Cytokines and cecal microbiome modulations conferred by a dual vaccine in *Salmonella*-infected layers

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ABSTRACT Zoonotic *Salmonella* infection is a critical and challenging issue for public health. Since human infections are mainly associated with consuming contaminated chicken products, strategies to reduce *Salmonella* carriage and shedding are essential. Here we investigate the mechanisms of the live attenuated *Salmonella* vaccine (AviPro *Salmonella* Duo) against *Salmonella* Enteritidis (**SE**) infection. We focused on inflammatory-related cytokine expressions and cecal microbiota modulations in specific-pathogen-free (**SPF**) and field layers. Forty-eight 2-day-old SPF layers were randomly allotted into S.SEvc, S.SEc, S.Vc, and S.Ct groups in trial 1. The equal number of filed layers at 25 wk were allocated into SEvc, SEc, Vc, and Ct groups in trial 2. Each group contained 12 layers. Groups were further assigned for vaccination (S.Vc and Vc groups), SE challenge (S.SEc and SEc groups), vaccination and the following SE challenge (S.SEvc and SEvc groups), or the placebo treatment (S.Ct and Ct groups). Cecal tissues and contents of layers on day 14 post-SE-challenges were collected for cytokine mRNA expression and 16S rRNA metagenomic analyses. We found that SE challenges significantly upregulated expressions of IFNγ, IL-1β, IL-12β, and NFκB1A in SPF layers. The vaccine notably counteracted the levels of IFNα, IFNγ, and NFκB1A activated by SE attacks. The vaccination, SE challenge, and their combination did not significantly affect alpha diversities but promoted dissimilarities in microbial communities between groups. *Eubacterium_ coprostanoligenes* and *Faecalibacterium_ prausnitzii* were identified as contributory taxa in the cecal microbiota of SE-challenged and vaccinated SPF layers. A significantly higher abundance of *Faecalibacterium_ prausnitzii* in the ceca further correlated with the vaccination conferred protection against SE infection. In contrast, *Oscillibacter_ valericigenes* and *Mediterraneibacter_ glycyrrhizinilyticus* were featured taxa in *Salmonella*-infected field layers. *Megamonas_ hypermegale* and *Megamonas_ rupellensis* were identified as featured taxa in vaccinated field layers compared to SE-infected layers. To conclude, applying a dual *Salmonella* vaccine in this study modulated expressions of inflammatory-related cytokines and the cecal microbiome in layers, contributing to protection against SE infection. The feature microbes are promising for developing predictive indices and as antibiotic alternatives added to feed to reduce the risk of *Salmonella* shedding and contamination.

Key words: *Salmonella*, cytokines, cecal microbiota, microbial shift, 16S rRNA metagenomics

INTRODUCTION

Salmonella Enteritidis (**SE**) is one of the most important foodborne pathogens worldwide (Varmuzova et al., 2015). Contaminated poultry products are relevant origin of the infection (Guo et al., 2011). Minimizing the

Salmonella carriage and shedding in the chicken flocks is key to reducing the risk of human foodborne illness. Several studies have reported that *Salmonella* infections in chickens commence with bacterial adhesion and invasions of the intestinal mucus membrane. Damaged intestinal epithelia and imbalanced electrolytes result in inflammatory diarrhea (Desmidt et al., 1998). The subsequent influx of macrophages and *Salmonella* pervasions contribute to intracellular infected phagocytes, systemic infections (Ruby et al., 2012), and prolonged fecal shedding (Marin et al., 2009). Hence, the effective control of *Salmonellae* requires multiple interventions. Reducing intestinal colonization and systemic invasion

through competitive exclusion and adaptive immunity is practical for controlling *Salmonella* loads in poultry (Mead, 2000; Desin et al., 2013).

The annual cost of salmonellosis in poultry is estimated at approximately $11.6 billion in the United States (Wernicki et al., 2017) and exceeds €3 billion in the European Union (Ehuwa et al., 2021). Although antibiotic treatment is the first choice for *Salmonella* infection, the ability to proliferate in *Salmonella*-containing vacuole supports *Salmonellae* to escape from antimicrobial and phagocytic activities, then developing resistance (Tierrez and García-del Portillo, 2005; Birhanu et al., 2021). Among several control tools, vaccines are valuable in decreasing the *Salmonella* incidence and shedding while simultaneously reducing the application of antibiotics. Based on the evidence that most *Salmonella* infections developed in newly hatched chickens (Beal et al., 2004), an early or concurrent immunization was suggested to effectively inhibit *Salmonella* colonization and invasion (Methner et al., 2011). For this purpose, a live attenuated vaccine showed competitiveness for field applications. It activated adaptive immunity (Varmuzova et al., 2016), but a synergistic effect on the prevention of systemic SE dissemination was further reported in an experiment applying the combined treatments of live vaccine and the competitive exclusion culture (Braukmann et al., 2016). The study also displayed that the systemic invasion elicited by the wild strain was regularly prohibited after 2 d post vaccinations. Those findings indicated that microbial competition or interaction, especially in the early age of chickens, played a role in preventing the progress of *Salmonella* infection.

Gut microbiota is associated with intestinal health, physiological development, immunological regulation, growth performance, and disease resistance in chickens (Clavijo and Flórez, 2018; Diaz Carrasco et al., 2019). Even though the mechanism for gut microbiota to grant resistance and protection against SE infection remains unclear, several studies have reported that SE infection affects microbial community composition in the ceca. The resident microbiota was also demonstrated to have a part in regulating host susceptibility to SE colonization and cloacal shedding (Mon et al., 2015; Liu et al., 2018; Zeng et al., 2018). Recently, the application of fecal microbiota transplant reduced mortality and liver inflammatory lesions in SE-infected SPF chickens (Wang et al., 2022). Those pieces of evidence highlighted that cecal microbiota exhibited specific protective effects against gut pathogens, particularly for SE. However, the key contributor to the microbiota exhibiting the protection was seldom addressed and discussed. Most studies either focused on the effect of *Salmonella* challenges on cecal microbiota modulations or applied probiotics and prebiotics to evaluate their efficacy in reducing the incidence of salmonellosis in chickens. Assuming that a chicken model showed low bacterial shedding or reduced tissue colonization after *Salmonella* incursion, it would be valuable to investigate what contributes to restoring the microbial community's resistance to SE. This would provide new insights into developing an alternative disease control strategy.

The application of a live attenuated vaccine, AviPro *Salmonella* Duo, in layers has been approved to reduce the incidence, cloacal shedding, and tissue colonization from SE infection (Lin et al., 2022). Here we applied this model to investigate the mechanisms of AviPro *Salmonella* Duo against SE infection by evaluating inflammatory-related cytokine expressions and cecal microbiota modulations in specific-pathogen-free (**SPF**) and field layers. The objective was to evaluate the roles of those cytokines, cecal microbiota, and the microbial biomarker in defending against SE infections through multiple analyses of different treatment arrangements.

