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Microbiota but not immune modulation by a pro- and postbiotic was associated with the diet-additive interaction in broilers

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ABSTRACT This study investigated the diet−additive interactions of a *Lactobacilli*-based probiotic (**Pro**) and postbiotic (**Post**) on immune parameters and cecal microbiota composition, with subsequent effects on the metabolome in broilers. A completely randomized block design was employed with 2 diets [standard (**SD**), and challenge (**CD**)] and 3 additive conditions (Control, Pro, Post) involving 1,368 one-day-old male Ross 308 broilers equally distributed among 36 pens in a 42 d study. Diets were formulated to contain identical nutrient levels, with CD higher than SD in non-starch polysaccharide content by including rye and barley. Total non-specific serum Ig A, M and G concentrations were determined weekly from d14 to 35. Following vaccination, titres of specific antibodies binding Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) were measured. Microbiota composition was analyzed by 16S rRNA gene sequencing at d14 and 35, and α- and β-diversity indexes (Observed, Chao1, Bray, Jaccard) were calculated. Cecal short-chain fatty acids and the semi-polar metabolome were determined in the Control SD and all CD groups at d35. At d35, a diet −additive interaction was observed on cecal microbiota composition. Within SD, Pro and Post did not affect operational taxonomic units (**OTU**) abundance (adjusted-$P > 0.05$) and diversity indexes ($P > 0.05$). Within CD, Pro and Post affected the relative abundances of 37 and 44 OTUs, respectively (adjusted-$P < 0.05$), with Post but not Pro affecting β-diversity indexes ($P = 0.041$ and 0.064 for Bray and Jaccard, respectively). Within CD, Post increased cecal acetate (21%; $P = 0.007$) and butyrate (41%; $P = 0.002$) concentration and affected the concentration of 2 metabolites (adjusted-$P < 0.05$), while Pro affected 240 metabolites (adjusted-$P < 0.05$). No diet−additive interactions were observed on serum Ig ($P > 0.05$), except for IgM at d14 ($P = 0.004$). Diet composition, but not the additives, affected immune status parameters. The Pro and Post affected cecal microbiota composition only under dietary challenging conditions as previously reported for growth.

Key words: dietary challenge, probiotic, postbiotic, microbiota, immunity

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INTRODUCTION

Since the ban on the use of antibiotic growth promoters in the European Union in 2006, and their limitation in many other countries, intensive efforts have focused on finding alternatives that can promote broiler health and performances (Ayalew et al., 2022). In addition to the ban, availability of feed ingredients is decreasing due to geopolitical instability, crop failures and conflicts, with high quality ingredients becoming scarcer and more expensive (Babatunde et al., 2021; Davis et al., 2021). As a result, the use of suboptimal feed ingredients is becoming more important, which can lead to dietary compositions challenging to broilers in terms of maintaining health, growth performance and welfare (Beski et al., 2015; Lannuzel et al., 2022; Polovinski-Horvatović, 2021).

Feed additives such as probiotics and their derived postbiotics have been shown to improve growth performance and health of broilers (Humam et al., 2019; Jha et al., 2020). Pro- and postbiotics are reported to affect the broiler gut microbiota (Zamojska et al., 2021) and immune parameters (Yazhini et al., 2018; Humam et al.,

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2019; Jha et al., 2020; Abd El-Ghany et al., 2022). The reported effects of these additives vary between strains, species and by the experimental design employed (Blajman et al., 2014; Humam et al., 2019; Ogbuewu and Mbajiorgu, 2022). In laying hens, mice, pigs and humans, the physiological effects of probiotics were shown to also depend on diet composition (Abd El-Hack et al., 2017; Liu et al., 2018; Larsen and Choi, 2023; Wastyk et al., 2023), providing evidence that diet can modulate effects of pro- and postbiotics.

The intestinal microbiota play a key role in chicken performance, gut health, immune education and development, and physiology (Kogut, 2019). It also influences nutrient digestion and absorption (Diaz Carrasco et al., 2019; Liao et al., 2020). Among all intestinal segments, the caeca contain the highest microbiota density and diversity and are also the major site of fermentation in chickens (Bindari and Gerber, 2022). The resident intestinal microbiota produce metabolites [e.g., short chain fatty acids (**SCFA**)], interact with immune and intestinal cells, and compete with potential pathogens (Bindari and Gerber, 2022). However, the microbiota also metabolize dietary nutrients which could have otherwise been absorbed and used by the chicken's metabolism. The intestinal microbiota are known to be strongly affected by diet (Bindari and Gerber, 2022) and by pro- and postbiotics in poultry (Zamojska et al., 2021). Considering the broad spectrum of activities of the gut microbiota (Kogut, 2019), it can be expected that the modification of the microbiota in terms of extent and composition by pro- and postbiotics depends on the diet composition which can, therefore, lead to different physiological effects.

In order to investigate effects of a specific *Lactobacilli*-based probiotic (**Pro**) and its derived postbiotic (**Post**), while considering the potential effect of diet composition, we performed an experiment under commercial conditions in broilers with different diet × additive combinations (Jansseune et al., 2024). Diet composition [standard and challenge (non-starch polysaccharide (**NSP**)-rich diet)] was found to affect the results of the 2 supplements on broiler growth performance, bone health and strength, and blood biochemical parameters. Here we report the effects of these specific Pro and Post supplements on microbiota composition with subsequent effects on the metabolome and immune parameters to obtain further insights into the diet − additive interactions reported by Jansseune et al. (2024). Since pro- and postbiotics were reported for their effects on the microbiome and immunity, which can be affected by dietary conditions, it was hypothesized that cecal microbiota composition and immune parameters are affected by a diet-additive interaction.

MATERIAL AND METHODS

Ethic Statement

The animal protocol for this research (ZT 2132) was approved by the Animal Welfare Committee of Zootest (Ploufragan, France) and complied with the guidelines of the European Union council directive 2010/63/EU for animal experiments. Animals were monitored daily, and handling and sampling took place under supervision of registered veterinarians.

Birds Housing and Management

One-day-old male Ross 308 broilers were purchased from a commercial hatchery of 43 weeks of age broiler breeders (Galina Vendée, Daviet Ets, Essarts-en-bocage, France), individually weighed and distributed across 36 pens with 38 broilers each, so that all pens had a similar average chick BW (∼43.0 g) and distribution. Pens were 1.90 × 1.25 × 0.8 m (L × W × H) with wood shavings as floor covering. All pens were located along the wall with air openings on one side of a commercial, 1200 m^2 Colorado type building. The water and feed per pen were provided ad libitum through nipple drinkers (5−6) and one 40 cm diameter Hung pan feeder (Josse, Montauban de Bretagne, France).

At d14, an individual eye vaccination against Newcastle disease virus (**NDV**) (Avinew NeO, Merial, Ancenis, France) was used as a model of a controlled immune challenge. Then, at d17, broilers were vaccinated through drinking water against infectious bursal disease virus (**IBDV**) (HIPRAGUMBORO G97, Laboratorios Hipra, Amer, Spain) as is common practice under commercial production.

Experimental Treatments

Briefly, a completely randomized 2 × 3 block design with a standard diet (**SD**) and challenge diet (**CD**) and 3 additive conditions [control (**Ctrl**), Pro and Post] with 6 pens of 38 chicks per treatment was used. The Ctrl SD contained wheat, corn and soybean meal, whereas the Ctrl CD additionally contained rye, barley and palm oil fat (ingredient and calculated composition are presented in Table 1). The commercial Pro was SORBIFLORE and its derived Post was METALAC (STI biotechnologie, Maen Roch, France). Pro and Post inoculums were included at 50 g and 500 g inoculum/t diet as is, respectively, from d1 to d11, and at 40 g and 400 g inoculum/t diet as is, respectively, from d12 to d42 according to manufacturer's recommendation.

Blood Samples Collection and Immune Parameters Evaluation

Blood Sampling. Four broilers/pen (n = 24) were randomly selected at d11, wing tagged, with blood collected by vein puncture at d14, 21, 28 and 35, alternating the right and left wing. Blood was collected in uncapped empty vacuum tubes (Labelians, Nemours, France), and then centrifuged at 1,800 × *g* for 5 min at room temperature (80-2 Electronic Centrifuge, YingTai Medical, Guangdong, China) to recover serum prior to storage at -20°C.

Table 1. Ingredient and calculated composition including energy content of the standard and challenge starter, grower and finisher diet for broilers.

