by bacterial infections from avian pathogenic *Escherichia coli* (**APEC**) and broad-host *Salmonella* serovars. Recombinant attenuated *Salmonella* vaccines (**RASV**) elicit cross-reactive immune responses against APEC in chickens; however, assessment of broad protection is lacking. Probiotics boost chicken immunity and improve vaccination responses. The objective of this study was to determine whether the RASV, the probiotics, or their combination had protection against APEC and *Salmonella*. White Leghorn chicks were randomly placed into 4 groups: no treatment (**CON**), probiotics (**PRO**), RASV (**VAX**), or both prophylactics (**P + V**). Chicks in the PRO and P + V groups were fed probiotics daily, beginning at the age of 1-day-old. Chicks in the P + V and VAX groups were orally inoculated with RASV at the age of 4 D and boosted 2 wks later. Total and antigen-specific IgY responses to *Salmonella* (lipopolysaccharide [**LPS**]) and *E. coli* (IroN and IutA) were measured in serum samples via ELISA.

Bactericidal potential of both serum and blood against 42 APEC isolates comprising 25 serotypes was assessed *in vitro*. *In vivo* protection against APEC was evaluated by air sac challenge with APEC  $\chi$ 7122 (O78:K80), gross pathological lesions were scored, and bacterial loads were enumerated. In a second similar study, birds were orally challenged with *S. Kentucky* (CVM29188), and feces were enumerated for *Salmonella* at multiple time points. Vaccination elicited significant LPS-specific antibodies regardless of probiotics ( $P < 0.0001$ ). Chicks in the P + V group demonstrated increased blood and serum bactericidal abilities against multiple APEC strains *in vitro* compared with the CON group. Following  $\chi$ 7122 challenge, P+V birds had less APEC in their blood ( $P < 0.001$ ) and lower signs of airsacculitis ( $P < 0.01$ ) and pericarditis/perihepatitis ( $P < 0.05$ ) than CON birds. Finally, only P + V birds were negative for fecal *Salmonella* at all time points. This study shows this combination treatment may be a feasible method to reduce infection by APEC and *Salmonella* in chickens.

**Key words:** probiotic, *Salmonella* vaccine, avian pathogenic *Escherichia coli*, IgY, poultry

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## INTRODUCTION

Bacterial infections are a major problem for poultry animals. Avian pathogenic *Escherichia coli* (**APEC**) causes systemic disease that is highly lethal in both broilers (Yogarathnam, 1995) and layers (Vandekerckhove et al., 2004) and is the leading cause of first-wk mortality in layers (Olsen et al., 2012). In addition, poultry products such as eggs are the common source of broad-host serovars of *Salmonella enterica*, the primary cause of

foodborne-associated hospitalizations and deaths in the United States (Center for Disease Control and Prevention, 2018). Furthermore, *Salmonella* Kentucky has broad host ranges, can transfer antibiotic resistance genes to commensal *Enterobacteriaceae*, and can effectively colonize the gastrointestinal tract of poultry (Fricke et al., 2009), making it a threat for promoting abundant drug-resistant microbes in commercial poultry operations. Coincidentally, antimicrobial-resistant *E. coli* (Brower et al., 2017) and *Salmonella* (Han et al., 2012; Folster et al., 2015; Thung et al., 2016) isolates are increasingly prevalent in poultry, necessitating alternative means to decrease bacterial load.

Live vaccines and probiotics are convenient and currently applied options to control bacterial infections. Recombinant attenuated *Salmonella* vaccines (**RASV**) are typically used to control *Salmonella* in poultry

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(Dórea et al., 2010; Muniz et al., 2017), however, they have also demonstrated a reduction of APEC mortality in chickens (Chaudhari et al., 2013; Lee et al., 2015). These studies used RASV delivering APEC surface antigens such as the aerobactin receptor IutA (Chaudhari et al., 2013), but protection was only evaluated for a single APEC strain (O2 serotype). Furthermore, RASV has cross-reactivity with *Enterobacteriaceae* (Curtiss et al., 2013), and our recent studies have demonstrated cross-reactivity between RASV strains and APEC antigens (Maddux et al., 2017) including IutA and the salmochelin receptor IroN (Stromberg et al., 2018). Thus, it is possible these RASV can be used to simultaneously decrease bacterial load of both *Salmonella* and APEC in chickens.

Certain probiotics boost host immune responses via cytokine activation (Brisbin et al., 2010), Toll-like receptor expression (Sato et al., 2009), improved antibody production (Yang et al., 2005; Haghghi et al., 2006), and short-chain fatty acid secretion (Pang and Iwasaki, 2012). Thus, probiotics have been proposed as potential vaccine adjuvants, but evidence is limited (Praharaj et al., 2015). Adding probiotics to an antigen-based coccidiosis vaccine improved *Coccidia* resistance in broilers (Ritzi et al., 2016; Nothhaft et al., 2017). However, use of both probiotics and RASV to protect against a broad spectrum of bacterial pathogens in layers has not been investigated.

This objective of the work was to determine whether the RASV, probiotics, or their combination provides resistance against APEC and *Salmonella* in layer hens. We hypothesized combining probiotics with RASV treatment in chickens will enhance host responses against both pathogens.

## MATERIALS AND METHODS

### Ethics Statement

Animal experiments were approved by Iowa State University Institutional Animal Care and Use Committee (log #1-16-8159-G). Animal distress was minimized during experimental procedures by providing animal enrichments. For acclimation purposes, no experimental treatments were performed within 3 D of receiving chickens. Open floor pens were implemented to enable social interactions between chickens. Euthanasia techniques (CO<sub>2</sub> asphyxiation) were in accordance with the American Veterinary Medical Association Guidelines (2013).

### Chicken Treatment Groups

The experimental timeline is summarized in Figure 1. One-day-old male and female specific pathogen-free White Leghorns (VALO, Adel, IA) were randomly placed into 4 groups: no treatment (CON), probiotics

(PRO), RASV (VAX), or both treatments (P + V). Based on the group, chicks were randomly placed into 4 pens (n = 10 birds/pen) and housed in separate rooms based on RASV treatment. Chickens were given *ad libitum* access to feed and water. One pen per room received a commercial probiotic supplement (GRO2, Gro-2-max, BioNatural America Institute, Royal Oak, MI) consisting of *Bacillus subtilis*, *Lactobacillus acidophilus*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Saccharomyces pastorianus* (confirmed by PCR), and this was thoroughly mixed with the feed (2.5 g of dry probiotic mix to 2.3 kg of feed; PRO and P + V). Fresh feed (3003484-324, Organic Starter-Grower, Purina, Gray Summit, MO) was evenly weighed and replaced in all pens at least every 2 D. The bedding was not replaced during the experiment.