MATERIALS AND METHODS

Salmonella Preparation, Vaccine, and Bacteriological Analyses

The SE strain 147 Nal[res] from Elanco Animal Health Co., Ltd. (Elanco Animal Health, Greenfield, IN) was augmented for oral challenges. The inocula were prepared by 10-fold dilution of the original SE stock (3.2×10^{10} CFU/ml) with PBS solution (Sigma, St. Louis, MO) to achieve the concentration of 10^9 colony forming units (**CFU**)/ml per chicken. Strain serovar and cells of challenge stocks were validated by serotyping and serial dilution cultures on xylose lysine deoxycholate (**XLD**) agar (Sigma, St. Louis, MO). For the vaccination, a commercial AviPro *Salmonella* DUO vaccine (Elanco Animal Health, Greenfield, IN) was applied. One dose of vaccine contained 1 to 6×10^8 CFU of SE strain Sm24/Rif12/Ssq and 1 to 6×10^8 CFU of ST strain Nal2/Rif9/Rtt. The SE vaccine strain is designed to be sensitive to erythromycin but resistant to streptomycin and rifampicin. Serotyping and serial dilution cultures confirmed vaccine strain serovar and their viable cell concentration. Antibiotic resistance profiles were tested and confirmed using AviPro Plate (Elanco Animal Health, Greenfield, Indiana). The isolation of *Salmonella* followed the procedures of ISO 6579:2002 (Microbiology of food and animal feeding stuffs-Horizontal method for detecting *Salmonella* spp.). Serotyping was conducted by plate agglutinations using antisera to O and H antigens. *Salmonella* with positive results for O9, Hg, and Hm was recognized as SE.

Study Design

Two trials were conducted in the animal biosafety level (**ABSL**)-2 poultry facility at the Animal Resource Center of National Taiwan University (**NTU**). The experimental designs are described in the following sections and summarized in Table 1. Through pairwise comparisons between treatments, cytokine expression profiles, cecal microbiota, and microbial biomarkers in layers carrying the protection against SE infection were explored. The study procedure was approved by NTU

Table 1. Experimental designs in SPF and field layers.

Trial	Group	N	Vaccination of AviPro Salmonella DUO	Bacterial Challenge	Date of sampling	
					Cecal tissues	Cecal contents
1 (SPF layers)	S.SEvc	12	[1]	[1] (SE 147 Nal^res)	14 dpc	14 dpc
	S.SEc	12	−	[1] (SE 147 Nal^res)	14 dpc	14 dpc
	S.Vc	12	[1]	−	14 dpc	14 dpc
	S.Ct	12	−	−	14 dpc	14 dpc
2 (Field layers)	SEvc	12*	[1]	[1] (SE 147 Nal^res)	14 dpc	14 dpc
	SEc	12	−	[1] (SE 147 Nal^res)	14 dpc	14 dpc
	Vc	12	+	−	14 dpc	14 dpc
	Ct	12	−	−	14 dpc	14 dpc

N: numbers of layers (6 cecal tissues and 6 cecal contents per group were collected at each sampling time).

SE stands for *Salmonella* Enteritidis.

[1]Performed; −: not performed; dpc: days post-SE-challenge.SE challenges were performed on 14 days post-arrival in 2 trials.Vaccinations were conducted on SPF layers on day 2 and on filed layers on day 5, week 8, and week 18.

*A chicken was found deceased before the sampling. The results of histopathological examinations and bacterial culture excluded the infection of *Salmonella*.

Institutional Animal Care and Use Committee (NTU-109-EL-00115 and NTU-109-EL-00160).

Trial 1 Forty-eight specific-pathogen-free (**SPF**) layers aged 2 d were randomly distributed into S.SEvc, S.SEc, S.Vc, and S.Ct groups with an equal number of chicks. During the trial period of 28 d, SPF layers of each group were housed in separate cages and provided with feed and water ad libitum. The temperature and humidity were maintained at $22 \pm 2°C$ and 60 to 80%. SPF layers aged 2 d in S.SEvc and S.Vc groups were orally administered 0.3 ml (one dose) of AviPro *Salmonella* Duo (Elanco Animal Health, Greenfield, Indiana). SPF layers aged 16 d in the S.SEvc and S.SEc groups were challenged with SE strain 147 Nal^res by oral routes. Layers in the S.Ct group were treated with the placebo as the negative control. All layers were manipulated with minimized distress and euthanized by CO_2 asphyxiation at the end of the trial.

Trial 2 Forty-eight field layers aged 25 wk were randomly selected from a commercial farm raising 2 flocks of layers. Half of them derived from the vaccinated flock, and 24 layers originated in the nonvaccinated flock. When these layers were reared in the commercial farm, layers in the vaccinated flock were vaccinated on day 5, week 8, and week 18. In the ABSL-2 facility, 24 vaccinated layers were randomly allocated into SEvc and Vc groups, and 24 unvaccinated layers were assigned to SEc and Ct groups with randomization. During the trial period of 28 d, field layers of each group were housed in separate cages and provided with feed and water ad libitum. The temperature and humidity were maintained at $22 \pm 2°C$ and 60 to 80%. All field layers in each group were sampled and approved negative for *Salmonella* by cloacal swabs. Afterward, field layers in the SEvc and S.SEc groups were gavaged with SE inocula with strain 147 Nal^res on day 14 post the date of arrival at the ABSL-2 facility. The Vc and Ct groups were treated with the placebo at the same time point. All field layers were euthanized by CO_2 asphyxiation on day 14 of post-SE challenges.

Sample Collections

Six layers were randomly selected per group in each trial, and cecal tissues were collected for analysis of the differential expression levels of inflammatory-related cytokine genes between groups. Sampled ceca were washed with PBS solution (Sigma, St. Louis, MO), trimmed to 0.5 cm × 0.5 cm, and then immersed into 0.5 ml of RNA-Later (ThermoFisher Scientific, Inc., Waltham, MA) at 4°C overnight. Cecal contents from those selected layers were also collected and frozen on dry ice immediately after the necropsy. All cecal tissues and contents were kept at −80°C for the following experiments.

Cytokine Gene Expression Analysis

RNA Extraction Collected cecal tissues were homogenized by MagNA Lyser (Roche, Basel, Switzerland) using the lysis buffer from MagNA pure compact RNA isolation kit (Roche, Basel, Switzerland). Total RNAs were subsequently extracted from the tissue solution by MagNA pure compact system (Roche, Basel, Switzerland). The quantity and quality of RNAs were measured and evaluated by Nanodrop One (ThermoFisher Scientific, Inc., Waltham, MA).

Quantitative RT-PCR Expressions of inflammatory-related cytokines were evaluated, including interleukin-1 beta (**IL-1β**), interleukin-6 (**IL-6**), interleukin-10 (**IL-10**), interleukin-12 beta (**IL-12β**), interferon-alpha (**IFNα**), interferon-gamma (**IFNγ**), lipopolysaccharide-induced tumor necrosis factor-alpha factor (**LITAF**), and nuclear factor-kappa-B-inhibitor alpha (**NFkB1α**). Qualified RNAs were reverse-transcribed at 37°C with a high-capacity cDNA reverse transcription kit (ThermoFisher Scientific, Inc., Waltham, MA) for 120 min according to the manufacturer's protocol. Primers, sequences, and sizes of amplicons are presented in Table 2. Glyceraldehyde 3-phosphate dehydrogenase was adopted as an internal control. The reaction mixture of quantitative PCR (**qPCR**) was prepared by mixing 2X Power SYBR Green PCR master mix (ThermoFisher Scientific, Inc., Waltham, MA) with 200 nM of primers and the template. Three technical replicates of the mixture were simultaneously run on Applied Biosystems 7900 HT real-time PCR system (ThermoFisher Scientific, Inc., Waltham, MA). Lastly, log transformed-

Table 2. Quantitative reverse transcription PCR (RT-qPCR) primers for cytokine gene expressions.