	Starter (0−11 d)		Grower (11−28 d)		Finisher (28−42 d)	
Composition	Standard	Challenge	Standard	Challenge	Standard	Challenge
Ingredient, % as is						
Corn	29.7	18.1	35.8	18.3	42.8	28.6
Wheat	30.0	25.0	30.0	24.9	30.0	15.0
Barley	-	10.0	-	10.0	-	10.0
Rye	-	5.00	-	10.0	-	15.0
Soybean meal	33.5	33.6	28.3	28.5	21.9	23.4
Limestone	1.61	1.61	1.11	1.10	0.85	0.85
Mono calcium phosphate dihydrate	1.37	1.30	0.90	0.83	0.78	0.72
Sodium chloride 99%	0.25	0.25	0.25	0.25	0.25	0.25
Sodium bicarbonate	0.15	0.15	0.15	0.15	0.15	0.15
Soy oil	2.42	3.00	2.51	2.00	2.23	2.00
Palm oil	-	1.00	-	2.98	-	3.00
DL-methionine 99%	0.25	0.25	0.22	0.24	0.20	0.23
Lysine HCL 98%	0.16	0.15	0.18	0.17	0.25	0.21
L-threonine 98%	0.09	0.09	0.08	0.08	0.09	0.09
Premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Calculated, % as is						
Dry matter	87.7	88.1	87.6	88.0	87.4	88.1
Crude protein	22.0	22.0	20.0	20.0	17.6	17.7
Crude fat	4.44	5.72	4.76	6.71	4.71	7.08
Starch	38.6	36.1	42.5	38.7	46.8	41.9
Ash	6.50	6.55	5.26	5.30	4.54	4.67
Cellulose	2.68	2.97	2.56	2.85	2.42	2.69
Dig. Methionine	0.56	0.56	0.51	0.51	0.46	0.48
Dig. Methionine+cystine	0.91	0.91	0.84	0.84	0.76	0.76
Dig. Lysine	1.16	1.16	1.05	1.05	0.95	0.96
Dig. Threonine	0.78	0.78	0.70	0.71	0.64	0.64
Dig. Valine	0.90	0.90	0.82	0.82	0.72	0.73
Dig. Arginine	1.32	1.33	1.18	1.19	1.00	1.03
Calcium	0.96	0.96	0.68	0.67	0.54	0.54
Available phosphorous	0.46	0.46	0.34	0.34	0.30	0.30
Chlorine total	0.23	0.23	0.24	0.24	0.25	0.24
Sodium total	0.15	0.15	0.15	0.15	0.15	0.15
Apparent metabolizable energy (MJ/kg as is)	12.05	12.05	12.63	12.63	12.87	12.87

[1]Supplied per kg premix: 2,000,000 IU retinyl acetate, 500,000 IU cholecalciferol, 10 g DL-a-tocopherol, 300 mg menadione, 400 mg thiamine, 1,500 mg riboflavin, 700 mg pyridoxine-HCL, 4 mg cyanocobalamin, 7 g niacin, 2.4 g D-pantothenic acid, 92 g choline chloride, 200 mg folic acid, 40 mg biotin, 53 g $FeSO_4 \cdot H_2O$, 9.6 g $CuSO_4 \cdot 5H_2O$, 28 g MnO, 33 g $ZnSO_4 \cdot H_2O$, 240 mg KJ, 66 mg Na_2SeO_3.

Measurement of Serum IgG, IgM and IgA. Immunoglobulin A, G, and M levels were determined in blood serum samples by indirect ELISAs at d14, 21, 28 and 35 as previously described by Lecoeur et al. (2024) using anti-chicken IgA, IgG or IgM as capture antibodies (Cat no. A30-103A, A30-104A and A30-102A, Bethyl Laboratories, Montgomery, USA) as well as conjugated to horseradish peroxidase as detection antibodies (Cat no. A30-103P, A30-104P and A30-102P, Bethyl Laboratories).

Assessment of Vaccine-Specific Antibodies. Serum titre against IDBV and NDV were assessed in sera at d21, 28 and 35 and NDV also at d14 by indirect ELISAs for the detection of antibodies. Kit ELISAs ID Screen IBD Indirect and ID Screen Newcastle Disease Indirect Conventional Vaccines (Innovative diagnostics, Grabels, France) were used for IBDV and NDV antibody titers, respectively. Antibody titers calculation and seropositivity threshold were performed following the manufacturer's instructions. Antibody titers were expressed as the antilog of the $\log_{10}$ titer obtained by calculating the sample to positive ratio (S/P = mean of test sample − mean of negative control / mean positive control - mean of the negative control) and using the equation $\log_{10}$ titer = 0.97 ($\log_{10}$ S/P) + 3.449 for IBDV, and the equation $\log_{10}$ titer = ($\log_{10}$ S/P) + 3.52 for NDV. Samples were considered seropositive when they had a titer above 993 and 875 for NDV and IBDV, respectively.

In addition, antibodies against NDV were also quantified using a hemagglutination inhibition (**HAI**) assay as previously described (Lecoeur et al., 2024). Briefly, 25 μL of NDV antigens diluted at 4 HA units (avian paramyxovirus type-1, ANSES, Ploufragan, France) was added to 25 μL of doubling dilutions of serum and left for 30 min at room temperature before the addition of 25 μL of 1% of chicken red blood cells. HAI titers were expressed as $\log_2$ of the reciprocal of the highest dilution of serum causing inhibition of hemagglutination.

Cecal Digesta Sample Collection

Cecal digesta were sampled from 3 and 4 randomly selected birds/pen at d14 and 35, respectively. Birds were euthanized by cervical dislocation prior to dissection. The content was obtained from both caeca by gentle squeezing of the segments before being individually homogenized and immediately frozen in liquid nitrogen and stored at -80°C. Cecal contents consistency was visually scored on a 5-points scale [from 0 (thick) to 4

(very liquid)] as well as the presence of visible structures associated with gas formation on the surface of cecal contents (e.g., foam-like structures, bubbles, etc.) on a 4-points scale (0: absence, 1: little, 2: middle and 3: highly foaming).

Cecal Microbiota DNA Extraction, Amplification, and Sequencing

For each sample, DNA from 200 mg cecal content was extracted following a protocol described by Borey et al. (2020). Then, DNA quantity was evaluated using a Qubit analyzer. Amplification of the V3-V4 hyper-variable region of the 16S rRNA coding gene was performed by the INRAE @BRIDGe platform (Jouy-en-Josas, France). In summary, universal V3-V4 primers (Nadkarni et al., 2002) were used for a first PCR reaction (denaturation: 95°C for 3 min; amplification: 30 cycles of 98, 68, and 72°C for 20, 60, and 60 s, respectively; final elongation 72°C for 10 min) with a T100 Thermal cycler (Biorad, Hercules, CA). Amplicons were purified using magnetic beads (Clean NA, GC biotech B.V., Waddinxveen, The Netherlands) and DNA concentration was controlled using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). In the second PCR, other primers were used (F: AATGATACGGCGACCACCGAGATCTACACT-CTTTCCCTACACGAC; R: CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACT-GGAGTTCAGACGTGT) with identical conditions as for the first PCR. Amplicons were purified and DNA concentration was controlled as described for the first PCR reaction. One run on an Illumina MiSeq was used to sequence amplicons (2×250 paired-end reads) according to the standard protocol.

Cecal Metabolome Analysis

Following the results on growth performances reported by Jansseune et al. (2024) and cecal microbiota composition, which both showed effects of CD vs. SD and of Pro and Post vs Ctrl in CD only, 4 treatments were selected (Ctrl SD, Ctrl CD, Pro CD and Post CD) for analysis of cecal SCFA concentrations and the metabolome at d35. Analyses were not performed at d14 for practical reasons (i.e., insufficient sample number and material) and because the microbiota alteration was weaker than at d35, as shown by the number of differentially abundant OTUs. Untargeted metabolomics was performed for identification and relative quantification of the water-soluble semi-polar metabolome.

Cecal and Quality Control Sample Preparation. An accurately weighed amount (50−100 mg) of each cecal content was mixed with 4 times w/v ultrapure water and homogenized by shaking for 2 min at 30 Hz. Each homogenized sample was then centrifuged ($16{,}000 \times g$, 4°C, 10 min, CENT 5430, Eppendorf, Hamburg, Germany), and the supernatant was collected and filtered by centrifugation ($15{,}000 \times g$, 4°C, 5 min, CENT 5430) using a 0.22 μm nylon Costar Spin-X filter (Corning, Corning, NY). A quality control (**QC**) sample was prepared by pooling small equal aliquots from each sample to create a representative average of the entire set which was analyzed every 5 samples.

SCFA Analysis. Analysis of SCFA was carried out by MS-Omics (Copenhagen, Denmark) following the procedure of Nielsen et al. (2018). Briefly, cecal digesta samples were acidified using HCl, and an internal standard containing deuterium-labelled fatty acids was added. All samples were analyzed in a randomized order. Analysis was performed using a high polarity column (Zebron ZB-FFAP, GC Cap. Column 30 m × 0.25 mm × 0.25 μm, Phenomenex Inc., Torrance, CA) installed in a gas chromatograph (7890B, Agilent, Santa Clara, CA) coupled to a quadrupole detector (5977B, Agilent). The system was controlled by ChemStation (Agilent). Raw data were converted to netCDF format using Chemstation (Agilent), before the data were imported and processed in Matlab R2021b (Mathworks, Portola Valley, CA) using the PARADISe software as described by Johnsen et al. (2017).