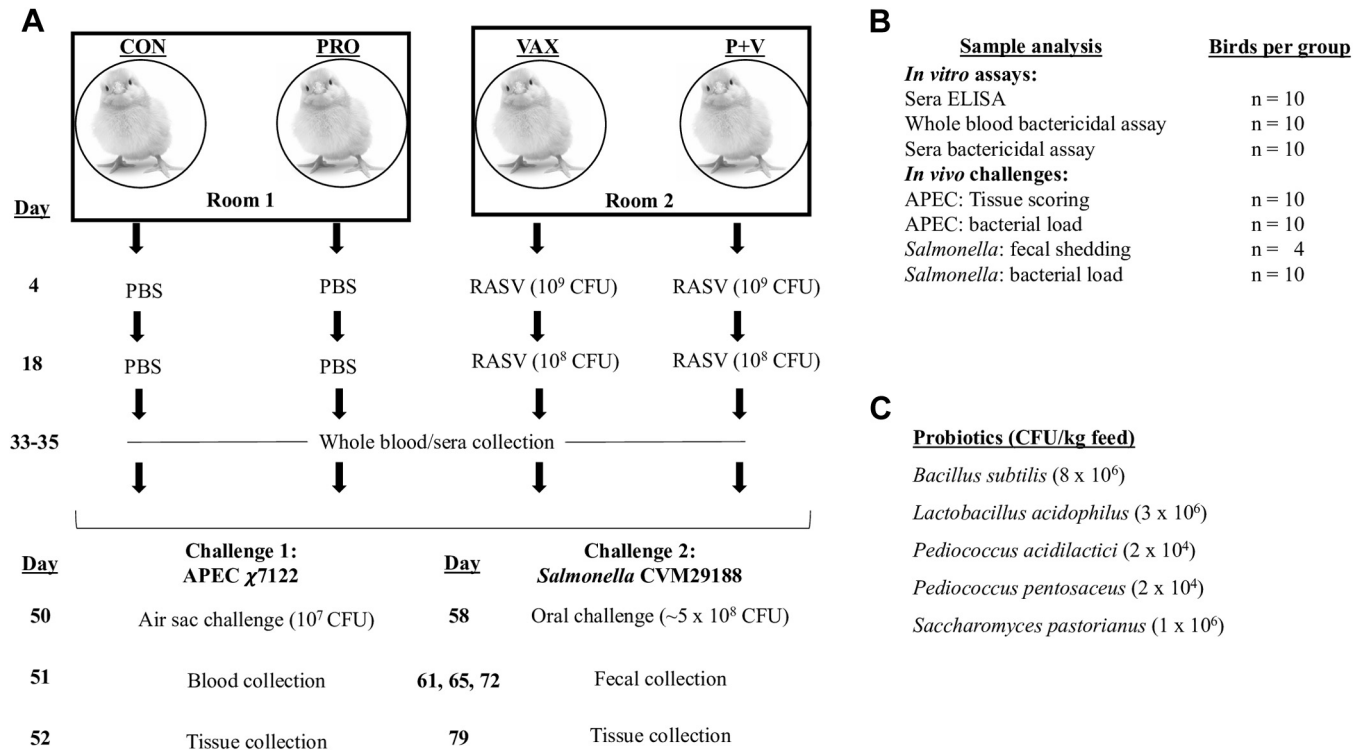
### Bacterial Strains

Reference *E. coli* strains, clinical APEC isolates (Stacy et al., 2014), and RASV  $\chi$ 9373 (Li et al., 2008) (Table 1) were stored in peptone-glycerol solution at  $-80^{\circ}\text{C}$ . In brief, *E. coli* isolates obtained in the study by Stacy et al. (2014) were originally retrieved from diseased poultry animals exhibiting signs of colibacillosis (thus their designation as APEC) by Dr. John Fairbrother at the University of Montreal. The reference APEC strain  $\chi$ 7122 (O78:K80) (Provence and Curtiss, 1992) and *S. Kentucky* CVM29188, isolated from chicken breast (Fricke et al., 2009), were used for *in vivo* challenges. RASV  $\chi$ 9373 is designed for delayed attenuation upon absence of environmental mannose via *pmi* deletion and complemented with the plasmid pYA3337 to introduce a functional *asd* gene missing in this RASV strain (annotated in Table 1) (Pei et al., 2014).

The strains were normally grown in Lysogeny broth (LB) or LB agar (0.1% glucose) overnight at  $37^{\circ}\text{C}$ . For immunization and challenge studies, strains  $\chi$ 9373,  $\chi$ 7122, and CVM29188 were grown by shaking ( $\chi$ 9373, CVM29188) or standing ( $\chi$ 7122) in LB until the bacterial suspension reached an optical density at 600 nm (OD<sub>600</sub>) of 0.8 and centrifuged for 20 min at  $4,000 \times g$  at room temperature. The pellet was resuspended and serially diluted in PBS. Bacterial concentrations were confirmed by plating on MacConkey agar (212123, BD Difco, Franklin Lakes, NJ).

### Immunization

At the age of 4 D, food and water were removed from pens of all birds 4–6 h before vaccination. Vaccine groups were orally immunized with 20  $\mu\text{L}$  of  $10^9$  cfu of RASV. Two wks after vaccination, the same chickens were given an additional 20  $\mu\text{L}$  of the oral RASV boost ( $10^8$  cfu). No treatment and PRO groups received 20  $\mu\text{L}$  of PBS as a control (Figure 1). Food and water were returned to pens 30 min after immunization.



**Figure 1.** Experimental design for treatments, sample collection, and analyses. (A) One-day-old chickens were split into 4 groups: probiotics only (PRO), vaccine only (VAX), both probiotics and vaccine (P + V), or no treatment (CON). Birds were placed in rooms based on vaccine treatment. Whole blood and sera were collected from birds before bacterial challenge, after which samples were collected to determine bacterial load. (B) Overview of analyses performed in this study. (C) Summary of the probiotics used in the present study. APEC, avian pathogenic *Escherichia coli*; RASV, recombinant attenuated *Salmonella* vaccine.