Gene	Sequence (5'→3')		Amplicon (bp)	Accession No.
	Forward	Reverse		
IL-1β	TGGGCATCAAGGGCTACA	TCGGGTTGGTTGGTGATG	244	Y15006
IL-6	AAATCCCTCCTCGCCAATCT	CCCTCACGGTCTTCTCCATAAA	106	NM_204628
IL-10	AATCACGGGCTGACTTTCAC	AACTCCCCCATGGCTTTGTA	64	AJ621614
IL-12β	CTGTGGCTCGCACTGATAAA	GGTGCTCTTCGGCAAATGG	84	NM_213571
IFNα	GACATCCTTCAGCATCTCTTCA	AGGCGCTGTAATCGTTGTCT	238	AB021154
IFNγ	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	259	NM_205149
LITAF	GGAATGAACCCTCCGCAGTA	CTGAACTGGGCGGTCATAGA	114	NM_204267
NFκB1A	GCAGATACTGCCCGAAAGTG	TGTCAGCTGTCTTCCTCCAA	109	NM_001001472

relative quantity (**Log-RQ**) was calculated from the results of the comparative C_T method.

Full-Length 16S rRNA Metagenomics

DNA Extraction and 16S rRNA Amplicon Generation Genomic DNA of cecal content was extracted by CatchGene Stool DNA Kit (QIAGEN, Valencia, CA) based on the recommended protocol. After analyzing the integrity of harvested DNAs by agarose gel electrophoresis, DNA concentration was measured by Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, MA) and adjusted to 1 ng/μl for the following applications. The full length of the 16S rRNA gene (V1−V9 regions) was then amplified by applying barcoded forward and reverse primers (forward primer-5′ GCATC/barcode/AGRGTTYGATYMTGGCTCAG 3′ and reverse primer-5′ GCATC/barcode/RGYTACCTTGT-TACGACTT 3′) and KAPA HiFi HotStart ReadyMix PCR kit (Roche, Basel, Switzerland) in line with the manufacturer's instructions. Briefly, the PCR program was launched with 2 ng of genomic DNA through a primary denaturation at 95°C for 3 min, followed by 25 cycles of denaturation, annealing, and extension at 95°C for 30 s, 57°C for 30 s, and 72°C for 60 s. After the additional cycle of an extension at 72°C for 5 min, 1 μl of PCR amplicon was analyzed on 1% agarose gel to visualize the expected size of approximately 1.5 kb and their band intensities. The bands around 1.5 kb were selected and purified using the AMPure PB Beads (Pacific Biosciences, Menlo Park, CA) for the library preparation.

SMRTbell Library Construction and HiFi Sequencing According to the document of amplification of full-length 16S gene with barcoded primers for multiplexed SMRTbell library preparation and sequencing procedure, an equal molar of each barcoded PCR product was pooled together. The SMRTbell libraries were constructed using the SMRTbell prep kit 3.0 (Pacific Biosciences, Menlo Park, CA) with 500 to 1,000 ng pooled amplicon sample. The thermocycler programs for introducing the universal hairpin adapters onto DNA fragments included end-repair and A-tailing for 35 min (step 1 at 37°C for 30 min and step 2 at 65°C for 5 min), adapter ligation at 20°C for 30 min and nuclease treatment at 37°C for 15 min. Afterward, libraries were purified with AMPure PB beads (Pacific Biosciences, Menlo Park, CA) to eliminate the adapter dimer. Libraries were then incubated with sequencing primer v4 and sequel II Binding Kit 2.1 (Pacific Biosciences, Menlo Park, CA) for the primer annealing and polymerase binding. Eventually, sequencing was conducted in the circular consensus sequence (**CCS**) mode on the PacBio Sequel IIe System (Pacific Biosciences, Menlo Park, CA). Highly accurate and long single-molecule reads with a Phred scale of 30 were produced for data analyses.

Data Analyses

Cytokine mRNA Expressions The data set was presented as Log-RQ and examined for normality by the Shapiro-Wilk test before the statistical analysis. According to the result, a significant difference between groups was analyzed by the parametric (ANOVA) test or non-parametric (Kruskal-Wallis) test utilizing SAS software version 9.4 (SAS Institute, Inc., Cary, NC). Statistical significance was determined based on the level of $P \leq$ 0.05.

16S rRNA Metagenomics The CCS reads with quality values ≥20 were classified as HiFi reads, holding base-level resolution with 99.9% of single-molecule read accuracy. After the demultiplexing procedure, the reads were processed with divisive amplicon denoising algorithm 2 (**DADA2**) version 1.14 (Callahan et al., 2016). Amplicons with single-nucleotide resolution were obtained through quality filtering, de-replication, ASV inference, and chimera removal. Reads trimming and filtering were applied with a maximum of two expected errors for each read. The same amplicon sequence variants (**ASVs**) with single-nucleotide resolution from the full-length 16S rRNA gene were achieved by this DADA2 algorithm. Each representative sequence was annotated with taxonomy classification from the NCBI database by applying the feature-classifier (Bokulich et al., 2018) and classify-consensus-blast (Camacho et al., 2009) algorithm in quantitative insights into microbial ecology (**QIIME**) software v2. For analyzing the similarities of sequences among ASVs, multiple sequence alignment was performed by using the QIIME v2 alignment MAFFT (Katoh and Standley, 2013) against the NCBI database (Balvočiūtė and Huson, 2017). Cecal community composition and dominant species in response to

different treatments were visualized and evaluated by stacked bar charts and bubble diagrams of relative microbial abundance at the different taxonomical levels. For analyzing alpha and beta diversity, the normalized ASVs abundance was used. Indices of Faith_pd, Pielou_evennesso, Shannon_entropy, and Simpson were calculated for evaluating community richness, evenness, and diversity. The significant dissimilarity of microbial community profiles (beta diversity) between groups was investigated using the multiple response permutation procedure (**MRPP**). Principal component analysis (**PCA**) was used to depict the hierarchical clustering of contributory species to treatments. The QIIME v2 with metagenomeSeq package was conducted to evaluate the differential abundance of contributory species among treatments. Featured taxon or biomarker for pairwise compared groups was identified by using the linear discriminant analysis effect size (**LEfSe**) algorithm (http://huttenhower.sph.harvard.edu/lefse/) with linear discriminant analysis (**LDA**) scores of 4.0 (Segata et al., 2011).

RESULTS

Cytokine Expressions in Response to Treatments

In SPF layers, SE challenges significantly upregulated the expressions of IFNγ, IL-1β, IL-12β, and NFκB1A in the SE-infected group compared to non-infected (S.Vc and S.Ct) groups. The vaccination significantly upregulated the level of IFNγ in the S.Vc group compared to the S.Ct group. One dose vaccination 14 d before the SE challenge significantly prevented the upregulated expressions of IFNα, IFNγ, and NFκB1A observed in the S.SEc group. The probabilities for the difference are shown in Figure 1A.

Compared to the Ct group in trial 2, SE challenges in field layers significantly downregulated the level of LITAF, and triple vaccinations significantly reduced the expressions of IL-1β, IL-6, IL-10, and IL-12β in field layers. Although the inhibitions on expressions of IFNα, IFNγ, and NFκB1A were also noted in field layers of the SEvc group, the differences between SEvc and SEc groups were not significant. The results are demonstrated in Figure 1B.

Microbial Structure and Shift Corresponding to Treatments

The phyla of Firmicutes (95.09%), Bacteroidetes (2.91%), and Tenericutes (1.77%) dominated the ceca of SPF layers. When the taxonomical structure was analyzed in detail, *Blautia_hominis* (9.52%), *Lacrimispora_saccharolytica* (5.32%), and *Negativibacillus_massiliensis* (4.33%) were identified as the most predominant species (Figure 2A). In contrast, the cecal communities of field layers were principally occupied by the phyla of Firmicutes (89.86%), Bacteroidetes (4.37%),

and Actinobacteria (2.30%). Among those taxa, *Lactobacillus_gallinarum* (5.98%), *Negativibacillus_massiliensis* (5.51%), and *Oscillibacter_valericigenes* (5.11%) were the dominant species (Figure 3A).