Semi-Polar Metabolites Analysis. Metabolites profiling in cecal digesta was performed by MS-Omics as described by Iribarren et al. (2021). Briefly, cecal digesta sample extracts were diluted 10 times in mobile phase eluent composed of 10 mM ammonium formate (VWR chemicals 84884.180, Rosny-sous-Bois, France) and 0.1% formic acid (VWR chemicals, 84865.180) and fortified with stable isotope labelled standards before analysis. The analysis was carried out using a Thermo Scientific Vanquish LC coupled to an Orbitrap Exploris 240 MS (Thermo Fisher Scientific, Waltham, MA). An electrospray ionization interface was used as ionization source. Analysis was performed in positive and negative ionization mode under polarity switching. The liquid chromatography MS/MS was performed using a slightly modified version of the protocol described by Doneanu et al. (2011). Peak areas were extracted using Compound Discoverer 3.3 (Thermo Fisher Scientific) and normalized to internal standards. Briefly, 4 steps were applied: 1) features corresponding to a peak characterized by one mass and one retention time are extracted from the raw data, 2) features belonging to the same compounds are grouped, 3) the molecular formula of each compound is determined, and 4) the collected information is used for compound identification at different levels (1, 2a, 2b, and 3). Identification criteria were for 1) retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra, 2a) retention times (compared against in-house authentic standards) and accurate mass (with an accepted deviation of 3 ppm), 2b) accurate mass (with an accepted deviation of 3 ppm) and MS/MS spectra, and 3) accurate mass alone (with an accepted deviation of 3 ppm) with the libraries Human metabolome database and CHEBI. Then, features absent from more than 10 samples in all treatments, absent in more than 5 samples of a treatment and with a coefficient of variation above 20% in the quality control samples were excluded. Missing values were

imputed with half of the minimal value of the feature and peak area data were log-transformed and Pareto-scaled.

Analysis of Cecal Microbiota Taxonomic Composition

Identification of swarm clustered operational taxonomic units (OTU) was performed with the FROGS [Find Rapidly OTUs with Galaxy Solution, (Escudié et al., 2018)] pipeline on galaxy (v. 4.0.1). The FROGS pre-process function was used to merge, denoise and dereplicate the sequenced reads with the following parameters: mismatch rate < 0.1 and sequence length 433-463. OTUs were identified and then chimera removed with the FROGS Clustering Swarm and FROGS Remove Chimera programs, respectively, using default parameters. Filtering of OTUs was performed with a minimum relative abundance threshold of 0.005% and a blast coverage > 0.95 with samples containing < 14,000 reads removed.

Statistical Analyses

All analyses were performed with R version 4.0.3 (R Core Team, 2023). Probability or adjusted probability values < 0.05 and $0.05 \leq p < 0.10$ were considered significant and a trend, respectively. Values deviating by more than 3 standard deviations from the mean were considered outliers and excluded from the dataset. Excluded values were restricted to Ig and cecal SCFA with a maximum of one excluded value per treatment.

Treatment effects were analyzed using the general linear model procedure and type 2 analysis of variance (ANOVA) (library lme4). Treatment effects for ordinal data were analyzed by logistic or ordinal logistic regression with the cumulative linked mixed model function for ordered logistic regression. Statistical models contained the fixed effects of diet and additive, their interaction and a random block factor. For blood Ig also BW and its interactions with diet and additive was included. Variance homogeneity was assessed with Levene's test and a White-Huber correction was applied in case of heteroscedasticity. If residuals were not normally distributed, as evaluated by the Shapiro test, data were boxcox transformed. If non-normality remained, data were analyzed by permutational analysis of variance (PERMANOVA) with treatment and random block effect and 5000 permutations (function adonis2, library vegan v. 2.6-4) and Euclidian distance as proposed by Anderson (2017). Intra-diet contrast (Pro vs Ctrl and Post vs Ctrl) and comparisons of controls (standard vs challenge) were performed for significant interaction effects. For significant main additive effects, inter-diet contrast analyses for Pro and Post against Ctrl were performed.

For the microbiota data, α-diversity (Observed and Chao 1) and β-diversity (Bray and Jaccard) indexes were determined with the Phyloseq package (v.1.42.0) and Diet, Additive and Diet × Additive effects by identified by ANOVA and PERMANOVA (vegan v.2.6.4), respectively. PERMANOVA was performed with 9,999 permutations. Differentially abundant OTU between treatments were identified with DESeq2 (v.1.38.2) with default parameters. The α- and β-diversity and differential abundance analyses were performed on rarefied data, obtained with the rarefy_even_depth function (phyloseq v.1.42.0) and an initial seed number of 10,000.

For the untargeted-metabolomic and SCFA data, single-dimensional statistical analyses were performed by ANOVA for the contrasts of interest. The models included a fixed treatment effect and a random block factor. When residuals normality was not confirmed, analyses were performed with rank ANOVA. Probability values tor the untargeted-metabolome were corrected for multiple testing by Benjamini-Hochberg correction and fold changes were calculated based on non-transformed peak area data.

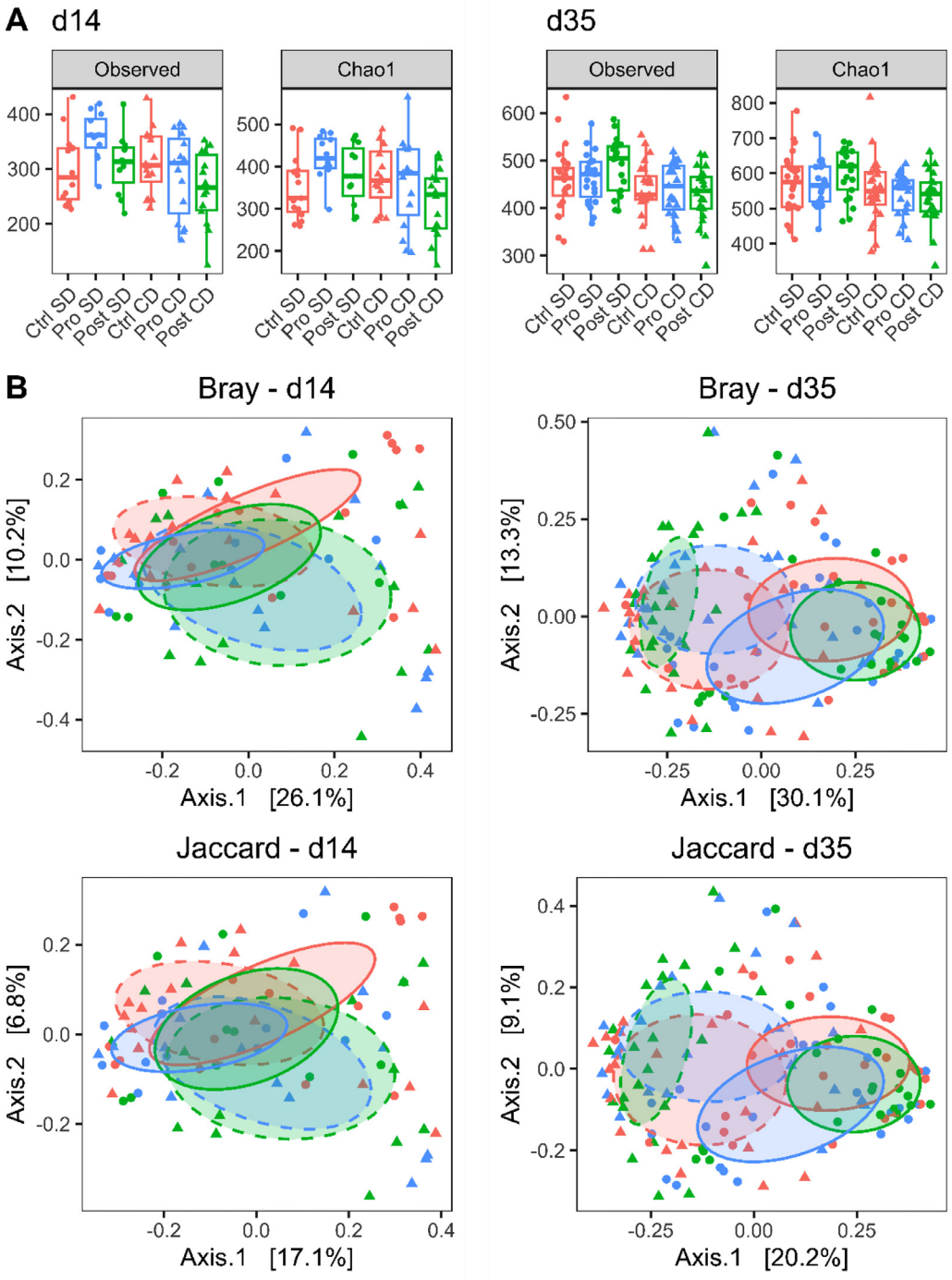
RESULTS

Diversity and Structure of the Cecal Microbiota

Sequencing of the cecal microbiota produced 11,881,627 reads and 1,944,205 clusters. After denoising, removing chimera and filtering low quality sequences, a total of 6,918,237 sequences were assigned to 1,114 OTUs out of which 2,771 sequences belonging to 4 OTUs were excluded based on blast coverage < 0.95. After removing samples with less than 14,000 reads (23 samples out of 107 evenly distributed between treatments, and 4 samples out of 143 at d14 and 35, respectively), the final dataset contained samples with, on average, 29,573 reads belonging to 1,050 OTUs at d14, and 31,835 reads belonging to 1,110 OTUs at d35, respectively.