## IgY Titers Measured by ELISA

Blood was collected from days 33 to 35 from the wing vein. After overnight coagulation at  $4^\circ\text{C}$  and centrifugation, serum was collected and stored at  $-80^\circ\text{C}$  until needed. ELISA were performed to compare antigen-specific and total IgY titers between the groups. In brief, 96-well plates were coated with  $2.0 \mu\text{g/mL}$  of the gram-negative envelope component lipopolysaccharide (LPS; *S. enterica* serovar Typhimurium, L6511, Sigma, St. Louis, MO) or siderophore receptors (IroN or IutA) overnight at  $4^\circ\text{C}$ . IroN and IutA antigens were purified from cultures of *E. coli* BL21 transfected with pET-101/D-TOPO vectors (Invitrogen, Carlsbad, CA) carrying *iroN* or *iutA*, respectively (Mellata et al., 2016). In addition,  $0.25 \mu\text{g/mL}$  of unlabeled mouse anti-chicken IgY (H + L, 8320-01, Southern Biotech, Birmingham, AL) was added onto separate 96-well plates to evaluate total IgY responses. Serum samples were diluted to a concentration of 1:50 in SEA blocking buffer (37,527, Thermo Fisher, Waltham, MA), serially diluted to a concentration of 1:2, and incubated for 1 h at room temperature. Goat anti-chicken IgY-AP (H + L, A16057, Invitrogen, Waltham, MA) was added, followed by the para-Nitrophenylphosphate (PNPP) substrate (34,047, Thermo Fisher, Waltham, MA). Absorbance was measured at 405 nm. To measure antibody titer, the reciprocal of the highest dilution values doubling the control value (i.e., nontreated birds) was considered positive. ELISA were performed

in duplicate per individual bird and independently replicated twice.

## Whole-Blood Bactericidal Assay

Reference *E. coli* strains were prepared as described previously in the Bacterial Strains section to attain  $10^2$  cfu/200  $\mu\text{L}$ . On days 34 and 35 after vaccination, 200  $\mu\text{L}$  of blood was collected from wing veins of birds using filter-sterilized heparin (1000 U/mL)-coated needles and placed on ice. Blood was evenly pooled into 2 groups per treatment and then diluted to a concentration of 1:8 in  $\text{CO}_2$ -independent media with 2 mmol L-glutamine (18045088, Gibco, Waltham, MA). Individual wells in 96-well plates were filled with whole blood solution and the bacterial inoculum (9:1, respectively) and incubated at  $40^\circ\text{C}$  for 30 min. After brief resuspension, the samples were serially diluted, plated on MacConkey agar, and incubated overnight at  $37^\circ\text{C}$ . The samples were tested in triplicate, and the experiments were independently replicated.

## Serum Bactericidal Assay

Forty-two APEC isolates, including APEC O1 (Johnson et al., 2007), APEC O2 (Johnson et al., 2006), and  $\chi$ 7122 (O78:K80), and non-pathogenic *E. coli* MG1655 (Barbieri et al., 2013) were streaked on LB agar (0.1% glucose), and colonies were mixed into PBS until OD reached 0.1, after which they were diluted

**Table 1.** Description of the bacteria and plasmid used in this study.

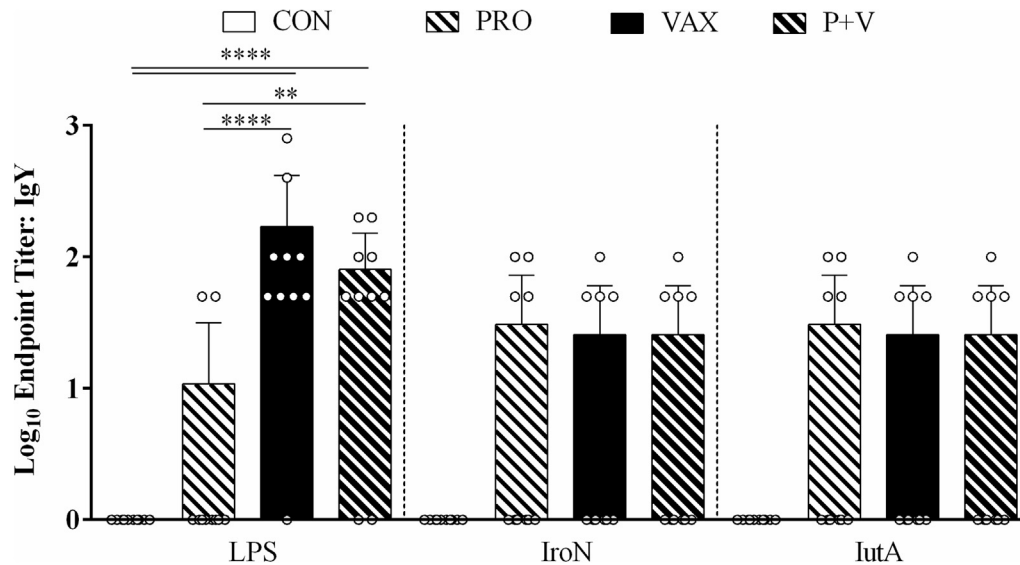
Strain or plasmid	Relevant genotype, phenotype, and characteristics	References
Recombinant attenuated <i>Salmonella</i> Typhimurium vaccine		
X9373	$\Delta pmi-2426 \Delta (gmd-fcl) 26 \Delta P_{furS1}::TT araC$ $P_{BAD fur} \Delta P_{crp527}::TT araC P_{BAD crp}$ $\Delta asdA2I::TT araC P_{BAD c2} \Delta araE25$ $\Delta araBAD23 \Delta relA198::araC P_{BAD lacITT}$	Pei et al., 2014
<i>Salmonella</i> Kentucky CVM29188	Tetracycline ( <i>tetRA</i> ) and streptomycin ( <i>strAB</i> ) resistance	Fricke et al., 2009
<i>Escherichia coli</i> : complement resistant		
<u>APEC O1</u>	APEC isolate, O1	Johnson et al., 2007
X7233	APEC isolate, O1	Stacy et al., 2014
X7254	APEC isolate, O1	Stacy et al., 2014
<u>APEC O2</u>	APEC isolate, O2	Johnson et al., 2006
X7245	APEC isolate, O2	Stacy et al., 2014
X7255	APEC isolate, O2	Stacy et al., 2014
X7533	APEC isolate, O10	Stacy et al., 2014
X7234	APEC isolate, O18	Stacy et al., 2014
X7501	APEC isolate, O22	Stacy et al., 2014
X7256	APEC isolate, O22	Stacy et al., 2014
X7531	APEC isolate, O23	Stacy et al., 2014
X7249	APEC isolate, O45	Stacy et al., 2014
$\chi$ 7520	APEC isolate, O55	Stacy et al., 2014
$\chi$ 7541	APEC isolate, O8/O60	Stacy et al., 2014
<u><math>\chi</math>7122</u>	APEC isolate, O78	Stacy et al., 2014
$\chi$ 7516	APEC isolate, O78	Stacy et al., 2014
$\chi$ 7547	APEC isolate, O21/O83	Stacy et al., 2014
$\chi$ 7509	APEC isolate, O115	Stacy et al., 2014
<i>E. coli</i> : complement sensitive		
<u>MG1655</u>	Laboratory strain, K-12, OR:K-H48	Barbieri et al., 2013
$\chi$ 7235	APEC isolate, O1	Stacy et al., 2014
$\chi$ 7504	APEC isolate, O6	Stacy et al., 2014
X7523	APEC isolate, O8	Stacy et al., 2014
X7499	APEC isolate, O9	Stacy et al., 2014
X7536	APEC isolate, O10	Stacy et al., 2014
X7260	APEC isolate, O11	Stacy et al., 2014
X7510	APEC isolate, O11	Stacy et al., 2014
X7507	APEC isolate, O15	Stacy et al., 2014
X7500	APEC isolate, O45	Stacy et al., 2014
X7511	APEC isolate, O55	Stacy et al., 2014
X7537	APEC isolate, O71	Stacy et al., 2014
X7548	APEC isolate, O71	Stacy et al., 2014
X7241	APEC isolate, O78	Stacy et al., 2014
X7544	APEC isolate, O78	Stacy et al., 2014
X7550	APEC isolate, O78	Stacy et al., 2014
X7546	APEC isolate, O83	Stacy et al., 2014
X7558	APEC isolate, O83	Stacy et al., 2014
X7552	APEC isolate, O103	Stacy et al., 2014
X7525	APEC isolate, O114	Stacy et al., 2014
X7514	APEC isolate, O131	Stacy et al., 2014
X7542	APEC isolate, O131	Stacy et al., 2014
X7518	APEC isolate, O138	Stacy et al., 2014
X7512	APEC isolate, O7/O157	Stacy et al., 2014
X7540	APEC isolate, O173	Stacy et al., 2014
Plasmid		
pYA3337	<i>asd</i> -based cloning vector (pSC101 <i>ori</i> ) with <i>P<sub>trc</sub></i> promoter	Torres-Escobar et al., 2010