For microbial shifts in response to treatments, the vaccination in SPF layers shifted the dominant species to *Faecalibacterium_prausnitzii* (14.43%), *Blautia_hominis* (6.84%), and *Subdoligranulum_variabile* (5.53%). SE challenges shifted the dominant species to *Lacrimispora_saccharolytica* (10.78%), *Eubacterium_coprostanoligenes* (9.25%), and *Blautia_hominis* (5.81%). In the cecal microbiota of SPF layers carrying the protection against SE infection (in the S.SEvc group), *Faecalibacterium_prausnitzii* and *Blautia_glucerasea* converted into dominant, and the abundance increased to 20.46% and 9.51% compared to the S.Ct group. On the other hand, the abundance of *Lacrimispora_saccharolytica*, the dominant species noted in SE-challenged SPF layers, was reduced from 10.78% to 6.95%. In field layers, vaccinations shifted the dominant species to *Subdoligranulum_variabile* (7.31%), *Negativibacillus_massiliensis* (4.39%), and *Mediterraneibacter_glycyrrhizinilyticus* (4.27%). SE challenges increased the dominance of *Oscillibacter_valericigenes* (7.10%) and *Negativibacillus_massiliensis* (5.76%) and promoted *Mediterraneibacter_glycyrrhizinilyticus* (5.8%) to be the second dominant species in the ceca. For field layers with protection against SE infection (in the SEvc group), cecal dominance of *Megamonas_rupellensis* (7.19%), *Megamonas_hypermegale* (5.90%), and *Lactobacillus_crispatus* (5.29%) was noted.

Analyzing differential taxa between samples showed that *Blautia_hominis* was the notably abundant species in SPF and vaccinated SPF layers (Figure 2B). *Faecalibacterium_prausnitzii* was the significantly abundant species in the vaccinated (S.Vc and S.SEvc) groups, and *Eubacterium_coprostanoligenes* was the significantly abundant species in the SE-challenged (S.SEc and S.SEvc) groups. On the other hand, *Lactobacillus_gallinarum* is represented as the notably abundant species in filed layers (Figure 3B). *Bacteroides_coprocola* was the significantly abundant species in the vaccinated (Vc and SEvc) groups. *Eubacterium_coprostanoligenes* and *Olsenella_profusa* were significantly abundant species in the SE-challenged (SEc and SEvc) groups.

Alpha and Beta Diversity Analyses

Faith's PD and Pielou's indices were applied to estimate the species richness and evenness. Shannon's entropy and Simpson indices were used to evaluate the species diversity. In the present study, none of the differential significance in these indices was detected between treatments, such as vaccination, SE challenge, and their combination, in trial 1 ($P > 0.05$) and trial 2 ($P > 0.05$). The results are summarized in Table 3.
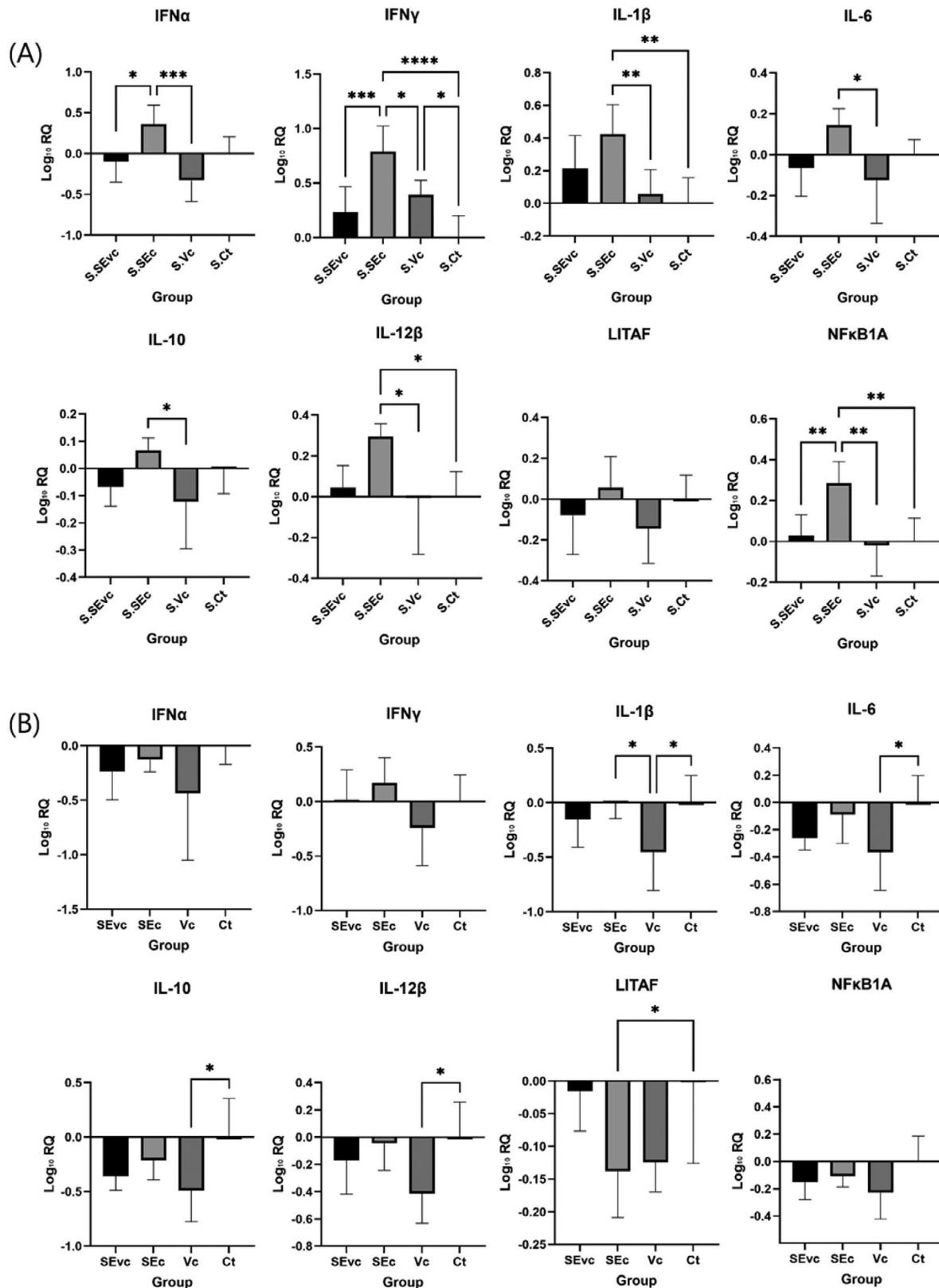
Figure 1. Relative gene expression levels of inflammatory-related cytokines in cecal tissue (n = 6 per group). The expression was presented as log transformed-relative quantity (Log-RQ). (A) Cytokine expressions between S.SEvc, S.SEc, S.Vc, and S.Ct groups in SPF layers. (B) Cytokine expressions between SEvc, SEc, Vc, and Ct groups in field layers. The data set was tested for normality by the Shapiro-Wilk test and then analyzed the significant difference between groups by ANOVA test or Kruskal-Wallis test: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

