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Dietary and phytogenic inclusion effects on the broiler chicken cecal ecosystem

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Dietary modulation in broilers is crucial for the establishment of beneficial microbiota and, subsequently, the promotion of intestinal health. In this trial, a 2 × 2 factorial design was used with two different specifications with respect to dietary metabolizable energy (ME) and crude protein (CP) levels (i.e., 95% and 100% of recommendations) and phytogenic levels (0 and 150 mg/kg). Levels of total bacteria, *Bacteroides* spp., *Lactobacillus* spp., and *Clostridium* cluster XIVa attached to the cecal mucosa and in the cecal digesta were lower in broilers fed the 95% ME and CP specification diets, as was the molar ratio of butyric acid. In addition, the relative activity of autoinducers-2 (AI-2) and the expression levels of *TLR4* and *AvBD6* were increased. Phytogenic supplementation reduced cecal digesta levels of *Escherichia coli* and *Clostridium* cluster I levels, and increased *Clostridium* cluster IV levels. Moreover, the butyric acid molar ratio and the relative activity of AI-2 were increased, whereas the concentration of branched VFAs and the expression of *AvBD6* and *LEAP2* were reduced by phytogenic administration. Dietary specifications and phytogenic interactions were shown for the cecal-attached microbiota composition, metabolic activity of digesta microbiota, relative expression of autoinducers-2, and relative expression of toll-like signaling molecules and host antimicrobial peptides. In conclusion, it has been shown that ME and CP dietary specifications, combined or not with phytogenics, modulate multilevel gut biomarkers ranging from microbiota composition and metabolic activity to microbial communications and host signaling, inflammation, and defense.

KEYWORDS

phytogenic, host defense peptides, microbiota, autoinducers-2, volatile fatty acids, gut barrier

1 Introduction

Good functioning of the gastrointestinal (GI) system is a basic requirement for the optimal productivity and health of broiler chickens. The GI system's proper function and health depends on the maintenance of a dynamic balance between the microbial populations that colonize the gastrointestinal tract, microbiota, and the cells of the host, such as immune and epithelial cells (Brisbin et al., 2008; Celi et al., 2017). The symbiotic relationship between chickens and their intestinal microbiota results in beneficial effects such as promotion of intestinal mucosa growth (Slawinska et al., 2019), exclusion of pathogenic microorganisms (Baba et al., 1991; Tierney et al., 2004), polysaccharides breakdown (Rehman et al., 2007), energy supply in the form of amino acids or volatile fatty acids (Van Immerseel et al., 2006; Elling-Staats et al., 2021), and homeostasis of the immune system (Brisbin et al., 2010; Brisbin et al., 2015; Hu et al., 2021).

A necessary condition for achieving a balanced symbiosis is the appropriate communication between the host cells and the microbiota and between the prokaryotic cells of the microbiota (Li et al., 2019). The latter communication in particular is considered a mechanism of bacterial homeostasis and cooperation, called quorum sensing (QS), mediated by small diffusible signal molecules termed autoinducers (AIs). For intraspecies communication, Gram-negative bacteria signal using acyl homoserine lactones (AI-1) and Gram-positive bacteria signal using small peptides. For interspecies communication, non-species-specific AIs are employed, such as AI-2. The AI-2 is a family of molecules, and a major signal-type molecule of QS, which is a derivative of 4,5-dihydroxy-2,3-pentanedione that is synthesized by AI-2 synthetase (LuxS) enzymes. AI-2 levels modulate the abundance of the major phyla of intestinal microbiota and could be a new biomarker for monitoring intestinal flora disorders (Li et al., 2019; Fu et al., 2020; Wu and Luo, 2021).

Diet is a determining factor for intestinal microbiota. It is known that energy and protein levels, as well as the overall composition of the diet, can influence the composition of the intestinal microbiota in broilers (Laudadio et al., 2012; Paraskeuas and Mountzouris, 2019b). Nutritional manipulation in broilers for the establishment of beneficial microbiota and/or the existing microbiota modification is a key tool for promoting gut health. More specifically, several studies have reported that the dietary addition of bioactive ingredients (e.g., probiotics, acidifiers, enzymes, phytogenics) can change the profile of bacterial populations in the intestinal tract (Mountzouris et al., 2010; Mountzouris et al., 2015; Palamidi and Mountzouris, 2018; Paraskeuas and Mountzouris, 2019b). In recent years, phytogenics are gaining importance in the scientific community. Phytogenics include a wide range of plant products, such as essential oils, herbs, and oleoresins (Mohammadi Gheisar and Kim, 2018), and act multifacetedly on the intestinal ecosystem, more specifically antimicrobially, with anti-inflammatory effects, and affecting the sensory mechanisms of the gut (Yang et al., 2015). However, their effects depend on both the composition and the level of addition to the final diet (Mountzouris et al., 2011; Mountzouris et al., 2020). It has been reported that plant extracts (α-pinene or carvacrol) *in vitro* exhibit anti-quorum-sensing effects against pathogens (Šimunović et al., 2020; Wagle et al., 2020). However, reports investigating the *in vivo* effects of plant extracts on QS on endogenous microbiota in broilers are scarce.