The diversity indexes of the cecal microbiota are presented in Figure 1. Results for α-diversity indexes and associated p-values are presented in Figure 1A and 1C, respectively. At d14, Observed and Chao1 α-diversity indexes showed a Diet × Additive effect. Contrast analyses revealed that Observed and Chao1 α-diversity indexes were 21% higher for Pro SD vs. Ctrl SD ($p = 0.023$ and 0.034 respectively), and 15% lower for Post CD vs Ctrl CD ($p = 0.036$ and 0.048, respectively). No further significant contrasts were observed. At d35, the α-diversity indexes were affected by diet only and were lower for CD compared to SD (-5.6 and -6.9% for Observed and Chao1 α-diversity indexes, respectively).

Results for β-diversity indexes and associated p-values are presented in Figure 1B and 1C, respectively. At d14, Bray and Jaccard β-diversity indexes showed only a Diet effect, whereas at d35 there was a Diet × Additive interaction (Figure 1C). At d35, within birds fed SD, Pro and Post had no effect on Bray and Jaccard β-diversity indexes. However, for birds fed the CD, Post affected Bray and Jaccard β-diversity indexes ($p = 0.041$ and 0.064, respectively), but Pro had no effect on these indexes. Moreover, the Ctrl SD vs Ctrl



C

	14				35			
	Alpha		Beta		Alpha		Beta	
Factor	Observed	Chao 1	Bray	Jaccard	Observed	Chao 1	Bray	Jaccard
Diet	0.019	0.072	0.028	0.021	0.003	0.017	<0.001	<0.001
Additive	0.080	0.125	0.456	0.454	0.937	0.838	0.749	0.773
Diet × Additive	0.015	0.038	0.261	0.267	0.442	0.540	0.021	0.021

Figure 1. Alpha (A) and MDS plot of Beta (B) diversity and associated p-values for treatment effects (C) of the cecal bacterial content in 14- and 35-day-old Ross 308 male broilers fed a standard (**SD**) or challenge diet (**CD**) unsupplemented (**Ctrl**) or supplemented with *Lactobacilli*-based probiotic (**Pro**) and postbiotic (**Post**) from d 1 onward. Each point represents an individual broiler. Red, blue and green symbols belong to Ctrl, Pro and Post, respectively. Dots and full ellipses belong to SD whereas triangles and dashed ellipses belong to CD.

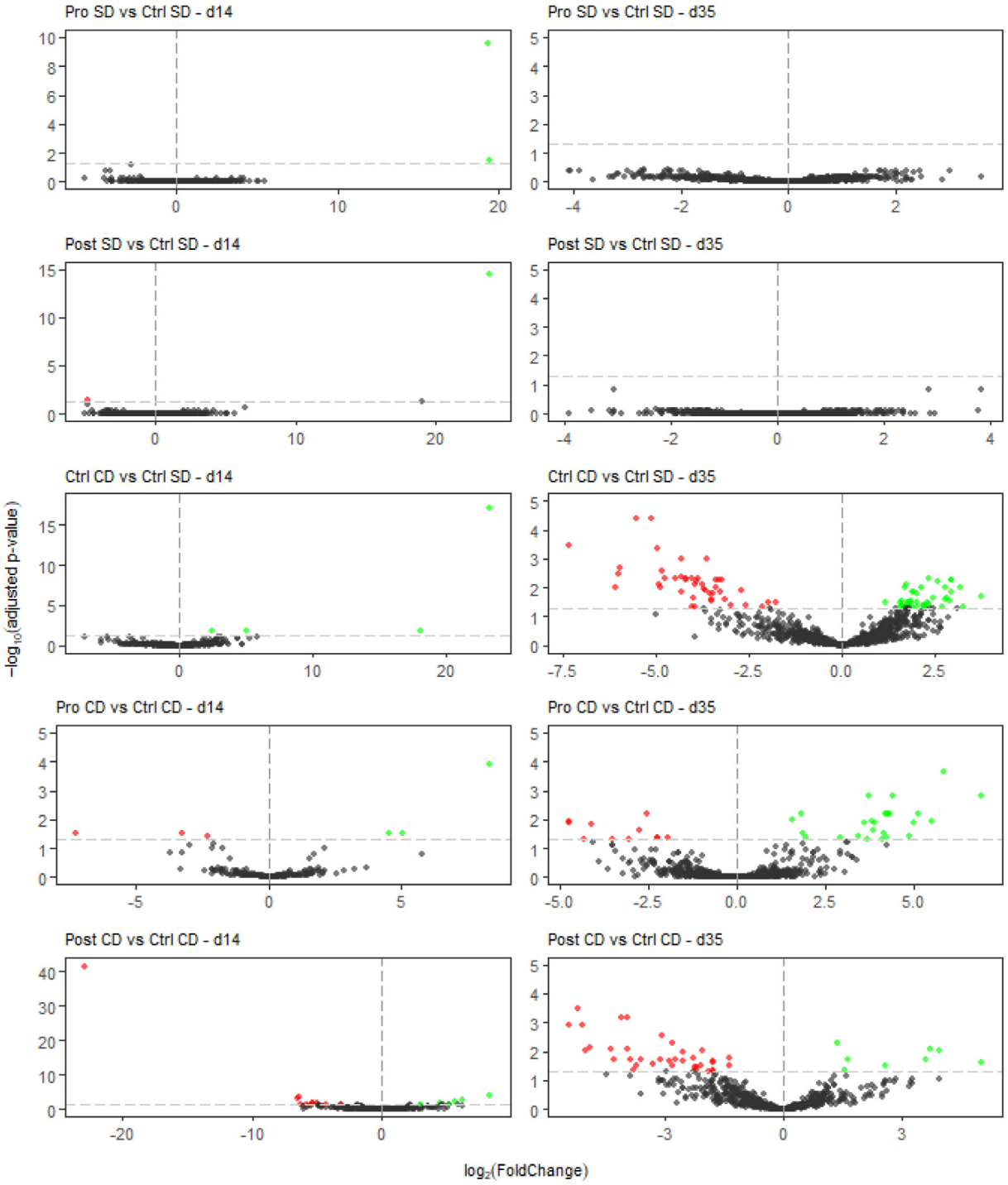


Figure 2. Volcano plots showing differentially abundant Operational Taxonomic Unit (**OTU**) in the cecal content of 14- and 35-day-old Ross 308 male broilers fed a standard (**SD**) or challenge diet (**CD**) unsupplemented (**Ctrl**) or supplemented with *Lactobacilli*-based probiotic (**Pro**) and postbiotic (**Post**) from d 1 onward. Each point represents an individual OTU. The position along the x-axis represents the abundance fold change. The horizontal dashed line represents adjusted $p = 0.05$. Green, red and grey dots represent significantly enriched OTUs, significantly depleted OTUs and not different OTUs, respectively.

CD comparison was significant for both β-diversity indexes.

Differential abundance analyses for OTUs are summarized in Figure 2 for mean fold change and associated adjusted p-value. Detailed results are available in Supplementary Table 1 for OTUs classification, mean and median abundance. Four (all up) and 86 (43 up; 43 down) OTUs were differentially abundant in Ctrl CD compared to Ctrl SD at d14 and 35, respectively. Most represented genus at d35 was Faeecalibacterium (20 up; 28 down) followed by Barnesiella (8 up), and 3 species were identified: *Gallibacterium anatis, Enterococcus cecorum* and *Anaerostipes butyraticus* with a log2 fold change (FC) of 3.8, 3.2 and 2.6, respectively. At d14, 2 OTUs were differentially abundant for Pro and Post compared to Ctrl for SD. For CD, Pro caused 6 differentially abundant OTU's (3 up; 3 down) and Post 17 (6 up; 11 down) compared to Ctrl. At d35, for SD, Pro and

Table 2. Mean cecal digesta concentration (**mM**) of short chain fatty acids in 35 day-old male Ross 308 broilers fed a standard (**SD**) or challenge diet (**CD**) without supplementation (**Ctrl**) or supplemented with Lactobacilli-based probiotic (**Pro**) and postbiotic (**Post**) from d 1 onward.