For beta diversity analysis, the results of MRPP demonstrated that the treatments promoted significant heterogeneity of microbial communities between groups in SPF layers by pairwise comparisons ($P \leq 0.05$), except for the observation between the S.Vc and S.Ct groups ($P > 0.05$). The findings indicated that specific taxa were contributing to the dissimilarities. Similar results were noted in pairwise comparisons of treatments in field
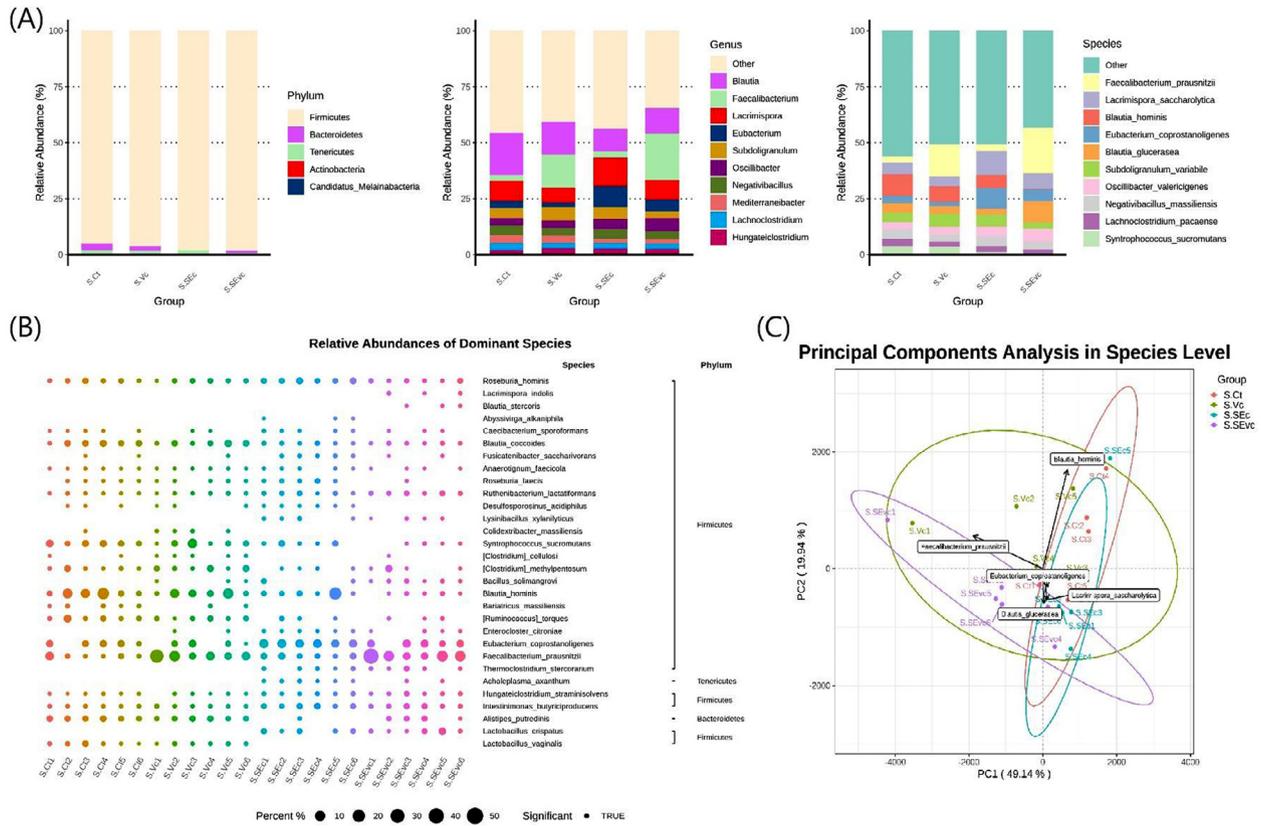
Figure 2. Microbial composition, dominant and contributory species in the ceca of SPF layers (n = 6 per group). (A) The distribution of the 10 most abundant taxa at the phylum, genus, and species level. (B) Relative microbial abundance at the phylum and species level in cecal samples (bubble diagram). The size of the circle indicates the relative abundance of species. A solid circle represents the significant difference between samples by the Kruskal Wallis test ($P \leq 0.05$). The hollow circle showed that the difference failed to reach the significance between samples ($P > 0.05$). (C) Hierarchical clustering of top five contributory species to treatments by principal component analysis.

layers. The differences in beta diversity were significant except for pairwise comparisons in the Ct-SEc and Vc-SEc ($P > 0.05$) groups. The results are summarized in Table 4.

Contributory Taxa and Biomarkers in Cecal Microbiota of Layers Treated With Vaccination, SE Inoculation, and Their Combination

The principal component analysis revealed that *Blautia_glucerasea*, *Blautia_hominis*, *Eubacterium_coprostanoligenes*, *Faecalibacterium_prausnitzii*, and *Lacrimispora_saccharolytica* were the most contributory species among samples-derived communities inn SPF layers (Figure 2C). On the contrary, *Akkermansia_glycaniphila*, *Megamonas_hypermegale*, *Megamonas_rupellensis*, *Oscillibacter_valericigenes*, and *Subdoligranulum_variabile* were the most contributory species to samples-derived communities in field layers (Figure 3C). After normalizing the taxonomic abundance, the differential significance of those contributory species among groups was analyzed by metagenomeSeq. The results further confirmed that *Eubacterium_coprostanoligenes* was the contributory taxon to the cecal

microbiota of SE-challenged SPF layers (Figure 4). Its abundance was significantly higher than SPF layers in the S.Vc and S.Ct groups ($P \leq 0.01$ and $P \leq 0.05$). *Faecalibacterium_prausnitzii* was the contributory taxon to the cecal microbiota of SPF layers exhibiting the protection against SE infection (S.SEvc group). The abundance was significantly higher than those in the S.SEc and S.Ct groups ($P \leq 0.05; P \leq 0.05$). *Blautia_hominis* may partially contribute to the cecal microbiota of non-SE challenged-SPF layers. Those layers possessed a significantly higher abundance of *Blautia_hominis* than the SPF layers in the S.SEvc group ($P \leq 0.05$ for the S.Vc group and $P \leq 0.01$ for the S.Ct group). Contrarily, the contributory species identified from the cecal microbiota of field layers did not exhibit differential significance in comparisons between treatment groups ($P > 0.05$).