Underlined strains indicate reference strains used as positive or negative controls.  
Abbreviations: APEC, avian pathogenic *E. coli*.

to reach  $10^2$  cfu/20  $\mu$ L. Equal volumes of chicken serum from each treatment group were split into 2 separate pools. Serum and the bacterial inoculum in the ratio of 9:1 ( $10^2$  cfu) was aliquoted into individual wells of 96-well plates and incubated for 6 h at 40°C to imitate *in vivo* challenge conditions in chickens (Van Goor et al., 2017). Serum and bacterial mixtures were serially diluted and plated on MacConkey agar. The samples were run in duplicate, and assays were independently repeated twice.

## In Vivo Bacterial Challenges

Challenge 1: APEC  $\chi$ 7122 Air Sac Challenge. On day 50, the chickens were challenged with inoculation of  $3 \times 10^7$  cfu of  $\chi$ 7122 in 100  $\mu$ L of PBS via the left caudal thoracic air sac using 26G  $\times$  9.5 mm needles (305110, BD, Franklin Lakes, NJ). The chickens were monitored for respiratory complications in the first 1 h immediately after inoculation. No birds exhibited any signs of respiratory distress immediately



**Figure 2.** Serum IgY antibody responses to treatments. Blood was collected from days 33 to 35, and serum was isolated to determine IgY antibody responses to treatments using ELISA. Chickens were placed into 4 groups: no treatment (CON), probiotics only (PRO), vaccine only (VAX), or both probiotics and vaccine (P + V). Each dot represents individual bird (n = 10 per group). Data are shown as mean  $\pm$  standard deviation of 2 experiments performed in duplicate. \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . LPS, lipopolysaccharide.

after inoculation. In addition, the birds were checked twice daily and sacrificed via CO<sub>2</sub> asphyxiation 48 h post-infection (hpi). At 24 hpi, blood was collected using heparinized needles, serially diluted in PBS, plated on MacConkey agar, and incubated overnight at 37°C. Tissues were scored for signs of inflammation and lesions in the air sac (0, normal; 1, slight edema; 2, slight diffuse thickening and neovascularization with slight fibrinous exudate; 3, moderate fibrinous exudate; and 4, severe extensive exudate), heart and pericardium (0, normal; 1, vascularization, opacity, cloudy fluid in the pericardial cavity; 2, acute pericarditis), and liver (0, normal; 1, mild fibrinous exudate; 2, severe perihepatitis), as described previously (Mellata et al., 2003a). Scores for heart plus pericardium and liver were combined for final analysis. At 48 hpi, tissues (spleen, liver, right lung, and heart) were aseptically collected from each euthanized bird for bacterial enumeration, performed as described previously.

**Challenge 2: *S. Kentucky* CVM29188 Oral Challenge.** In a separate experiment using the same treatment groups, feces from birds were plated on TC-S MacConkey agar (15  $\mu$ g/mL of tetracycline and 30  $\mu$ g/ml of streptomycin) to enumerate resistant *Enterobacteriaceae* 1 wk before the *Salmonella* challenge. At day 58, the birds were orally challenged with 500  $\mu$ l of CVM29188 ( $4.6 \times 10^8$  cfu) in PBS. At days 3, 7, and 14 after the challenge, feces were collected from all birds, resuspended in PBS, and plated on TC-S MacConkey agar to track CVM29188 and resistant *Enterobacteriaceae*. At day 21 after the challenge, intestinal contents (jejunum, ileum, cecum, and colon) and extraintestinal tissues (spleen and liver) were collected, homogenized in PBS, and plated on TC-S MacConkey agar for bacterial enumeration.