	SD	CD				Probability value		
Fatty acid	Ctrl	Ctrl	Pro	Post	pooled SEM	Ctrl SD vs. Ctrl CD	Pro vs. Ctrl CD	Post vs. Ctrl CD
Acetic acid	15.5[1]	14.7	17.0	17.8	1.8	0.600	0.055	0.007
Propanoic acid	2.59	2.36	2.68	2.44	0.39	0.472	0.192	0.748
2-Methylpropanoic acid	0.239	0.251	0.228	0.207	0.043	0.724	0.468	0.110
Butyric acid	3.21	3.50	4.31	4.94	0.68	0.552	0.131	0.002
3-Methylbutanoic acid	0.180	0.203	0.185	0.198	0.028	0.303	0.402	0.806
Pentanoic acid	0.278	0.275	0.316	0.320	0.40	0.932	0.131	0.095

[1]Mean of 24 animals per treatment, excluding outliers and values below the limit of detection.

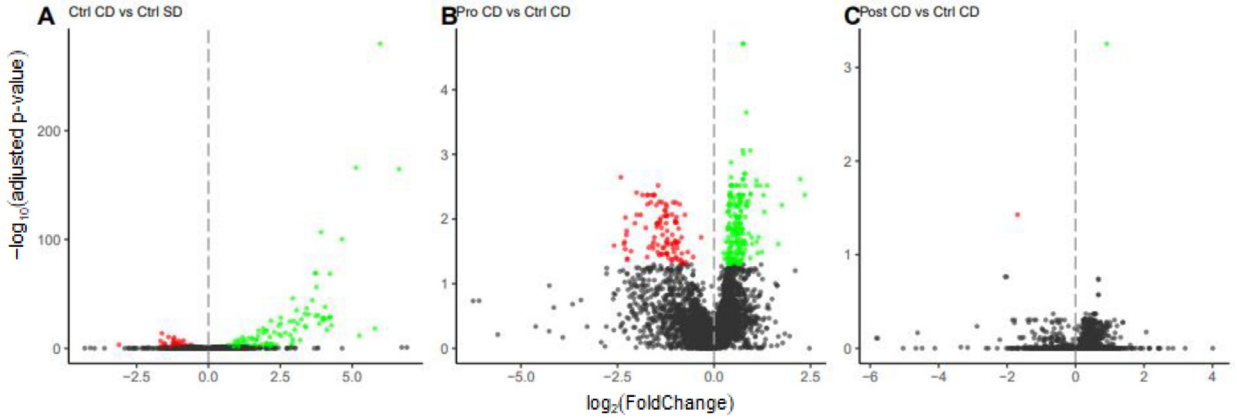
Post had no differentially abundant OTUs compared to Ctrl. By contrast, for CD, Pro had 36 (25 up; 11 down) and Post had 44 (8 up; 36 down) differentially abundant OTUs compared to Ctrl. Among the 36 OTUs affected by Pro in CD at d35, most represented genera were *Faecalibacterium* (16 up; 1 down) and *Lachnospiraceae* GCA-900066575 (3 up), with 1 identified specie, *Butyricocccus pullicaecorum* being decreased by a log2(FC) of -3.1. Among the 44 OTUs affected by Post in CD at d35 most represented genera were *Faecalibacterium* (6 up; 8 down) and *Bacteroides* (5 down) and 3 species were identified: *Intestinimonas timonensis, E. cecorum* and *Pseudoflavoni-fractor capillosus* with log2 (FC) of 1.6, -2.8 and -2.1, respectively.

Short Chain Fatty Acids

Concentrations of acetic acid, propionic acid, butyric acid, 3-methyl butyrate and pentanoic acid in cecal digesta are presented in Table 2. The concentrations of all SCFA were unaffected by diet (Ctrl SD vs Ctrl CD). Pro only tended to increase acetic acid (+15.6%; $p = 0.055$), whereas Post increased acetic acid (+21.1%) and butyric acid (+41.1%) and tended to increase pentanoic acid concentration (+16.4%; $p = 0.095$). Formic acid, 4-methyl-propanoic acid, hexanoic acid heptanoic acid concentrations were below the limit of detection in 94.6, 90.3, 98.9, and 100% of the samples, respectively, with the positive samples evenly distributed among the 6 treatment groups (Ctrl, Pro and Post × SD and CD). For 2-methyl propanoic acid, 73.1% of the samples were above the detection limit with an even distribution among the 6 treatment groups.

Semi-Polar Metabolome

A total of 4,270 features were detected in the samples of which 69 could be unambiguously annotated (Level 1). Additionally, 72 features were annotated by accurate mass and matched to the retention time of reference standards run on the same system (Level 2a). Also, 129 features could be annotated by matching them to the accurate mass and MS/MS fragmentation of reference spectra (Level 2b) with 2,225 features able to be



B	Foam-like formations d14	Foam-like formations d35	Consistency d35
Diet	0.002	0.067	0.002
Additive	0.957	0.194	0.358
Diet × Additive	0.883	0.688	0.688

Figure 3. Volcano plot showing differentially abundant features in the cecal content of 35-day-old Ross 308 male broilers fed a control (**Ctrl**) standard (**SD**) or challenge diet (**CD**) supplemented with *Lactobacilli*-based probiotic (**Pro**) and postbiotic (**Post**) from d 1 onward. Each point represents an individual feature. The position along the x-axis represents the abundance fold change. Green, red and grey dots represent significantly enriched features (adjusted $p < 0.05$), significantly depleted features (adjusted $p < 0.05$) and not different features (adjusted $p > 0.05$), respectively.

annotated by exact mass and isotope pattern and reference count (Level 3). Finally, 1,775 features were distinguished from the background and were not identified.

Univariate statistics (summarized in Figure 3) revealed that 169 features were different between Ctrl SD and Ctrl CD (36 down, 133 up), 240 features were different between Pro CD and Ctrl CD (107 down, 133 up), and 2 features were different between Post CD and Ctrl CD (1 down, 1 up). Among affected metabolites, which were unambiguously named (Level 1 and 2a), Ctrl CD increased acetylcholine by a Log2(FC) of 1.56 ($p = 0.016$) compared to the Ctrl SD. For Pro CD vs. Ctrl CD, anserine, carnosine, cytidine, diaminopimelic acid, 5-metylcytosine and propionyl carnitine were decreased by a Log2(FC) of -0.80, -2.41, -2.05, -1.01, -1.55 and -1.74, respectively ($p = 0.048$, 0.002, 0.016, 0.022, 0.019, and 0.039, respectively) while dodecanedioic acid and tetradecanedioic acid increased by a Log2 (FC) of 0.57 and 0.75 ($p = 0.031$ and 0.035, respectively).

Cecal Digesta Score

Cecal digesta scores were affected by diet only (Figure 4). The presence of foam-like formation was greater in the SD than in the CD group at both sampling ages, while there were no additive nor interaction effects. Birds fed SD had also a more liquid cecal contents at d35, with no additive nor interaction effects. Cecal digesta consistency could not be evaluated at d14 due to too low contents.

Serum Immunoglobulins A, M, and G

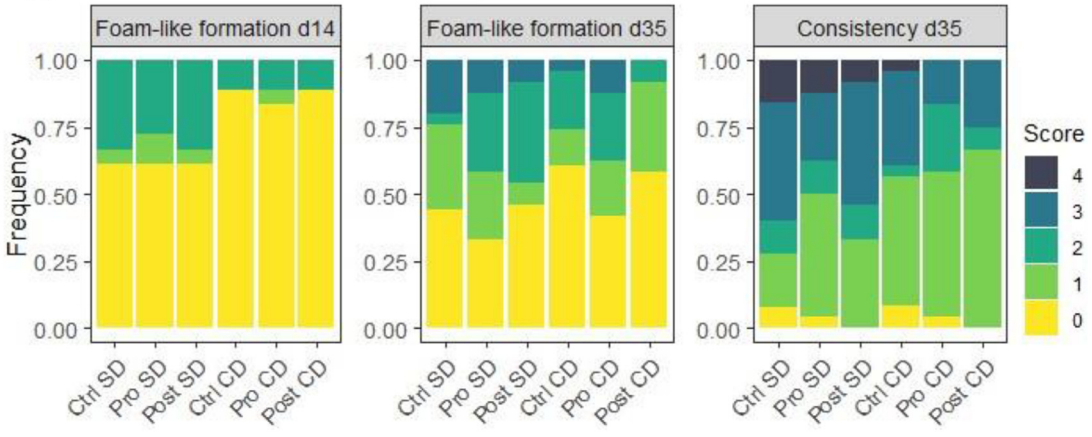
Neither diet nor additive, nor their interaction had a consistent effect on serum IgA, IgM and IgG concentrations at d14, 21, 28, and 35 (Figure 5). The only interaction effect was observed for IgM at d14 which yielded significant contrasts for Pro and Post in SD but not CD with a reduction of IgM concentration compared to the Ctrl. The Diet effect was significant on IgM at d14 and 28 and showed a tendency at d21, with CD eliciting lower IgM concentration than SD. An effect of the additive was only observed on IgA at d21 with a contrast for Pro ($p = 0.006$) but not Post ($p = 0.096$), and a reduction compared to the Ctrl.