When four groups were compared by LEfSe analysis, *Eubacterium_coprostanoligenes* and *Lacrimispora_saccharolytica* were differential or featured taxa (biomarkers) in the SPF layers of the S.SEc group. *Faecalibacterium_prausnitzii* was the biomarker in the S.SEvc group. *Blautia_hominis*, *Blautia_coccoides*, *Syntrophococcus_sucromutans*, *Ruminococcus_torques*, and *Alistipes_putredinis* were biomarker in the S.Ct group (Figure 5A and B). Pairwise group
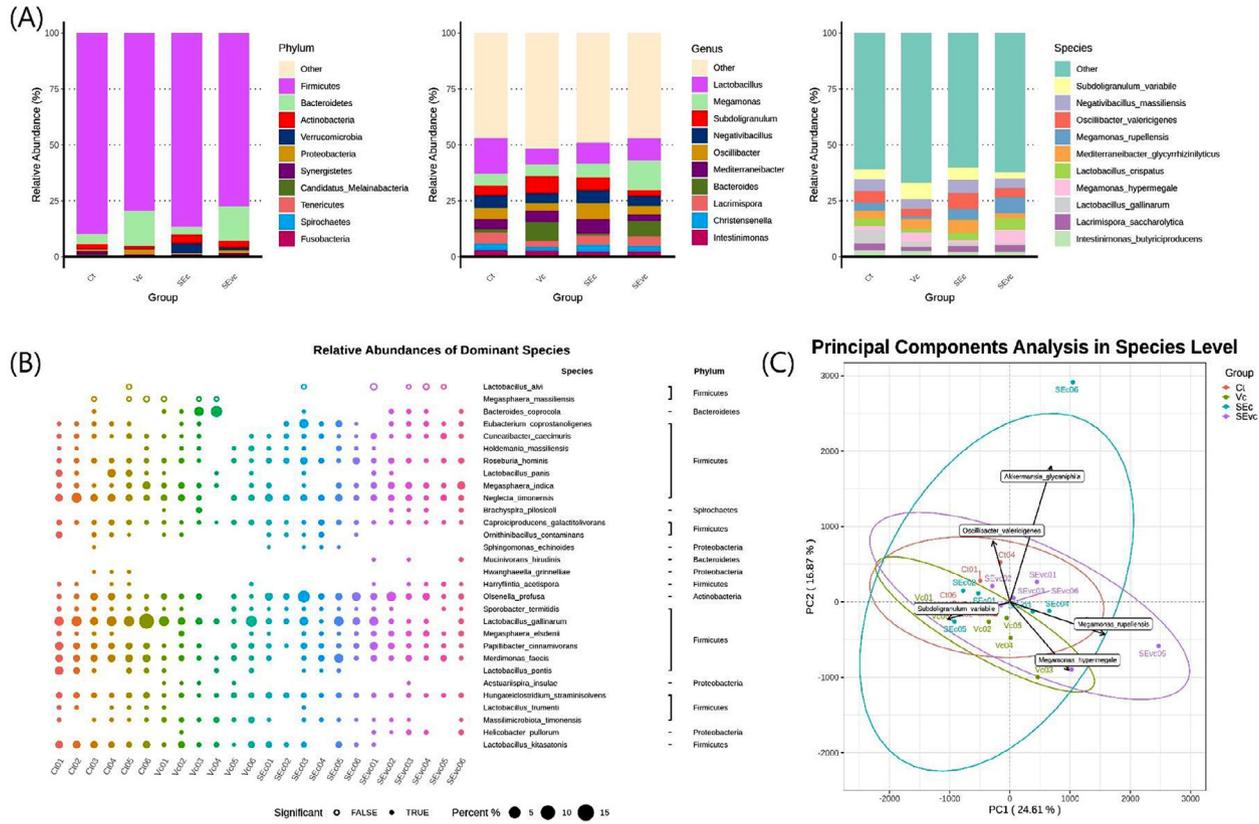
Figure 3. Microbial composition, dominant and contributory species in the ceca of Field layers (n = 6 per group). (A) The distribution of the ten most abundant taxa at the phylum, genus, and species level. (B) Relative microbial abundance at the phylum and species level in cecal samples (bubble diagram). The size of the circle indicates the relative abundance of species. A solid circle represents the significant difference between samples by the Kruskal Wallis test ($P \leq 0.05$). The hollow circle showed that the difference failed to reach the significance between samples ($P > 0.05$). (C) Hierarchical clustering of top 5 contributory species to treatments by principal component analysis.

comparisons were applied in the field layers due to insufficient taxon information in four group comparisons. *Oscillibacter_valericigenes* and *Mediterraneibacter_glycyrrhizinilyticus* were differential taxa in the SEc group compared to the Ct group (Figure 5C). *Akkermansia_glycaniphila*, *Lactobacillus_cripatus*, *Megamonas_hypermegale*, and *Megamonas_rupellensis* were biomarkers in the SEvc group compared to the SEc group (Figure 5D).

DISCUSSION

Despite a high concentration of SE inoculum being applied, infected layers did not present symptoms of illness during the period of trials. The application of SE strain 147 Nal^{res} was well-established for local and systemic infections in layers with persistent shedding (Lin et al., 2022). This observation indicated that SE-challenged layers converted into asymptomatic carriers. The pathogen persisted in the body and played a significant role in *Salmonella* circulation and contamination of poultry products (Kogut and Arsenault, 2017). Therefore, reducing the *Salmonella* load in the host was invariably the critical point for controlling the disease and the risk to public health. In our previous findings, one dose of AviPro *Salmonella* Duo significantly inhibited the degree of SE (strain 147 Nal^{res}) shedding and tissue invasions in layers. When complete immunity was achieved

in commercial layers by triple vaccinations, no bacterial shedding was noted, and *Salmonella* loads in tissues were notably reduced (Lin et al., 2022). The present study used the identical model to explore the role of host immunity and gut microbiota in the vaccinated layers carrying the protection against SE shedding and tissue invasion. Although the small intestines were the initial location for *Salmonella* pervasion, the ceca were the main sites involving host susceptibility to SE colonization and bacterial shedding (Cazals et al., 2022). The featured shifts and microbes in the cecal microbiota may involve or contribute to the protection. A recent study demonstrated that gut microbiota composition before *Salmonella* infection determined chicken's super- or low-shedder phenotypes (Kempf et al., 2020). It is noted that many studies highlighted *Salmonella*'s effect on gut microbiota. However, few addressed the contributory taxa linked with SE-infected layers and layers carrying the protection against SE attacks. The discovery of those feature microbes will be promising for being predictive biomarkers and antibiotic alternatives to reduce the risk of contaminating layer flocks and egg products.

IL-1β, IL-6, IL-12β, and TNF-α exert pro-inflammatory properties and activities of innate immunity. IL-10 inhibits cytokine production and mononuclear cell function, exhibiting anti-inflammatory effects (Zhang and An, 2007). The IFN-α has been demonstrated to be crucial in augmenting dendritic cell response and increasing the life of activated lymphocytes (Tough et al., 1996). In
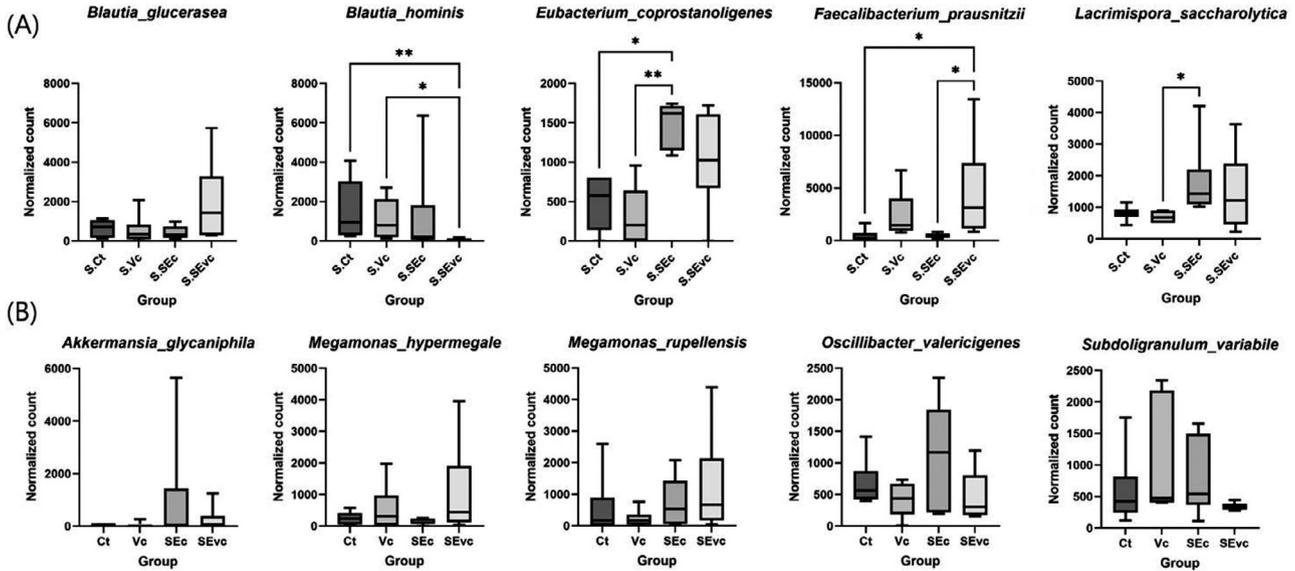
Figure 4. MetagenomeSeq analysis of the most contributory species in groups of SPF layers (A) and field layers (B). The x and y axes represented the group and the normalized count of species, respectively. Results are shown as mean ± SEM (n = 6 per group). Tukey test: [**]$P \leq 0.05$, [**]$P \leq 0.01$.