## Statistical Analysis

Prism software version 6.0 (GraphPad, San Diego, CA) was used to calculate significance for all statistical analyses. One-way ANOVA followed by Tukey's test for multiple comparisons of means was used to compare differences between the groups, depending on the experiments. For ELISA, differences were compared between the groups within the antigen tested. For bactericidal assays, the groups were compared within the strain tested. For APEC challenge data, differences were compared within the tissue type. For *Salmonella* challenge data, differences were compared for groups both within and between time points.  $P$  values  $< 0.05$  were considered significant.

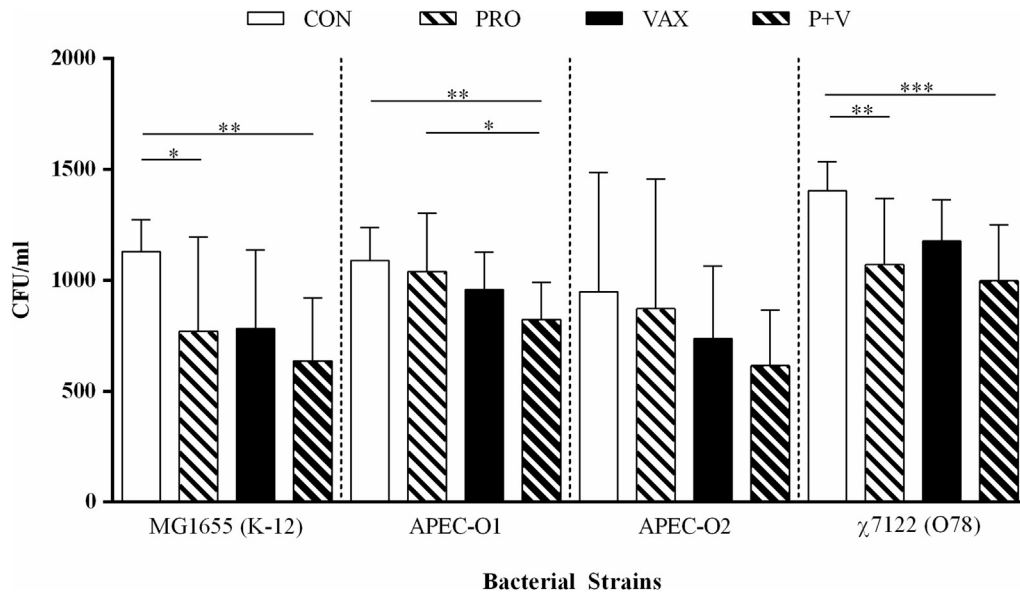
## RESULTS

### Specific and Nonspecific IgY Responses in Serum

In Figure 2, serum samples of the PRO group did not elicit significantly higher specific antibody titers against any antigen tested than those of the CON group. Serum samples of the RASV-immunized groups demonstrated higher IgY titers against LPS than those of the CON (VAX and P + V,  $P < 0.0001$ ) and PRO (VAX,  $P < 0.0001$ ; P + V,  $P < 0.01$ ) groups. No significant differences were observed for anti-IutA, anti-IroN (Figure 2), or total IgY (data not shown).

### Bactericidal Ability Against Multiple APEC Strains in Vitro

Whole blood samples of the PRO group exhibited enhanced bactericidal ability against MG1655



**Figure 3.** Whole-blood killing assay results with reference avian pathogenic *Escherichia coli* (APEC) strains and MG1655. Whole blood was pooled ( $n = 5$ ), with each assay performed in triplicate, and 2 pools were performed per experimental replicate (days 34 and 35). Chickens were placed into 4 groups: no treatment (CON), probiotics only (PRO), vaccine only (VAX), or both probiotics and vaccine (P + V). Bars are shown as mean  $\pm$  standard deviation of 2 experiments performed in triplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

( $P < 0.05$ ) and  $\chi 7122$  ( $P < 0.01$ ) compared with those of the CON group (Figure 3). Blood of vaccinated birds yielded no significant bactericidal ability against any strain tested. However, blood samples of the P + V group demonstrated the highest broad killing activity against MG1655 ( $P < 0.01$ ), APEC O1 ( $P < 0.01$ ), and  $\chi 7122$  compared with those of the CON group ( $P < 0.001$ ). Furthermore, APEC O1 growth was found to be suppressed in the blood samples of the P + V group when compared with those of the PRO group ( $P < 0.05$ ).

In serum, APEC growth inhibition occurred in a strain-dependent manner by treatment (Figure 4). Serum samples of the PRO group yielded significant killing ability against  $\chi 7233$  (O1) compared with those of the CON group (Figure 4A,  $P < 0.05$ ). Serum samples of the PRO, VAX, and P + V groups demonstrated increased killing activity against  $\chi 7234$  (O18) compared with those of the control group (Figure 4A,  $P < 0.05$ ). Serum samples of the VAX ( $P < 0.05$ ) and P + V ( $P < 0.01$ ) groups demonstrated increased bactericidal activity against  $\chi 7256$  (O22) compared with those of the CON group (Figure 4A). The isolate  $\chi 7531$  (O23) was found to be resistant to bactericidal activity in the serum samples of the P + V group only when compared with those of all other treatment groups (Figure 4B,  $P < 0.01$ ). Serum samples of the PRO group demonstrated increased killing activity against  $\chi 7249$  (O45) compared with those of the CON, VAX, and P + V groups (Figure 4B,  $P < 0.0001$ ). Serum samples of the VAX group demonstrated increased killing activity against  $\chi 7520$  when compared with those of the CON group (Figure 4B,  $P < 0.05$ ).  $\chi 7122$  was completely eliminated in the serum samples of the PRO and P + V groups when compared with those of the CON group (Figure 4B,  $P < 0.0001$ ). Most APEC isolates tested

failed to grow in serum (complement sensitive) obtained from any group (Table 1).