Nowadays, due to the increased cost of feeding broilers and environmental concerns related to high nitrogen excretion, there is a tendency to reduce the energy density and protein level in the diet. Incorporation of a mixture of carvacrol, cinnamaldehyde, and capsicum oleoresin (Bravo et al., 2014) or a phytogenic based on carvacrol, anethol, and limonene (Paraskeuas et al., 2016) appear to compensate for the effects of the reduction in energy and protein levels and restore broiler performance.

The aim of this study was to generate new knowledge on the effects of dietary energy and protein levels with or without phytogenic addition on the cecal microbiota composition and its metabolic activity, signaling of cecal microbiota, and the expression of critical genes relevant for inflammation and defense in the cecal mucosa of broilers.

2 Materials and methods

2.1 Ethics statement

The experimental protocol was in compliance with the current European Union Directive on the protection of animals used for scientific purposes (Directive, 2007; Directive, 2010) and was approved by the relevant national authority Department of Agriculture and Veterinary Policy, General Directorate of Agriculture, Economy, Veterinary and Fisheries (Approval 1130/290216).

2.2 Birds and experimental treatments

This study forms part of our previous research work (Griela et al., 2021). To avoid excessive repetition, a brief description of the experimental treatments is given below. A total of 540 1-day-old male Cobb 500 broilers vaccinated for Marek disease, infectious bronchitis, and Newcastle disease were acquired from a local hatchery. Birds were arranged according to a 2×2 factorial design in four treatments, with nine (n=9) replicate pens of 15 chicks per treatment for a 42-d study. Depending on diet specifications (i.e., 95% and 100%) and phytogenic supplementation (i.e., 0 and 150 mg/kg), the four experimental treatments were D95 (95% of optimal ME and CP specifications

with no phytogenic supplementation), D95+ (95% of optimal ME and CP specifications with phytogenic supplementation), D100 (100% of optimal ME and CP specifications with no phytogenic supplementation), and D100+ (100% of optimal ME and CP specifications with phytogenic supplementation).

A three-phase feeding program with starter (1–10 d), grower (11–22 d), and finisher (23–42 d) diets was followed (Table S1). In particular, for each growth phase, two diets were formulated to meet 95% and 100% of optimal Cobb 500 metabolizable energy (ME) and protein (CP) specifications. The phytogenic used was a commercial product; a blend of bioactive compounds such as carvacrol, thymol, carvone, methyl salicylate, and menthol (Digestarom® Biomin Phytogenics GmbH, Germany), as previously described (Griela et al., 2021).

Each replicate was assigned to a clean floor cage (1 m^2), and the birds were raised on rice hull litter. The temperature program was set at 32°C at week 1 and gradually reduced to 23°C by week 6. Heat was provided with a heating lamp per cage. Except for day 1, an 18-h light to 6-h dark lighting program was applied during the experiment to ensure adequate access to feed and water.

The ingredient (g/kg) and calculated chemical composition (g/kg as fed) of the basal diets (i.e., 95% vs 100%) was as follows (Table S1). Starter diet: AMEn (11.97 vs. 12.60 MJ); crude protein (204.3 vs. 215.0 g); lysine (12.5 vs. 13.2 g); methionine + cysteine (9.4 vs. 9.9 g); threonine (8.2 vs. 8.6 g); calcium (9 g); and available phosphorus (4.5 g). Grower diet: AMEn (12.27 vs. 12.92 MJ); crude protein (185.3 vs. 195 g); lysine (11.3 vs. 11.9 g); methionine + cysteine (8.6 vs. 9.0 g); threonine (7.5 vs. 7.9 g); calcium (8.4 g); and available phosphorus (4.2 g). Finisher diet: AMEn (12.59 vs. 13.26 MJ); crude protein (175.8 vs. 185.0 g); lysine (10.0 vs. 10.5 g); methionine + cysteine (7.8 vs. 8.2 g); threonine (6.8 vs. 7.1 g); calcium (7.6 g); and available phosphorus (3.8 g).