contrast, IFN-γ was shown to stimulate macrophages, modulate the balance of cytokine production, and promote cell-mediated immunity (Zhang and An, 2007). For LITAF, it exhibits a biological response similar to TNF-α in mammalians and serves as TNF-α in poultry (Lillehoj et al., 2001). In this study, SE challenges significantly promoted the release of IFNγ, IL-1β, IL-12β, and NFκB1A in SPF layers, coinciding with the findings of other studies (Crhanova et al., 2011; Wang et al., 2022). During the early stages of infection, phagocytes were recruited and activated by the inflammation of the affected tissues. Large amounts of IFN-γ were also generated from a variety of cells, mainly from NK cells, to promote the adaptive immunity. The SE colonization correlated the expression of IL-1β and IL-12β to recruit macrophages and monocytes, activating innate immunity. NFκB1A functioned to promote the secretions of pro-inflammatory cytokines (Ziglam, et al., 2004; Iwasaki and Medzhitov, 2010). The results suggested that the defense mechanism against SE started with innate immunity and converted into adaptive immune responses by the lead of those upregulated cytokines. However, vaccination in the S.SEvc group (vaccination and the following SE challenge) notably downregulated the IFN-α, IFN-γ, and NFκB1A elicited by SE attacks. It is speculated that the vaccine strain may exert competitive exclusion over the challenged strain, reducing challenge strain colonization on the ceca. Therefore, the expressions of those cytokines in layers carrying the protection were lower than those in SE-challenged SPF layers. In addition, significantly decreased IL-6 and IL-10 expressions were noted in vaccinated SPF layers compared to the SE-challenged layers. IL-6 has been demonstrated to induce B cell proliferation and differentiation into antibody-forming cells. The function of IL-10 aims to prohibit cytokine secretion, stimulate B cell proliferation and antibody production, and suppress cellular

immunity (Zhang and An, 2007). The suppressions of IL-6 and IL-10 may indicate that this dual vaccine favored the cellular immunity pathway to develop host immunity. The downregulations of IL-6 and IL-10 were also noted in vaccinated layers compared to the control field layers. However, the modulations on cytokine expression in field layers were not as significant as in SPF layers.

Gut microbiota significantly affects intestinal morphology, nutrition, immunity, productivity, and pathogen resistance (Khan et al., 2020). In this study, Firmicutes and Bacteroidetes were the most abundant phyla in the cecal microbiota of layers, consistent with findings in the Sergeant et al. study (Sergeant et al., 2014). It was also concordant that the abundance of Bacteroidetes increased in older age, while the amount of Firmicutes reduced (Sun et al., 2021). The present study adopted the full-length 16S rRNA sequencing to enhance the specificity of taxon identification at the species level on microbial shifts, contributory taxa, and featured microbes (biomarkers). Several studies indicated that the infection of SE promoted the abundance of the *Enterobacteriaceae* family in juvenile layers, significantly reducing the overall diversity of gut microbiota (Mon et al., 2015; Lee et al., 2020). Nonetheless, SE challenges in this study did not notably affect the richness and diversity of microbial communities in the ceca. Based on the heterogeneity of microbial communities detected between groups, we analyzed the taxa in the cecal microbiota that may contribute to the dissimilarities. As a result, *Eubacterium_ coprostanoligenes* and *Faecalibacterium_ prausnitzii* were recognized contributory and featured taxa in the cecal microbiota of SE-challenged and vaccinated SPF layers carrying the protection, respectively. *Eubacterium_ coprostanoligenes* is an anaerobe colonizing the gut. It converts cholesterol to coprostanol in the gastrointestinal system of layers
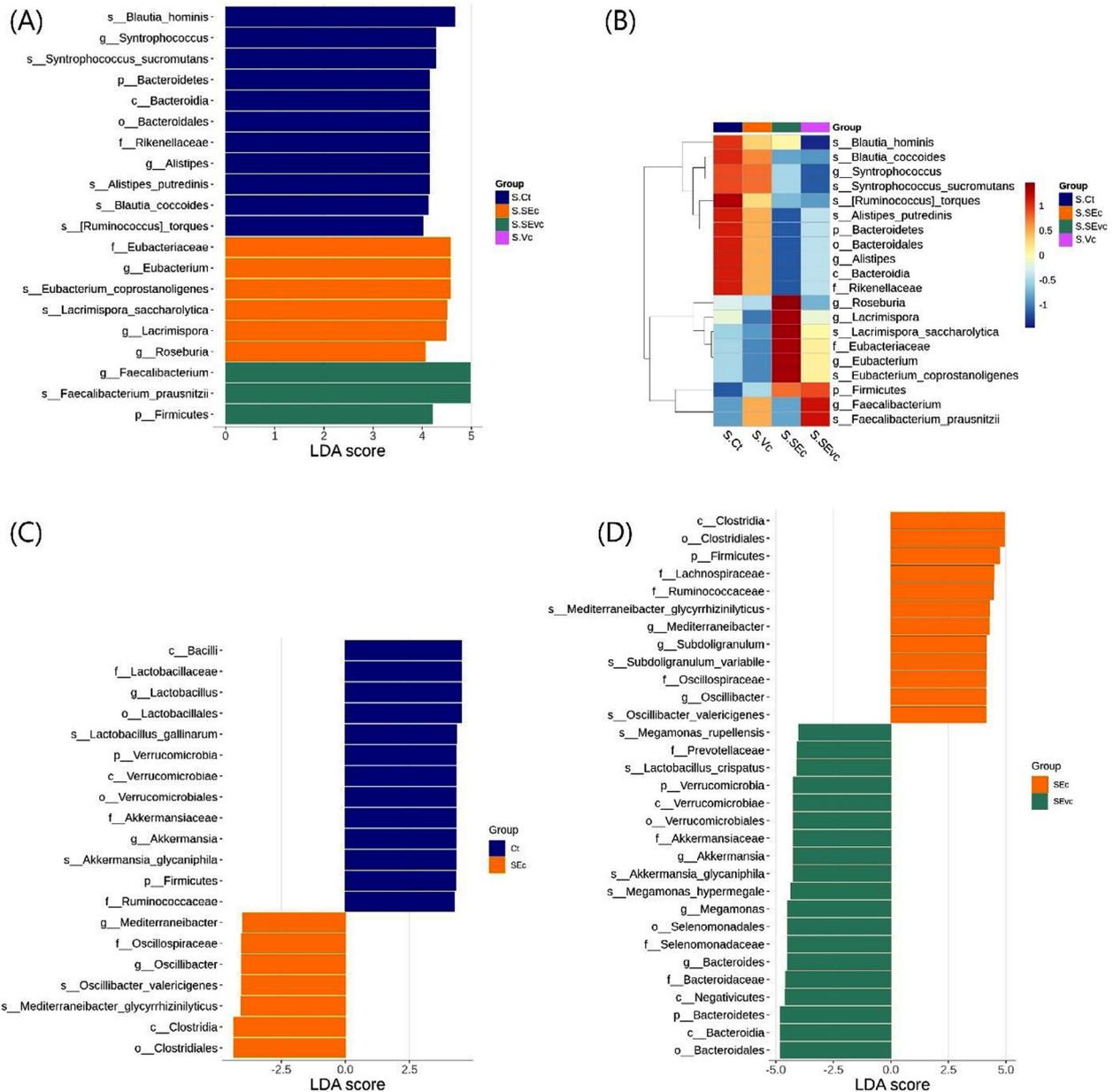
Figure 5. Differential taxa between groups identified by linear discriminant analysis effect size (LEfSe) using the linear discriminant analysis (LDA) score of 4 (n = 6 per group). The most differentially abundant clades were shown at all taxonomic levels. (A) Biomarkers identified by differential comparisons between four groups in SPF layer trial. (B) Heat map of identified biomarkers in SPF layer groups. (C) Biomarkers identified by pairwise comparisons between the SEc and Ct groups in field layer trial. (D) Biomarkers identified by pairwise comparisons between the SEvc and SEc groups.