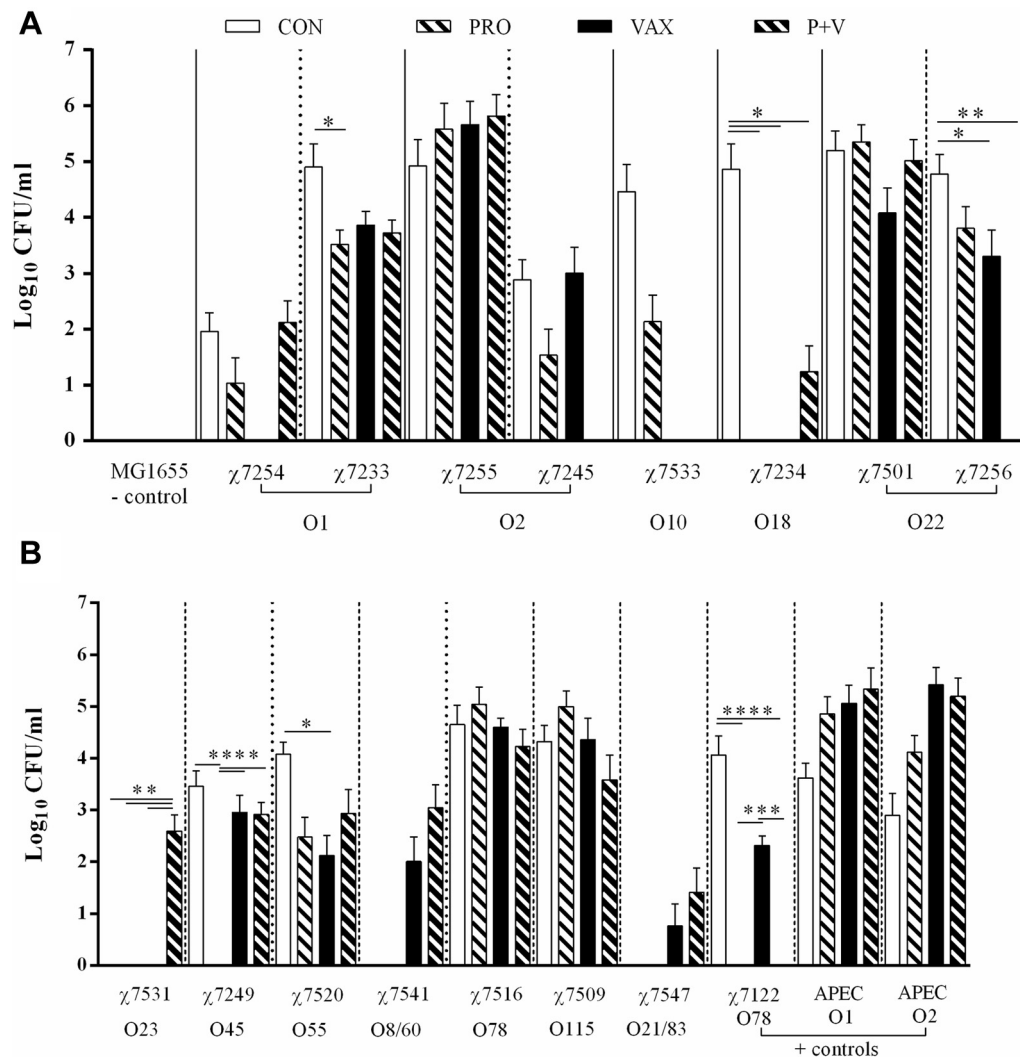
### In Vivo Protection Against APEC Challenge

For lesion scores, the PRO and P + V groups yielded significantly lower signs of airsacculitis (Figure 5A,  $P < 0.05$  and  $P < 0.01$ , respectively) than the CON group. Lesion scores for heart and liver were significantly lower in the P + V and VAX groups ( $P < 0.05$ ) than in the CON group. At 24 hpi, blood from RASV-immunized birds demonstrated low levels of  $\chi 7122$  *in vivo* (Figure 5B,  $P < 0.01$  and  $P < 0.001$  for VAX and P + V, respectively).

For bacterial loads, reduced cfu of  $\chi 7122$  were seen in the spleen tissues of the P + V group when compared with those of the VAX group ( $P < 0.05$ ), although a significantly lower cfu was not observed when compared with that of the CON group. Numerical but insignificant decreases in cfu of  $\chi 7122$  in liver and lung samples were seen in the P + V group. A higher proportion of tissue samples were negative for  $\chi 7122$  in the P + V group than in the other groups, reaching significance in the lung (Figure 5,  $P < 0.05$ ). There were no significant differences between the groups in bacterial cfu in heart tissue.

### In Vivo Protection Against *S. Kentucky* CVM29188

No *Salmonella* was detected in feces of any bird before the CVM29188 challenge (data not shown). After the challenge, CVM29188 was not detected in feces at days 3 and 14 after the challenge. However, CVM29188 was shed the highest at day 7 when compared with other



**Figure 4.** Serum bactericidal assay against multiple avian pathogenic *Escherichia coli* (APEC) isolates and reference strains. (A) MG1655 was the negative control owing to complement sensitivity. (B)  $\chi$ 7122, APEC O1, and APEC O2 were positive controls owing to known complement resistance. APEC isolates of various serogroups (O1, O2, O10, O18, O22, O23, O45, O55, O8/60, O78, O115, and O21/83) were used in this assay. Serum was pooled from 5 birds, and 2 pools were performed per experimental replicate. Chickens were placed into 4 groups: no treatment (CON), probiotics only (PRO), vaccine only (VAX), or both probiotics and vaccine (P + V). Data are shown as mean  $\pm$  standard deviation of the mean of 2 experiments performed in triplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