2.3 Sample preparation for microbiological analysis

For the determination of the luminal- and mucosa-associated microbiota composition and the digesta volatile fatty acids (VFAs) concentration, one bird per replicate pen (i.e., nine birds per treatment) were euthanized *via* electrical stunning prior to slaughter. Subsequently the abdomen was opened and the ceca removed and immediately snap frozen in liquid nitrogen and stored at –80°C. For the purpose of this study, one cecum was thawed on ice and opened longitudinally. Firstly, cecal digesta were removed carefully and stored at –30°C until further analysis; then, to remove remaining digesta and bacteria not attached to the gut mucosa, ceca tissue was washed three times in ice-cold saline by gentle agitation. Subsequently, cecal-mucosa-attached bacteria were removed from the mucosa following a protocol of 3 × 1 min vigorous hand-shaking washes (15 ml) in saline containing 0.1% (wt/wt) Tween 80, according to Li et al. (2003). Finally, the washes were pooled and centrifuged at 10,000 × g for 30 min at 4°C to precipitate cells (cell pellet).

2.4 DNA extraction

Cecal digesta and cell pellets from caecum were used for DNA extraction using a manual protocol adopted by the International Human Microbiome Standards project (Dore et al., 2015; http://www.microbiome-standards.org). For each sample, the extracted DNA was eluted in 200 ml of TE buffer, and the quality and quantity of the DNA preparations were determined by spectrophotometry (Q3000, Quawell Technology, Inc.) and stored at –30°C.

2.5 Quantitative real-time PCR for bacteria enumeration

For the quantification of total bacteria (domain bacteria), *Lactobacillus* spp., *Escherichia coli*, *Bacteroides* spp., *Clostridium* cluster I (*Clostridium perfringens* group), *Clostridium* cluster XIVa (*Clostridium coccoides* group), and *Clostridium* cluster IV (*Clostridium leptum* group), suitable primers were used targeting the 16S rRNA gene (Table 1). Primer specificity was confirmed using BLAST (NCBI) and the ProbeMatch programs.

Real-time PCR was performed in microplates with the SaCycler-96 (Sacace Biotechnologies Srl). Reactions were made to a final volume of 10μl and consisted of a 5μl of 2 × FastGene IC Green universal mix (Nippon Genetics, Tokyo, Japan), forward and reverse primers each at a final concentration of 300– 450 nmol/l, and 1 μl of DNA template (i.e., 20 ng of sample DNA/reaction). The reactions were incubated at 95°C for 5 s, at the primer-specific annealing temperature for 20 s, and at 72°C for 33 s. This was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in duplicates. Depending on whether the sample was from mucosa or luminal digesta, results were expressed as log-cells/g mucosa-associated cell pellet or as log-cells/g wet digesta contents, respectively.

2.6 Bacterial strains and calibration curves

Reference bacterial strains that were used to control the specificity of the primers and to construct standard curves are shown in Table 2. Each of the reference strains was cultured on selective broth under suitable conditions. Bacterial genomic DNA from each culture was extracted using a manual protocol adopted by the International Human Microbiome Standards project (Dore et al., 2015; http://www.microbiome-standards.org). For each sample, the extracted DNA was eluted in 100 μl of TE buffer,

TABLE 1 Primers targeting 16S rRNA gene used for determination of luminal- and mucosa-associated microbiota composition by real-time PCR.

Target group or organism	Primer sequence (5′-3′)	Annealing temperature	Reference
All bacteria (domain bacteria)	F: ACTCCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	60°C	Clifford et al., 2012
Bacteroides spp.	F: GAGAGGAAGGTCCCCCAC R: CGCTACTTGGCTGGTTCAG	58°C	Peinado et al., 2013
Lactobacillus spp.	F: GAGGCAGCAGTAGGGAATCTTC R: GGCCAGTTACTACCTCTATCCTTCTTC	60°C	Delroisse et al., 2008; Peinado et al., 2013
Escherichia coli	F: CATGCCGCGTGTATGAAGAA R: GGGTAACGTCAATGAGCAAAGG	60°C	Silkie and Nelson, 2009
Clostridium cluster I (*C. perfringens* subgroup)	F: TACCHRAGGAGGAAGCCAC R: GTTCTTCCTAATCTCTACGCAT	56°C	Goodarzi Boroojeni et al., 2014
Clostridium cluster XIVa (*C. coccoides* subgroup)	F: ACTCCTACGGGAGGCAGC R: CTTCTTAGTCAGGTACCGTCAT	60°C	Schwiertz et al., 2010
Clostridium cluster IV (*C. leptum* subgroup)	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	52°C	Matsuki et al., 2004

and the quality and quantity of the preparations were determined by spectrophotometry (Q3000, Quawell Technology, Inc) and stored at -30°C.

For the quantification of bacterial species and groups, a quantification method similar to the one described by Joly et al. (2006) was used. In more detail, an appropriate standard curve using 10-fold serial dilutions of a known concentration of genomic DNA was included in each 96-well plate. The concentration of genome copies, from each bacterial species in the initial purified DNA solution used to construct the standard curves, was calculated by assuming an average molecular mass of 660 Da for 1 bp of double-stranded DNA and using the following equation: concentration of genome copies ¼ Quantity of DNA (fg)/Mean mass of the corresponding genome (fg). The amount of genomic copies correspond to an equal amount of bacterial cells. Genome sizes for all bacteria species and groups used in this study are presented in Table 2.