The correlations between broiler BW and serum Ig concentration at all ages (d14, 21, 28, and 35) were also assessed (Figure 6A) and the ones which showed an effect of BW alone or in interaction with the treatments with $p < 0.1$ are presented in Figure 6B. A Diet × BW effect was present for IgA at d14, 21, and 28 ($p = 0.086$, 0.001, and 0.057, respectively) and for IgG at d28 and 35 ($p = 0.022$ and 0.091, respectively). In the latter conditions, within SD, BW was not correlated with serum Ig concentration, whereas in birds receiving the CD, BW was negatively correlated with the serum Ig concentration. Body weight alone showed a negative correlation with IgA at d35 ($p = 0.077$) and IgM at d28 ($p < 0.001$).

Immune Response to Vaccination

The antibody response to NDV vaccination was assessed by HAI and ELISA. Overall median HAI scores were 1 at d14 and 21 (Figure 7A), with no treatment nor interaction effects (Figure 7D). An interaction effect was observed at d28 and 35 (Figure 7D) yielding contrasts for Post CD vs. Ctrl CD at d21 ($p = 0.096$), Post SD vs. Ctrl SD at d35 ($p = 0.042$) and Pro CD vs. Ctrl CD at d35 ($p = 0.078$). No other significant contrasts or trends were

A

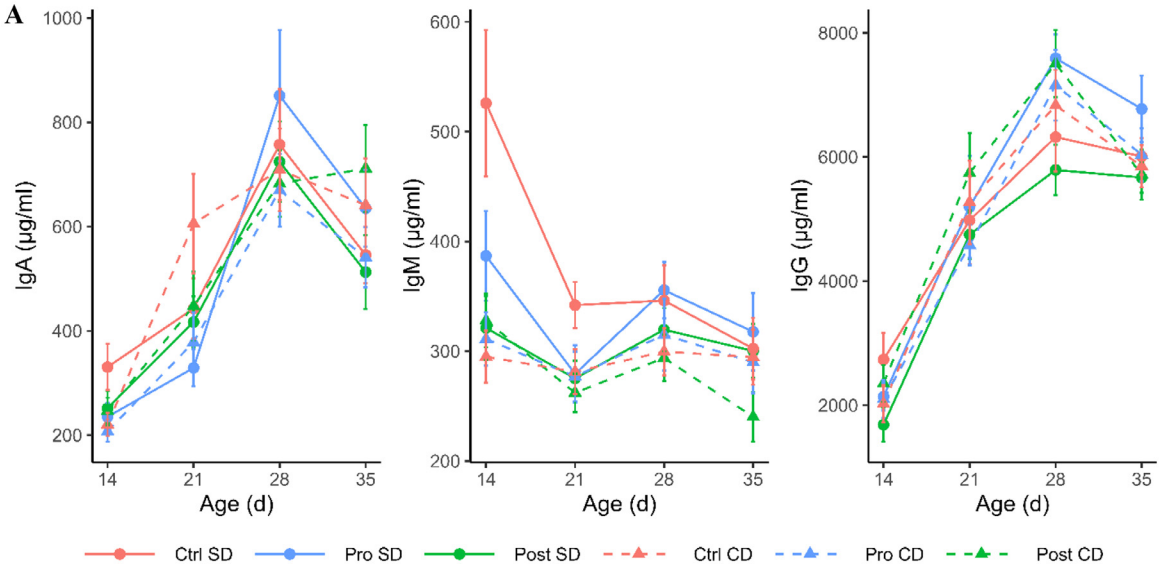


B

	IgA				IgM				IgG			
	d14	d21	d28	d35	d14	d21	d28	d35	d14	d21	d28	d35
Diet	0.105	0.194	0.245	0.186	0.007	0.070	0.031	0.160	0.898	0.316	0.254	0.146
Additive	0.227	0.024	0.801	0.872	0.154	0.077	0.844	0.417	0.613	0.657	0.077	0.136
Diet × Additive	0.158	0.385	0.704	0.127	0.004	0.214	0.654	0.308	0.052	0.892	0.052	0.762

Figure 4. Caeca digesta scores (A) and associated p-values (B). Caeca content scores (consistency and foam-like formation) of 14- and 35-day-old Ross 308 male broilers fed a standard (**SD**) or challenge diet (**CD**) unsupplemented (**Ctrl**) or supplemented with *Lactobacilli*-based probiotic (**Pro**) and postbiotic (**Post**) from d 1 onward.

A



B

Parameter		*P*-value						
Ig	Age (d)	Diet (D)	Additive (A)	Body weight (BW)	D×A	D×BW	A×BW	A×D×BW
A	14	0.203	0.148	0.219	0.446	0.086	0.796	0.341
M		0.013	0.263	0.329	0.028	0.321	0.788	0.937
G		0.771	0.900	0.205	0.079	0.282	0.959	0.197
A	21	0.028	0.018	0.006	0.626	0.001	0.82	0.084
M		0.246	0.103	0.107	0.221	0.222	0.723	0.177
G		0.334	0.629	0.630	0.879	0.427	0.647	0.177
A	28	0.765	0.502	0.000	0.399	0.057	0.410	0.402
M		0.092	0.649	0.000	0.656	0.519	0.361	0.424
G		0.217	0.089	0.101	0.030	0.022	0.996	0.652
A	35	0.071	0.309	0.077	0.573	0.375	0.612	0.255
M		0.251	0.381	0.224	0.108	0.530	0.109	0.109
G		0.107	0.708	0.079	0.788	0.091	0.416	0.682

Figure 5. Mean total immunoglobulin (**Ig**) A, M and G concentrations (A) and associated p-values for treatment effects (B) in 14, 21, 28, and 35-day-old male Ross 308 broilers fed a standard (**SD**) or challenge diet (**CD**) unsupplementated (**Ctrl**) or supplemented with *Lactobacilli*-based probiotic (Pro) and postbiotic (Post) from d 1 onward. Values are the mean of 24 animals, excluding outliers.

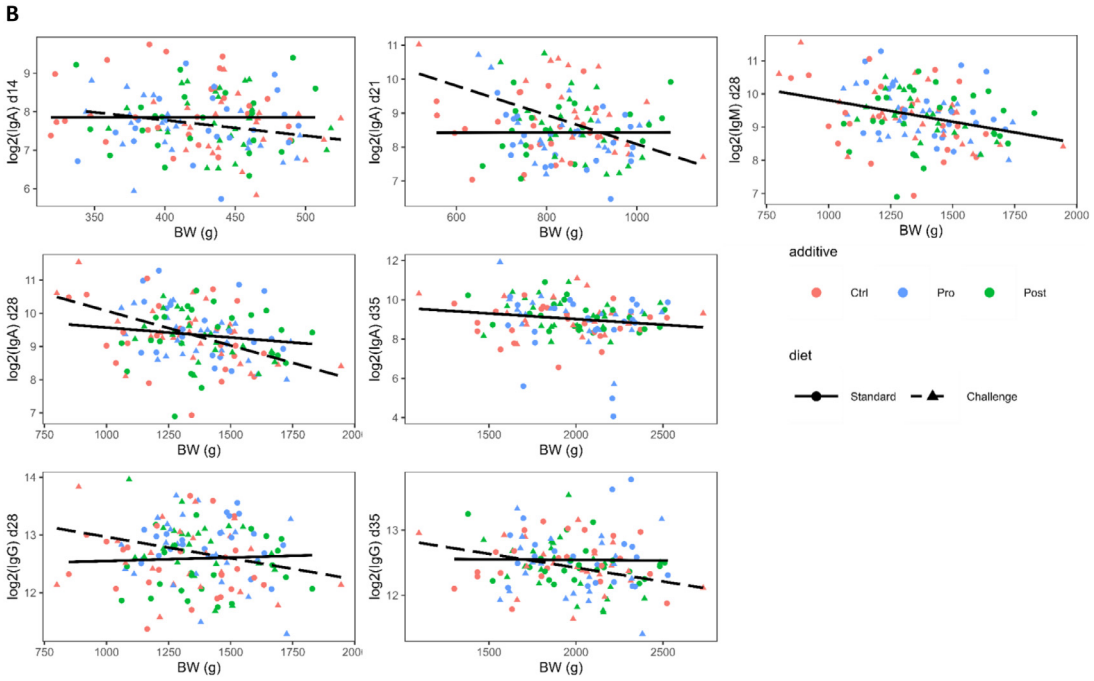
found. Based on the ELISA analyses, 74.3% of the birds were seropositive for NDV at d14 (Figure 7B) due to maternal antibody transfer. One week after vaccination (d21), only one bird was seropositive. At 2 wk after vaccination (d28), 25% of the birds were seropositive with no treatment nor interaction effects. At 3 wk after vaccination (d35), only a Diet effect was observed for the CD group which had 50% seropositive broilers, compared to the SD group (19 vs. 38%).