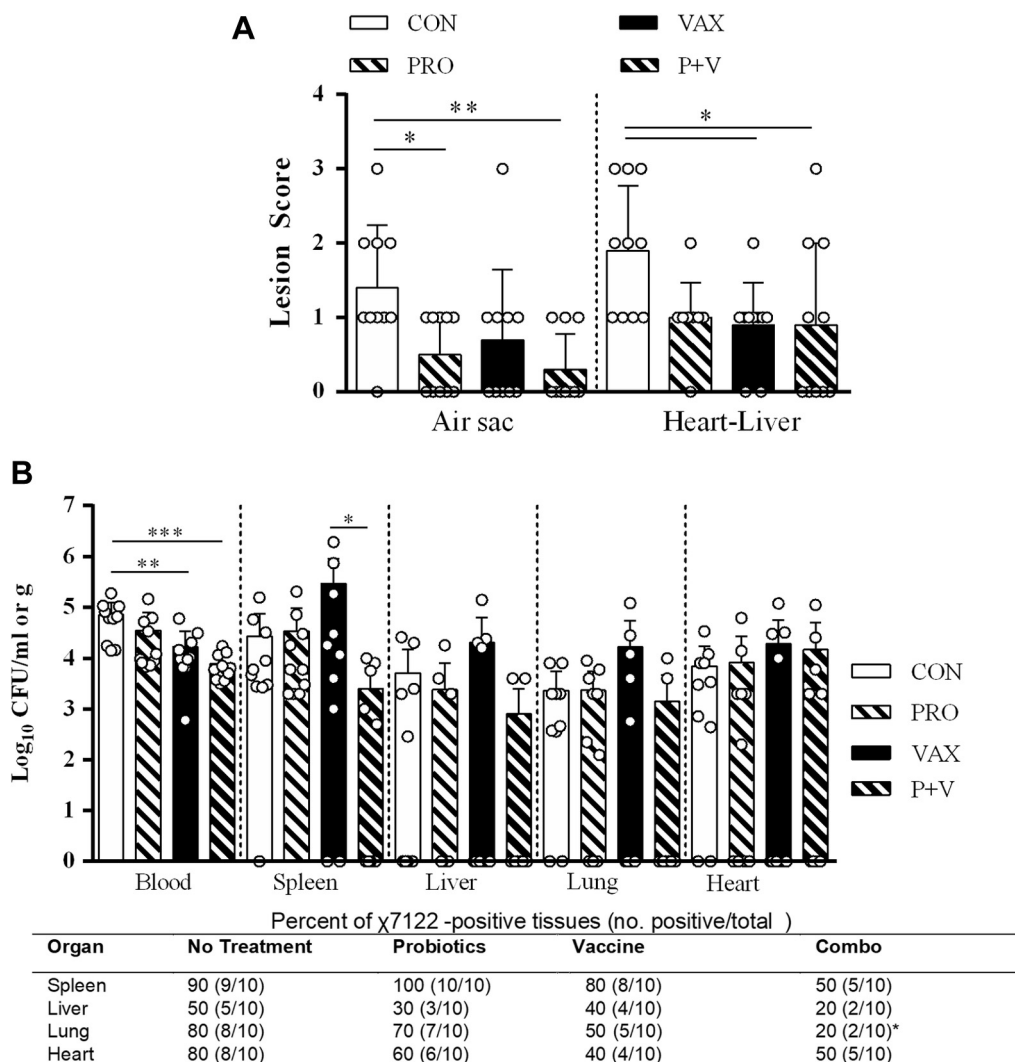
time points (CON and VAX,  $P < 0.05$ ), although only birds of the P + V group were all negative for CVM29188 (Figure 6). No significant differences were seen between the groups at day 7. Furthermore, CVM29188 was not detected in any intestinal contents and extraintestinal tissue samples collected at necropsy from any bird (data not included).

## DISCUSSION

The recent rise of bacterial infections in poultry necessitates a prophylactic that protects against a broad spectrum of pathogens. APEC is a commensal in the chicken gut (Ewers et al., 2009), but if inhaled can translocate from the lung epithelium into the bloodstream to cause systemic infections such as colibacillosis (Mellata, 2013). These antigenically and genetically diverse APEC strains are primed for outbreaks in densely populated chicken facilities upon aerosolizing from feces

(Dziva and Stevens, 2008). A recent study genotyping *E. coli* isolates from broiler carcasses in Spanish farms characterized 26 different serotypes, with most strains containing multiple APEC-associated virulence factors and antimicrobial resistance (Solà-Ginés et al., 2015). In addition, although colonization of broad-host *Salmonella* serovars in older laying hens is asymptomatic (Pande et al., 2016), they can be shed in feces, having major consequences on food safety and human health. *Salmonella* can contaminate egg surfaces via biofilms or the egg yolk itself via shell penetration or invasion into the oviduct before laying (Gantois et al., 2009; Chousalkar and Gole, 2016). Altogether, a prophylactic option that can reduce loads of a broad spectrum of bacterial pathogens is imperative for optimal poultry production.

Recently, recombinant antigen-based vaccines have elicited broad APEC protection (Van Goor et al., 2017). However, these vaccines require subcutaneous



**Figure 5.** Gross colibacillosis lesions and bacterial loads after *in vivo*  $\chi$ 7122 challenge. Animals were challenged via air sac with  $\chi$ 7122 and humanely euthanized 48 hpi. Tissues were (A) scored for signs of colibacillosis and (B) screened for bacterial enumeration. Bacterial enumeration was carried out in the blood at 24 hpi and in organs (spleen, liver, lung, and heart) at 48 hpi. Chickens were placed into 4 groups: no treatment (CON), probiotics only (PRO), vaccine only (VAX), or both probiotics and vaccine (P + V). Each dot represents an individual animal, and bars represent mean  $\pm$  standard deviation. \* $P < 0.05$ ; \*\* $P < 0.01$  \*\*\* $P < 0.001$ . hpi, h post-infection.

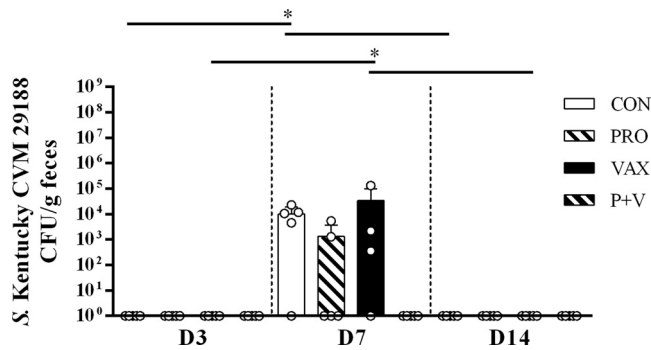
injections, which is inconvenient for commercial operations. Given orally, RASV translocate from the gut epithelium to lymphoid tissues such as the spleen, enabling memory T-cell development (Curtiss et al., 2010). This study is novel in the attempt to assess adjuvant potential of probiotics to improve host responses to RASV immunization. Importantly, both are currently used in poultry farms (Dórea et al., 2010; Tellez et al., 2012; Muniz et al., 2017) and can be simply orally administered (e.g., food and water). A mixture of probiotics, opposed to individual strains, increases the range of beneficial activities (Adhikari and Kim, 2017). Furthermore, the addition of *Anaerospobacter mobilis* and *Lactobacillus reuteri* enhanced efficacy of a live *E. coli*-based vaccine carrying a *Campylobacter jejuni* antigen (Nothhaft et al., 2017), suggesting that a probiotic mixture can enhance live vaccine efficacy in poultry animals.