2.7 Cecal volatile fatty acid concentration

For the determination of cecal VFA concentration, digesta were homogenized following a 10-fold dilution (i.e., 10% wt/vol) in sterile ice-cold phosphate-buffered saline (0.1 mol/l, pH 7.0). Homogenates were subsequently centrifuged at 12,000 × g for 10 min at 4°C and the resulting supernatants were stored at –80°C until their analysis by capillary gas chromatography (GC). Samples of 2 µl were injected into a gas chromatograph (Agilent 6890GC System, Agilent Technologies, Santa Clara, CA, USA) equipped with a Stabilwax®-DA Capillary GC Column (size x I.D. 30m × 0.25 mm, df 0.25 µm) (Restek Corporation, Bellefonte, PA, USA) and a flame ionization detector. The injector and detector temperature were set at 200°C and 220°C, respectively, and the temperature program was run from 140°C to 200°C with a temperature ramp rate of 5°C/min. Helium

TABLE 2 Reference strains and genome sizes.

Reference strains	Target bacterial group(s)	NCBI reference sequence	Genome size (Mbp)
Escherichia coli (ATCC 25922)	*Escherichia* sp. & domain bacteria	NZ_CP009072.1	5.13
Bacteroides vulgatus (ATCC 8482)	*Bacteroides* spp.	NC_009614.1	5.16
Lactobacillus acidophilus (ATCC 314)	*Lactobacillus* spp.	NC_006814.3	1.99
Clostridium perfringens (ATCC 13124)	*Clostridium* cluster I (*C. perfringens* subgroup)	NC_008261.1	3.26
Clostridium clostridioforme (DSM933)	*Clostridium* cluster XIVa (*C. coccoides* subgroup)	NZ_FOOJ00000000.1	5.47
Clostridium leptum (DSM 753)	*Clostridium* cluster IV (*C. leptum* subgroup)	NZ_ABCB00000000.2	3.27

was the carrier gas with a column flow of 20 ml/min. The VFAs determined were acetic, propionic, isobutyric, butyric, isovaleric, and isohexanoic. Results were expressed as mmol/kg wet digesta for total VFA and as molar ratios (% of total VFA) for acetic, propionic, butyric, and branched VFAs (b-VFA; sum of isobutyric, isovaleric, and isocaproic).

2.8 Sample preparation for gene expression studies/RNA isolation and reverse transcription to cDNA

For the determination of gene expression studies, the second cecum was used. In brief, the whole cecum was exposed and the cecal digesta were removed. The cecum was washed completely in 30 ml cold phosphate-buffered saline (PBS)–ethylenediaminetetraacetic acid (EDTA; 10 mmol/l) solution (pH = 7.2). Total RNA was extracted from the cecal tissue or the cecal tonsil using the NucleoZOL reagent, as reported by the manufacturer's protocol (Macherey-Nagel GmbH & Co. KG, Duren, Germany). The purity and quality of RNA were evaluated using spectrophotometry (Q3000, Quawell Ltd) based on 260/230 nm wavelength ratios. Genomic DNA was removed using DNase I (M0303, New England Biolabs Inc, Ipswich, UK). A concentration of 500 ng of total RNA of each sample was reverse transcribed using the PrimeScript RT reagent kit (Perfect Real Time, Takara Bio Inc., Shiga, Japan), according to the manufacturer's protocol. cDNAs were stored at –20°C.

2.9 Quantitative polymerase chain reaction for relative expression

For the relative expression of mucin 2 (*MUC2*), zonula occludens-1 (*ZO1*), zonula occludens-2 (*ZO2*), claudin-1 (*CLDN1*), occludin (*OCLN*), toll-like receptor 2 type-2 (*TLR2B)*, toll-like receptor 4 (*TLR4*), interferon gamma (*IFNG*), nuclear factor kappa B subunit 1 (*NFKB1*), inducible nitric oxide synthase 2 (*NOS2*), free fatty acid receptor 2 (*FFAR2*), free fatty acid receptor 4 (*FFAR4*) was used produced cDNA from cecal tissue. For the relative expression of avian beta-defensin 1 (*AvBD1*) and cathelicidin 2 (*CATH2*), cDNA from the cecal tonsils was used as the relative expression from the cecal tissue cDNA was very low.

Primers not originating from the scientific literature were designed with the PerlPrimer program v.1.1.19 (Marshall, 2004) using the GenBank sequences, and their sequences are shown in Table 3. Primer specificity and efficiency were determined using pooled samples.