Following the IBD vaccination at d17, few broilers were seropositive at d21 (1.5%) but none at d28 (0%), with overall, 29% being seropositive at d35 (Figure 7C). A d35, only a Diet effect was observed, with the CD group having less seropositive broilers to IBD than the SD group (21 vs. 37%) (Figure 7D).

DISCUSSION

We previously reported (Jansseune et al., 2024) that diet composition (i.e., standard SD vs challenge CD) affected the effects of Pro and Post additives on broiler growth and physiology. The current study reports additional results on broiler immune parameters, cecal microbiota and metabolome. The cecal microbiota with subsequent effects on the cecal metabolome, but not the immunological parameters paralleled the Pro and Post effects on growth performance reported by Jansseune et al. (2024) with effects restricted to CD. As stated by Bindari and Gerber (2022), diet composition is one of the most important factors influencing gut microbiota composition in livestock animals. As such, it may impact the mechanisms by which feed additives like pro- and postbiotics affect the gut microbiota and hence the variations of the traits of interest like growth or health.

Of note are the microbiota composition analyses at d35 which revealed a strong diet-additive interaction, with the latter being already present at d14 but less pronounced. This diet-dependent effect of Pro and Post was also observed on broiler growth at d35, since Pro and Post had no effect in SD, whereas they increased BW in CD (reported in Jansseune et al., 2024). Pro- and



D	NDV								IBD		
	HAI titre				ELISA				ELISA		
Factor	14	21	28	35	14	21	28	35	21	28	35
Diet	0.901	0.828	0.404	0.010	0.319	0.212	0.700	0.020	0.085	1	0.036
Additive	0.199	0.257	0.672	0.412	0.189	0.307	0.895	0.794	0.111	1	0.376
Diet × additive	0.629	0.926	0.022	0.003	0.490	1.000	0.146	0.393	1.000	1	0.160

Figure 6. Correlation between broiler body weight (**BW**) and serum immunoglobulins (**Ig**) A, M and G levels in Ross 308 male broilers fed a standard (**SD**) or challenge diet (**CD**) unsupplemented (**Ctrl**) or supplemented with *Lactobacilli*-based probiotic (Pro) and postbiotic (Post) from d 1 onward. A: p-values for treatment effect on serum Ig per age. B: Regression lines represent the main BW effect alone (one regression line) or in interaction with the diet (2 regression lines) when $p < 0.1$.

postbiotics have been previously studied (Li et al., 2017; Rodrigues et al., 2020; Danladi et al., 2022a; Chai et al., 2023; Yang et al., 2023) for their effects on the gut microbiota in broilers, which can include modifications of microbiota richness, diversity, composition and activity, with the study here being the first to report a diet-dependent effect. The gut microbiota and its metabolites have major effects on host physiology by modulating the host immune development and defense, brain function, metabolism, gut health and functioning (Wu et al., 2022; Wickramasuriya et al., 2022). Moreover, it is known that under different growth conditions, such as available nutrients, bacteria adapt their metabolism and, as a consequence, produce different metabolites (Vernocchi et al., 2020).

Diet composition had a major impact on caeca digesta characteristics, microbiota and metabolome characteristics but not on SCFA concentration. The CD effect is in line with previous research since the soluble NSP present in greater proportion in CD are known to have a water holding capacity and to increase digesta viscosity (Nguyen et al., 2021). The lower foam-like formation score in caeca digesta in the CD group is indicative of a lesser gas production during fermentation. Lower α-diversity in birds fed CD may be associated with a lower robustness and resilience of the microbiota to external stressors (e.g., antibiotic and dietary challenge) (Dogra et al., 2020), which may have contributed to the deleterious effect observed for CD on growth performance (Jansseune et al., 2024). The CD also affected β-diversity indexes at both ages, indicating a dissimilarity between SD and CD microbiota, which was associated with many differentially abundant OTUs and metabolites, and high fold change. Among the semi-polar metabolites affected by CD, only acetylcholine was named and was increased by the CD. Acetylcholine is a cholinergic neurotransmitter, produced and metabolized by intestinal cells and the microbiota, which regulate intestinal motility and secretion (Chen et al., 2021). Surprisingly, although the CD was formulated to contain more NSP fibers than SD, birds fed the Ctrl CD did not have a higher cecal SCFA concentration when compared to birds fed the Ctrl SD. In poultry, dietary fibers are commonly reported to increase SCFA production and concentration through fermentation by the microbiota (Liu et al., 2021). The absence of difference in SCFA concentration in our study is surprising, but it must be kept in mind that the concentration of SCFA or any metabolite in the gut is the result of multiple dynamic processes, including SCFA and metabolites production, absorption, and catabolism by the complex microbial community and by the host. The CD-

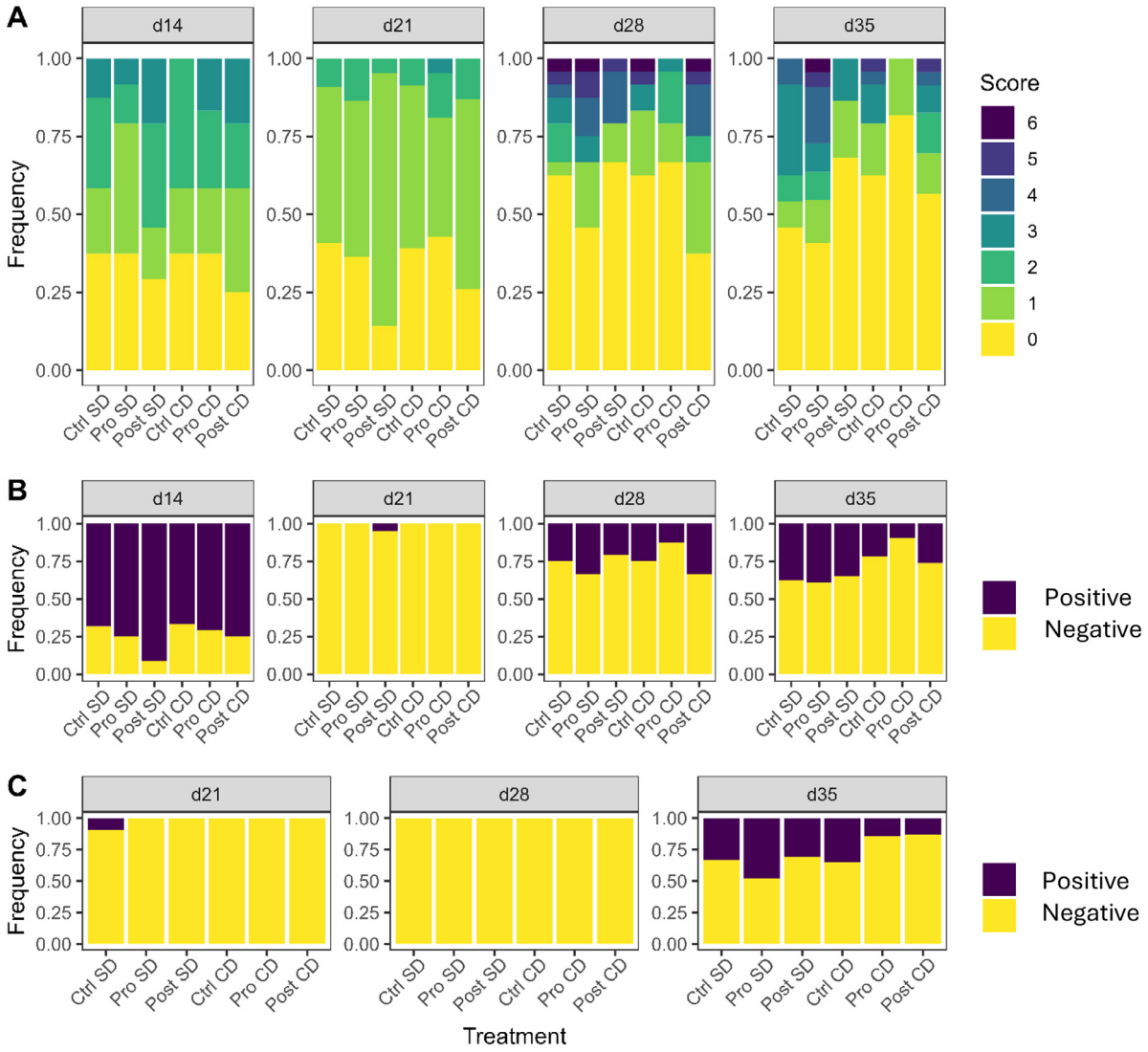


Figure 7. Response to Newcastle disease virus (**NDV**) and infectious bursal disease (**IBD**) vaccine in Ross 308 male broilers fed a standard (**SD**) or challenge diet (**CD**) unsupplemented (**Ctrl**) or supplemented with *Lactobacilli*-based probiotic (**Pro**) and postbiotic (**Post**) from d 1 onward. Anti-NDV hemagglutination inhibition (HAI) score (A), ELISA-determined NDV (B) and IBD (C) seropositivity, and associated *p*-values for treatment effects (D) following NDV and IBD vaccination at d14 and 17, respectively.