(Li et al., 1996). An increase in its abundance was noted in pigs while time-restricted feeding was conducted (Wang et al., 2021). It was considered relative to the host's nutrition and metabolism, but its role in *Salmonella* infection was seldom addressed. *Faecalibacterium prausnitzii* is one of the highly abundant microbes in healthy human colons and is considered a bioindicator of human health. The decrease in its abundance favored inflammatory processes and metabolic diseases, such as inflammatory bowel disease and type 2 diabetes (Verhoog et al., 2019). The function of this microbe was documented to produce butyrate as the energy for colonocytes, maintain the balance of Th17/Treg cells, and

exert anti-inflammatory activity (Ferreira-Halder et al., 2017; Zhou et al., 2018). A recent study showed that antibiotic growth promoter use in chicken flocks increased the abundance of *Faecalibacterium prausnitzii, Bacillus fragilis*, and some *Lactobacillus*, positively relating to the growth performance (Broom, 2018). *Faecalibacterium_ prausnitzii* was demonstrated as a significantly predominant and featured species in the cecal microbiota of vaccinated SPF layers carrying the protection, linking with the phenotype displaying low cloacal shedding and tissue invasions. Accordingly, manipulating the abundance of *Faecalibacterium_- prausnitzii* in the ceca is considered beneficial for

Table 3. The results of alpha diversity analysis.

Group	Faith_pd	Pielou_evenness	Shannon_entropy	Simpson
S.SEvc	13.393	0.831	6.827	0.977
S.SEc	14.052	0.862	7.272	0.983
S.Vc	14.767	0.852	7.145	0.985
S.Ct	15.178	0.877	7.378	0.989
SEvc	25.989	0.903	8.062	0.991
SEc	23.425	0.890	7.857	0.986
Vc	23.783	0.901	7.951	0.991
Ct	23.935	0.905	8.071	0.991

Table 4. Pairwise comparison of between-group species composition by multiple response permutation procedure (MRPP).

Group	A	Observed-delta	Expected-delta	P value
S.Ct - S.Vc	0.020	0.625	0.638	0.068
S.Ct - S.SEc	0.130	0.624	0.717	0.004*
S.Ct - S.SEvc	0.109	0.665	0.746	0.003*
S.Vc - S.SEc	0.141	0.637	0.741	0.002*
S.Vc - S.SEvc	0.112	0.679	0.764	0.006*
S.SEc - S.SEvc	0.055	0.677	0.716	0.007*
Ct - Vc	0.009	0.891	0.899	0.002*
Ct - SEc	0.004	0.871	0.875	0.110
Ct - SEvc	0.022	0.862	0.881	0.003*
Vc - SEc	0.003	0.906	0.909	0.148
Vc - SEvc	0.009	0.897	0.905	0.034*
SEc - SEvc	0.011	0.877	0.886	0.018*

A represents the effect size of within-group homogeneity as compared to the random expectation.
A > 0 indicates the difference between groups is higher than the difference within groups, and vice versa for A < 0.
Observed-delta and expected-delta represent the level of difference within groups and between groups, respectively.
*Indicated P-value ≤ 0.05.

conferring adequate protection against SE infection. Nonetheless, further experiments should be conducted to test the hypothesis.

In field layers, several biomarkers were identified in the cecal microbiota after treatments. *Oscillibacter_valericigenes* is a Gram-negative rod and valerate producer, first isolated from the alimentary tract of a Japanese corbicula clam (*Corbicula japonica*) and also found in human intestinal microbiota. Its abundance seemed relative to specific health benefits in pigs (Pajarillo et al., 2015) but associated with Crohn's disease and septicemia in humans (Broutin et al., 2020). *Mediterraneibacter_glycyrrhizinilyticus* is a Gram-positive, nonmotile anaerobe regularly observed in cecal microbiota (Wongkuna et al., 2021). Its role in host health or disease has not yet been determined. Conversely, *Megamonas_hypermegale* played a critical part in polysaccharide degradation and regulation of short-chain fatty acids (**SCFA**s) concentration (Sergeant et al., 2014). It was shown to competitively exclude *Salmonella* (Barnes, et al., 1979) and was associated with *Campylobacter* exclusion in poultry (Scupham et al., 2010). *Megamonas_rupellensis* was an anaerobe isolated from the cecum of a duck (Chevrot et al., 2008). Unlike *Megamonas_hypermegale*, the function of *Megamonas_rupellensis* remains unspecified. A higher abundance of genus *Megamonas* generally improved the permeability of the gut microbiota, modulated immunity, and prohibited the release of inflammatory response factors (Koh et al., 2016). Overall, the affluence of the genus *Megamonas* is

regarded as beneficial for the host. The effective control of zoonotic salmonellosis focuses on reducing *Salmonella* carriage and bacterial shedding. Evidence showed that cecal microbiota composition might determine infected chickens' shedding capacity and bacterial loads. The present study provided insight into the cecal microbiota's contribution to SE infection in layers and identified feature microbes associated with the protection. It is worth evaluating the potential and feasibility of applying biomarkers in layers carrying the protection as antibiotic alternatives to prevent and control SE infections.

In conclusion, applying a dual *Salmonella* vaccine modulated expressions of inflammatory-related cytokines and the cecal microbiome in layers to protect against SE infection. Based on the profile of cytokine expressions, the vaccine elicited mild inflammatory responses to stimulate host immune systems and counteracted the levels of IFN-α, IFN-γ, and NFκB1A activated by SE attacks. Through analyzing the dissimilarities noted in cecal microbiomes between treatment groups, *Faecalibacterium_prausnitzii* was contributory and featured taxon for vaccinated SPF layers. On the contrary, *Megamonas_hypermegale* and *Megamonas_rupellensis* were biomarkers in the vaccinated filed layers. A significantly higher abundance of *Faecalibacterium prausnitzii* in the ceca correlated with the vaccination that conferred protection against SE infection. Those findings demonstrated that not merely inflammatory-related cytokines but the cecal microbiome are involve in developing and defending against SE infection. The featured microbes are promising for developing

predictive indices and antibiotic alternatives to reduce the risk of contaminating layer flocks and products.

DISCLOSURES

The authors declare no conflict of interest.

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