The relative expression of the above genes was detected using quantitative real-time PCR (qPCR) using the SaCycler-96 (Sacace Biotechnologies Srl) with FastGene IC Green 2x qPCR universal mix (Nippon Genetics, Tokyo, Japan). Each reaction contained 5 ng of RNA equivalents, as well as 200–450 nmol/l of forward and reverse primers for each gene. The reactions were incubated at 95° C for 5 s, 59–65°C (depending on the target gene) for 20 s, and 72° C for 33 s. This was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in duplicates. Relative expression ratios of target genes were calculated according to Pfaffl (2001), adapted for the multi-reference genes normalization procedure according to Hellemans et al. (2007), using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyltransferase (*HPRT*) as reference genes (Table 3).

2.10 Cecal AI-2 relative activity

The relative expression of AI-2 was investigated by using the *Vibrio harveyi* bioluminescence assay (Taga and Xavier, 2011; Hsiao et al., 2014). In brief, previously frozen cecal digesta were suspended in 2034 Autoinducer bioassay (AB) medium, centrifuged, and the supernatant was filtered using a 0.2-μm pore-size syringe filter (Macherey-Nagel GmbH & Co. KG, Duren, Germany) to create cecal cell-free fluids (CCFF). The samples were stored at –30°C.

An overnight culture of *V. harveyi* BAA-1117 (sensor 1^-, sensor 2^+) was diluted 1:1.000 with fresh AB. A total of 90 μl of this cell suspension was mixed with 10 μl of the CCFF in a 96-well polystyrene microplate (Nunc® MicroWell™, Merck KGaA, Darmstadt, Germany). A total of 10 μl of sterile growth medium was used as the negative control. In addition, 10 μl of the cell-free culture fluids of *V. harveyi* BAA-1119 was used as the positive control to verify the bioassays. The microplates were incubated at 30°C with shaking and luminescence measured every 30 min with a Synergy HT multimode microplate reader (BioTek, Winooski, VT, US) for 7 hours. AI-2-like activity is expressed as relative AI-2-like activity, which was calculated as the ratio of luminescence of the test sample (CCFE) to that of the control (negative) sample.

2.11 Statistical analysis

Data were analyzed using a general linear model (GLM): a general factorial ANOVA procedure using dietary ME and CP specifications (i.e., 95% and 100%) and phytogenic inclusion level (i.e., 0 and 150 mg/kg diet) as fixed factors on individual broiler basis. In addition, data were tested for normality using the Kolmogorov–Smirnov test and found to be normally distributed. Moreover, the statistically significant effects were further analyzed, and means were compared using Tukey's honest significant difference multiple comparison procedure. Statistical significance was determined at a P-value <0.05. All statistical analyses were done using SPSS for Windows Statistical Package Program (SPSS Inc., Chicago, IL, US).

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TABLE 3 Oligonucleotide primers used for quantitative RT-PCR.

Gene	Primer sequence (5′-3′)	Annealing temperature	GenBank accession no
Barrier function genes			
MUC2	F: GCTGATTGTCACTCACGCCTT R: ATCTGCCTGAATCACAGGTGC	60°C	NM_001318434.1
ZO1	F: AAAGCCATTCCTGTAAGCC R: GTTTCACCTTTCTCTTTGTCC	60°C	XM_040706827.2
ZO2	F: TAAAGCCATTCCTGTAAGCC R: GTTTCACCTTTCTCTTTGTCC	60°C	XM_025144668.3
CLDN1	F: CTGATTGCTTCCAACCAG R: CAGGTCAAACAGAGGTACAAG	59°C	NM_001013611.2
OCLN	F: TCATCGCCTCCATCGTCTAC R: TCTTACTGCGCGTCTTCTGG	62°C	NM_205128.1
TLR pathway			
TLR2B	F:CTTGGAGATCAGAGTTTGGA R:ATTTGGGAATTTGAGTGCTG	62°C	NM_001161650.1
TLR4	F: GTCTCTCCTTCCTTACCTGCTGTTC R: AGGAGGAGAAAGACAGGGTAGGTG	65°C	NM_001030693.1
IFNG	F: AGCTCCCGATGAACGAC R: CAGGAGGTCATAAGATGCCA	62°C	NM_205149.1
NFKB1	F: TGTGGTTGTCAGGATGGTC R: GGTCTGGTAAAGGTCATTTCTC	62°C	NM_205134
NOS2	F: AAAGAAAGGGATCAAAGGTGGT R: CAAGCATCCTCTTCAAAGTCTG	60°C	NM_204961.1
Nutrient-sensing genes			
FFAR2	F: GCTCGACCCCTTCATCTTCT R: ACACATTGTGCCCCGAATTG	62°C	XM_046904776.1
FFAR4	F: CCCATTCATCGCCATCGT R: ACAAATGTGAATCTCCTCACCA	60°C	XM_003641481.6
Host defense peptide (HDP) genes			
AvBD1	F: GAGTGGCTTCTGTGCATTTCTG R: TTGAGCATTTCCCACTGATGAG	60°C	NM_204993.1
AvBD6	F: TACCTGCTGCTGTCTGTCCT R: AGTCCACTGCCACATGATCC	60°C	NM_001001193.1
CATH2	F: CGACTGCGACTTCAAGGAGAA R: GATCTCGGGAGTGTCCTGC	60°C	NM_001024830.3
LEAP2	F: CTCAGCCAGGTGTACTGTGCTT R: CGTCATCCGCTTCAGTCTCA	60°C	NM_001001606.2
Reference genes			
GAPDH	F: GCTGAATGGGAAGCTTACTG R: AAGGTGGAGGAATGGCTG	60°C	NM_204305.1
HPRT	F: CCCAAACATTATGCAGACGA R: TGTCCTGTCCATGATGAGC	60°C	XR_003074757.3