In this study, vaccination with RASV  $\chi$ 9373 triggered significant anti-LPS IgY levels in the serum compared

with control or probiotic treatment alone. The presence of anti-LPS IgY in birds not vaccinated with RASV could be due to a cross-reactivity with anti-LPS of other *Enterobacteriaceae* species, such as *E. coli* (Aydingug et al., 1989). However, anti-lipid A antibodies are not protective (Mullan et al., 1974), suggesting that elevated levels may have little benefit to poultry animals. Importantly, the present study supports this claim by the poor association between serum bactericidal responses and anti-LPS IgY titers.

Protection against multiple serogroups is crucial as APEC isolates from chickens are highly diverse within and between birds (Solà-Ginés et al., 2015; Paudel et al., 2016). Blood samples from the P + V group elicited high bactericidal effects against 2 of the 3 APEC isolates tested *in vitro*. Although whole blood of probiotic-supplemented birds reduced bacterial loads of MG1655 and  $\chi$ 7122, blood samples from the VAX group did not decrease bacterial levels, suggesting that anti-LPS IgY was not a major factor in these responses.





**Figure 6.** *Salmonella* Kentucky shedding in feces after challenge. Animals were orally inoculated with *S. Kentucky* CVM29188, and feces were collected from birds at 3, 7, and 14 days after challenge for bacterial enumeration. Chickens were placed into 4 groups: no treatment (CON), probiotics only (PRO), vaccine only (VAX), or both probiotics and vaccine (P + V). Each dot represents an individual animal, and bars represent mean  $\pm$  standard deviation. \* $P < 0.05$ .

Instead, this suggests probiotics could have improved bactericidal activities of the innate effector cells, such as monocytes and heterophils, present in whole blood. However, RASV immunization improved probiotic-stimulated bactericidal activities in blood as APEC O1 elimination was higher in the P + V group than in the PRO group. Blood leukocytes from mice that were given probiotics exhibited higher levels of phagocytosis and respiratory burst (Dvorožňáková et al., 2016), supporting the evidence that probiotics modulate systemic immunity (Belkaid and Hand, 2014).

Serum resistance was highly variable between APEC isolates, even among those of the same serotype. However, the PRO and VAX groups generated protective serum against multiple APEC isolates, although this range in *in vitro* protection was not increased by combining treatments. Peculiarly, serum from the P + V group stimulated survival of the APEC isolate  $\chi$ 7531 (O23) compared with that of the other groups. Heat-treated serum from nontreated birds enabled growth of  $\chi$ 7531 similar to MG1655 (data not shown), suggesting this strain is complement sensitive. Complement C3 instructs adaptive responses in mice (Kim et al., 2018), suggesting an interaction between host complement and adaptive responses induced by vaccination. Thus, we hypothesize the complement pathway may be altered via synergism between the RASV and probiotics, which may enhance serum survival of certain APEC strains and/or serotypes.

We challenged birds with  $\chi$ 7122 because of its unique susceptibility to both serum and blood of the P + V and PRO groups in our *in vitro* study and its high virulence capacity (Provence and Curtiss, 1992). The precise mechanism to serum resistance in this strain is unclear (Mellata et al., 2003a). It is possible that other immune effectors such as antimicrobial peptides specifically elicited under probiotic treatment are responsible; however, why only this particular isolate exhibited susceptibility in this manner warrants further investigation. In addition, although bacterial enumerations in blood were the lowest in the P + V group when

compared with the CON group 24 hpi, this trend was not significant for tissues 48 hpi. This suggests tissue survival may be important in  $\chi$ 7122 pathogenesis. Mellata et al., 2003b demonstrated not only the capability of  $\chi$ 7122 to survive in professional phagocytes for more than 48 h but also the capability of its O78 antigen to enhance intracellular survival. In addition, loss of biofilm formation significantly attenuates APEC survival in tissues (Wang et al., 2011).  $\chi$ 7122 possesses genetic potential for biofilm formation, although this phenotype was not demonstrated *in vitro* (Stacy et al., 2014).

Previous studies with broad-host *Salmonella* in laying hens have shown that *Salmonella* can be naturally cleared within 2 wks (Pande et al., 2016), which is supported by our data for CVM29188 shedding. In the present study, CVM29188 was only detected 7 days after inoculation. Although birds of the CON, PRO, and VAX groups were all positive for CVM29188, birds of the P + V group were negative at every time point, suggesting the combination is efficacious against *Salmonella* persistence. In the VAX group, highest levels of *Salmonella* were observed at day 7 when compared with 3 and 14 days after the challenge. Although  $\chi$ 9373 is a live *Salmonella* vaccine, it is derived from a Typhimurium strain (Pei et al., 2014) and may not provide broad protection against *S. Kentucky* when given alone. The CVM29188 challenge strain was isolated from poultry, and the animal's intestinal tract may serve as a reservoir for exchanging virulence and resistance genes horizontally (Shterzer and Mizrahi, 2015; Zeng and Lin, 2017). CVM29188 possesses a ColV plasmid, which improves its ability to colonize the gut and cause extraintestinal disease in 1-day-old broilers (Johnson et al., 2010). However, our birds were much older (58-day-old) and appeared to naturally resist colonization and disease to a greater degree, likely due to a more mature gut microbiota (Varmuzova et al., 2016). Furthermore, this is the first study to inoculate this strain in layers, so immune differences in the production phenotype may also impact CVM29188 colonization (Koenen et al., 2002).

In conclusion, our data show that RASV  $\chi$ 9373 and probiotics did not synergistically improve antibody responses in serum, but strain-specific synergistic protection against APEC was observed in whole blood and recapitulated via superior protection against  $\chi$ 7122 *in vitro* and *in vivo*. Furthermore, *Salmonella* shedding in feces at day 7 was not found in the group treated with this combination, whereas other groups exhibited highest shedding, suggesting this combination can effectively reduce risk of infection and colonization by multiple pathogenic bacteria. Future studies aim to uncover how effector cells may contribute to these responses and the role of individual probiotic strains.

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