associated modification of the microbiota and metabolome may have contributed to the deleterious effects of this diet on growth performances as we reported previously (Jansseune et al., 2024), and may have resulted in conditions where Pro and Post effects could be observed.

Despite difference in OTUs abundances, the absence of β-diversity modification reflected that Pro had a minor effect on microbiota composition. Nevertheless, Pro could have induced a high shift in microbiota or host metabolism as suggested by the 240 differentially abundant metabolites. Among OTUs affected by Pro, the *Faecalibacterium* genus was particularly represented with all except one being increased. Also, only one OTU was identified at the species rank, *B. pullicaecorum,* and was decreased by Pro. This bacterium can produce butyrate under the conditions within the gut (Geirnaert et al., 2014); however Pro had no effect on cecal content butyrate and other SCFA concentrations. The effect of a single bacterial species can be masked by the other microbiota as well as the host's metabolism. Eight metabolites affected by Pro were identified, including anserine, carnosine, propionylcarnitine, cytidine, diaminopimelic acid and 5-metylcytosine which decreased, and dodecanedioic acid and tetradecanedioic acid which increased. Lauric acid (dodecanedioic acid) and myristic acid (tetradecanedioic acid) have antibacterial effects (Arellano et al., 2023), with lauric acid being especially effective against Gram-positive bacteria (Zentek et al., 2011). Diaminopimelic acid is part of the peptidoglycan layer of most Gram-negative and some Gram-positive bacteria, and is a ligand for the nucleotide oligomerization and binding domain (NOD)-like receptor 1, which is particularly expressed in the gut and which activates pro-inflammatory immune pathways promoting local and systemic inflammation (Jiao et al., 2022). However, whether peptidoglycan monomers can induce inflammation is not well understood (Iyer and Coggeshall, 2011). An interesting finding was the presence of anserine, carnosine and propionyl-carnitine in the caeca, with a reduction of 43, 81 and 70 %, respectively, in Pro-supplemented birds. Since anserine and carnosine are not

produced by plants (Li and Wu, 2020; Wu, 2020), these metabolites must be from endogenous origin and synthesized *de novo* in skeletal muscle of the broilers. Similarly, propionyl-carnitine, a derivative of carnitine is highly specific for skeletal and cardiac muscle (Ferrari et al., 2004). The role of anserine, carnosine and propionyl carnitine received high interest for their cardioprotective properties in humans (Ferrari et al., 2004; Mingorance et al., 2011; Wu, 2020). To our knowledge the endogenous secretion of these compounds in the gut of humans and animals has not been reported and further research is required to investigate the underlying mechanisms and physiological implications.

Within the CD, Post modified the microbiota composition, as shown by the different β-diversity and abundances of several OTUs. However, despite these changes in microbiota composition, it seems that the overall activity of the microbiota or the host or both may not have been much affected, since only 2 unknown metabolites next to butyrate and acetate were affected. Post lowered *E. cecorum* following an increase by the CD. This effect may be beneficial since *E. cecorum* is frequently associated with diseases in poultry, such as locomotor disorders, septicaemia and co-infections (Souillard et al., 2022). Post increased cecal butyrate and acetate, which are known for multiple effects, such as a bacteriostatic activity and a reduction of the pH in the gut, creating an unfavourable environment for pathogenic bacteria (Liu et al., 2021). Butyrate can be produced by multiple bacterial species, most of which belong to the phylum *Firmicutes* (Singh et al., 2023), a phylum representing 89% of the OTUs affected by Post. Butyrate can have effects on the endocrine and immune systems and intestinal metabolism which can be associated with beneficial effects on growth performances, when present in the caeca (Moquet et al., 2016).

The titration of total concentrations of IgA, M and G revealed limited and non-persistent effects of the treatments. Many previous studies investigated broiler humoral immune responses with probiotic supplementation, but studies with postbiotics are scarcer. Studies with *Lactobacillus* pro- and postbiotics reported inconsistent effects, either increasing or not affecting humoral antibody responses. This inconsistency is potentially due to differences in bacterial strain, dosage, preparation and condition of the broilers (Koenen et al., 2004; Brisbin et al., 2011; Humam et al., 2019; Danladi et al., 2022b). However, despite the lack of effects on systemic antibody levels, pro- and postbiotics may modulate intestinal secretory IgA levels (Amerah et al., 2013; Danladi et al., 2022b). Unfortunately, in our study, only serum Ig levels were evaluated, while many other immune parameters could be modulated, reflecting other types of immunity (i.e., gut secretory Ig and innate immune cells activation).

Broilers' specific antibody response against IBDV and NDV vaccine showed that the additives had no effects on these responses, while the CD hampered NDV and IBD vaccination antibody responses, although the overall seropositivity to the vaccine was very low. *Lactobacilli* probiotics were reported to increase Ig response to specific antigens after vaccination but this must be tempered as their effect can be specific to the antigens, Ig type and measurement location (blood vs gut) (Koenen et al., 2004; Haghighi et al., 2005; Brisbin et al., 2011). The CD-induced reduction of vaccine antibody responses highlights that it may have reduced the broiler's ability to cope with immune challenges factors.

An interesting finding of the current study was an association of broiler BW, either alone or in interaction with the diet, on serum Ig. In the SD, Ig concentrations were mainly not correlated with BW, whereas in CD, heavier broilers had lower Ig concentrations. The CD created a challenging situation for the broilers as reported by Jansseune et al. (2024). Our data suggest that a suboptimal dietary condition, allowed the expression of individual variation for the humoral immune response. Higher Ig production and the associated mechanisms can be detrimental to the broiler's performance in the absence of an immune challenge due to their cost in terms of nutrients (Broom and Kogut, 2018). Moreover, higher Ig levels can reflect a higher infection pressure in these birds, which would have impaired growth (Broom and Kogut, 2018). Such higher infection pressure is likely more pronounced under the CD due to rye and barley. Indeed, these 2 ingredients can promote an environment favorable to the growth of harmful bacteria (Lazaro et al., 2003; Mehrabadi and Jamshidi, 2019).

Of note, our study was conducted under commercial conditions and not within the high sanitary environment of an experimental facility, which can impact microbiota composition and functionality and their response to dietary interventions (Kers et al., 2019). This effect on the microbiota may have influenced the bird's immune status since the gut microbiota are known to influence host immunity (Kogut, 2022). Moreover, because of the inherent presence of a higher antigenic diversity in a commercial poultry barn compared to an experimental station, the basal level of immune stimulation in our study was probably higher with more variability, which could render the evaluation of the treatment effects more difficult. The difference in growth conditions (commercial vs experimental) makes results difficult to compare between these 2 environments. However, the same reasoning applies regarding results obtained from commercial growth conditions and their practical application.

CONCLUSIONS

The results of the present study support the Diet × Additive effects reported on growth by Jansseune et al (2024) and provide a first indication that diet composition can have a major impact on the effect of pro- and postbiotics on the cecal microbiome. Under CD, the Pro-induced modifications of the cecal microbiota were associated with effects on the cecal semi-polar metabolome, while Post effects on the cecal microbiota were associated with effect on SCFA concentrations and to a lesser extent on the semi-polar metabolome. Humoral

immune parameters showed non consistent effects but revealed an interaction between broiler BW and dietary conditions. Consequently, diet formulation should be considered to evaluate the effects of microbes-based additives and humoral immune response.

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Data Availability: The sequencing data analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) database under the Bioproject accession number PRJNA1096845. All data generated and analyzed during this study are included in this published article and its additional files.

DISCLOSURES

The authors declare no conflicts of interest.

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