3 Results

3.1 Mucosa-associated and digesta microbiota

A significant interaction of diet specifications × phytogenic ($P_{D\times P} \leq 0.01$) was noted for the concentration of *Lactobacillus* spp. and *Clostridium* cluster IV. In particular, treatments D100 and D100+ were associated with a higher concentration of *Lactobacillus* spp. In addition, treatment D100+ was associated with higher *Clostridium* cluster IV levels than treatment D95+, whereas treatments D100 and D95 were intermediate. Diet specifications were the main factor significantly affecting cecal mucosa-associated microbiota (Figure 1). Broilers with diets formulated with the 100% ME and CP specifications had significantly higher concentrations of total bacteria ($P_D \leq 0.05$), *Bacteroides* spp. ($P_D \leq 0.05$), *Lactobacillus* spp. ($P_D \leq 0.01$), and *Clostridium* cluster XIVa ($P_D \leq 0.01$). Phytogenic addition did not significantly affect ($P > 0.05$) the cecal mucosa-associated microbiota.

There were no significant ($P > 0.05$) interactions of diet specifications × phytogenic at cecal digesta. Diet specifications were the main factor that significantly affected *Clostridium* cluster XIVa levels in cecal digesta (Figure 2). In particular, broilers fed diets formulated with the 100% specifications had significantly higher concentrations ($P_D \leq 0.05$) of *Clostridium* cluster XIVa. Phytogenic addition significantly decreased *E. coli* ($P_P \leq 0.05$) and *Clostridium* cluster I ($P_P \leq 0.05$) levels and significantly increased the *Clostridium* cluster IV ($P_P \leq 0.01$) concentration.

3.2 Volatile fatty acid concentration

A significant interaction of diet specifications × phytogenic was noted for acetic acid ($P_{D\times P} \leq 0.01$). In particular, the acetic

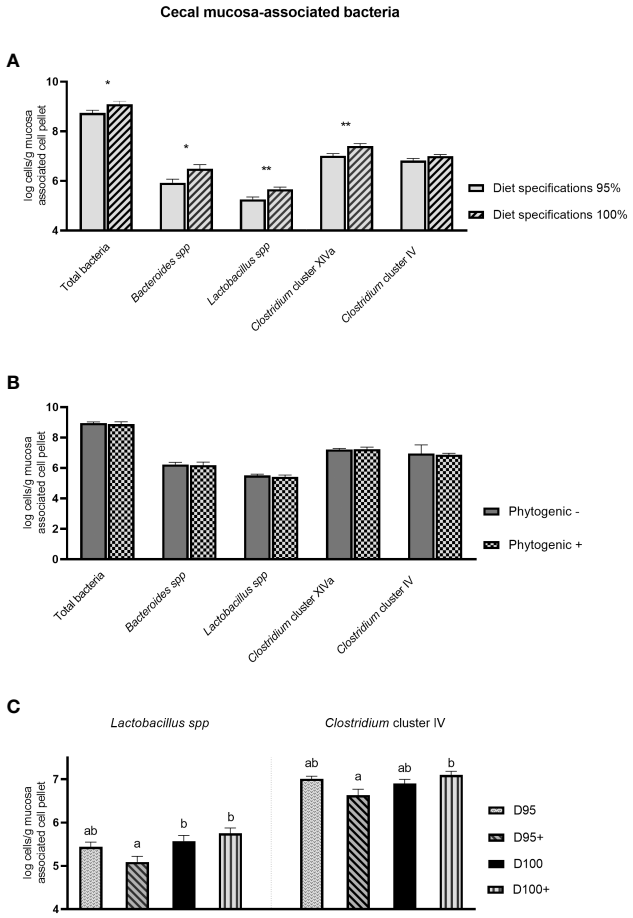

FIGURE 1
Effect of diet specifications **(A)** and phytogenic inclusion **(B)** and interaction effect of diet specifications × phytogenic inclusion **(C)** in cecal mucosa-associated bacteria of 42-day-old broilers. Columns indicate means + SE and the asterisks denote level of statistical difference *P ≤ 0.05, **P ≤ 0.01. Treatment means with different superscripts a, b differ significantly ($P < 0.05$).

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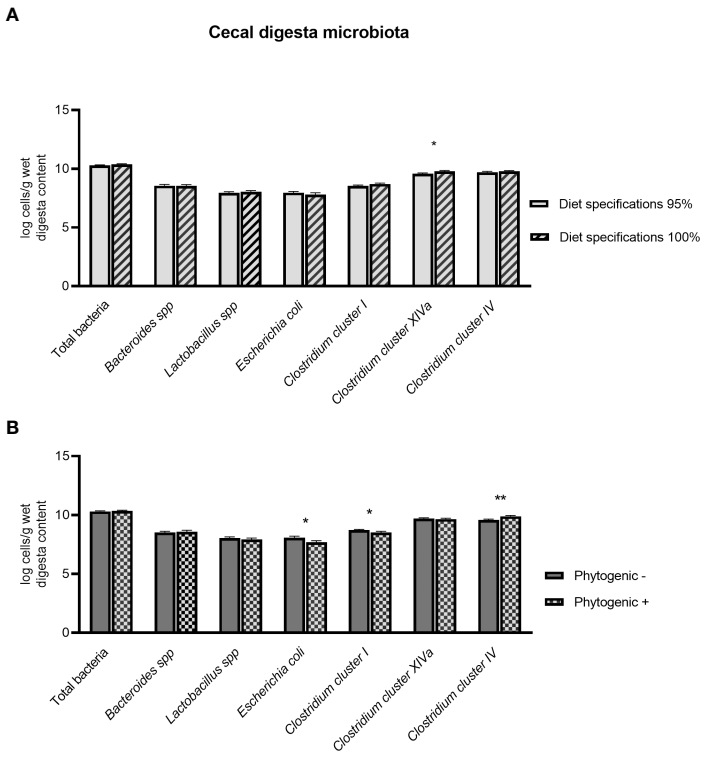

FIGURE 2
Effect of diet specifications **(A)** and phytogenic inclusion **(B)** in cecal digesta microbiota of 42-day-old broilers. Columns indicate means + SE and the asterisks denote level of statistical difference *P ≤ 0.05, **P ≤ 0.01.

molar ratio was higher in treatments D100 and D95+ than in treatment D95, whereas treatment D100+ was intermediate. Diet specifications significantly affected the cecal digesta VFA pattern (Table 4). In particular, the butyric acid molar ratio was significantly higher ($P_P \leq 0.05$) in broilers fed diets formulated with the 100% specifications. Phytogenic addition significantly increased the butyric molar ratio ($P_P \leq 0.05$) and significantly decreased the branched VFA ($P_P \leq 0.05$) molar ratios.

TABLE 4 Effects of diet specifications %, phytogenic inclusion, and their interaction on the volatile fatty acid (VFA) concentration and molar ratios in the cecal digesta of 42-day-old broilers.

Component	Diet specifications[1]		phytogenic inclusion[2]		Treatments[3]				Statistics			
	95	100	No	Yes	D95	D95+	D100	D100+	s.e.m[4]	P_D	P_P	$P_{D\times P}$
Total VFA (mmol/kg of wet cecal digesta)	49.16	52.17	49.17	52.15	42.56	55.75	55.79	48.55	5.890	NS	NS	NS
Acetic (%)	65.39	66.57	65.28	66.69	61.28^{a}	69.50^{ab}	69.28^{b}	63.87^{ab}	2.066	NS	NS	**
Propionic (%)	5.53	4.83	5.13	5.23	5.90	5.16	4.36	5.30	0.523	NS	NS	NS
Butyric (%)	18.27	23.04	17.86	23.46	15.72	20.81	19.99	26.10	1.944	*	**	NS
Branched VFA (b-VFA, %)	4.85	3.90	5.96	2.78	7.00	2.69	4.92	2.87	1.292	NS	*	NS

[1]Diet specifications: 95 (i.e., 95% of recommended ME and CP specs) and 100 (i.e., 100% of recommended ME and CP specs). [2] phytogenic inclusion (No = 0 mg/kg diet and Yes = 150 mg/kg diet). [3] Means within the same row with different superscripts (a–c) differ significantly (P < 0.05). [4] Pooled standard error of means. Total VFA, acetic + propionic + butyric + branched VFA; branched VFA, isobutyric + isovaleric + isocaproic.
NS, P > 0.05, *P ≤ 0.05, **P ≤ 0.01.

3.3 Relative gene expressions in the broiler ceca

3.3.1 Cecal tissue

An interaction of diet specifications × phytogenic ($P_{D\times P} \leq 0.01$) was noted for the relative expression of *TLR2B* ($P_{D\times P} \leq 0.05$) and *TLR4* ($P_{D\times P} \leq 0.05$). The relative expression of *TLR2B* was lower in treatment D100+ than in the other treatments and the relative expression of *TLR4* was lower in treatment D100+ than in treatments D95 and D95+ (Table 5). Diet specifications as a main factor significantly affected ($P_D \leq 0.05$) *TLR4* relative expression. In particular, *TLR4* expression was significantly lower in broilers fed diets formulated with the 100% specifications. Phytogenic supplementation did not affect ($P_P \leq 0.05$) relative gene expressions in the broiler ceca tissue.

3.3.2 Cecal tonsils

An interaction of diet specifications × phytogenic was noted for relative expression of *LEAP2* ($P_{D\times P} \leq 0.05$) and *AVBD6* ($P_{D\times P} \leq 0.01$). In particular, broilers with a diet formulated with 100% specifications and supplemented with phytogenic (D100+) had lower expression levels of *LEAP2* than broilers with a diet formulated with 100% specifications and no phytogenic addition (D100+). Transcripts of *AVBD6* were lower in treatments D100 and D100+ than in treatment D95, whereas treatment D95+ received intermediate values and differed from the rest of the treatments. *AVBD6* expression was significantly affected ($P_D \leq 0.001$) by diet specifications. Broilers with a diet formulated with 100% specifications had lower *AVBD6* transcripts. Broilers with a diet supplemented with phytogenic had significantly lower values of *LEAP2* ($P_P \leq 0.05$) and *AVBD6* ($P_P \leq 0.05$) transcripts.

TABLE 5 Effects of diet specifications %, phytogenic inclusion, and their interaction on mRNA expression of, barrier function, immune and nutrient sensing genes in chicken cecal mucosa.

Gene	Diet specifications[1]		phytogenic inclusion[2]		Treatments[3]					Statistics		
	95	100	No	Yes	D95	D95+	D100	D100+	s.e.m[4]	P_D	P_P	$P_{D\times P}$
Barrier function genes												
MUC2	3.87	4.99	3.75	5.11	3.94	3.79	3.55	6.44	0.845	NS	NS	NS
ZO1	1.46	1.54	1.49	1.50	1.53	1.38	1.45	1.63	0.182	NS	NS	NS
ZO2	1.45	1.45	1.60	1.31	1.54	1.36	1.64	1.26	0.227	NS	NS	NS
CLDN1	1.67	1.75	1.69	1.73	1.80	1.55	1.91	1.58	0.217	NS	NS	NS
OCLN	1.38	1.45	1.48	1.36	1.60	1.17	1.35	1.53	0.165	NS	NS	NS
TLR pathway												
TLR2B	0.76	0.60	0.76	0.60	0.73^{b}	0.79^{b}	0.78^{b}	0.41^{a}	0.088	NS	NS	*
TLR4	0.77	0.61	0.73	0.65	0.74^{b}	0.80^{b}	0.72^{ab}	0.50^{a}	0.061	*	NS	*
IFNG	1.03	1.57	1.23	1.37	1.38	1.76	1.08	0.97	0.299	NS	NS	NS
NFKB1	0.93	1.01	0.97	0.97	1.01	1.01	0.94	0.92	0.080	NS	NS	NS
NOS2	0.80	0.83	0.93	0.70	0.95	0.71	0.91	0.69	0.128	NS	NS	NS
Nutrient-sensing genes												
FFAR2	1.40	1.52	1.42	1.50	1.59	1.45	1.25	1.54	0.378	NS	NS	NS
FFAR4	1.84	1.37	1.61	1.60	1.05	1.70	2.18	1.51	0.227	NS	NS	NS
Host defense peptide (HDP) genes												
AvBD1	0.48	0.59	0.62	0.45	0.60	0.35	0.64	0.55	0.118	NS	NS	NS
AvBD6	0.71	0.30	0.61	0.41	0.92^{c}	0.51^{b}	0.29^{a}	0.31^{a}	0.046	***	*	**
CATH2	0.77	1.06	0.64	1.19	0.50	1.04	0.79	1.03	0.306	NS	NS	NS
LEAP2	1.40	1.41	1.71	1.09	1.39^{ab}	1.40^{ab}	2.03^{b}	0.78^{a}	0.275	NS	*	*

[1]Diet specifications: 95 (i.e., 95% of recommended ME and CP specs) and 100 (i.e., 100% of recommended ME and CP specs). [2] phytogenic inclusion (No = 0 mg/kg diet and Yes = 150 mg/kg diet). [3] Means within the same row with different superscripts (a-c) differ significantly ($P < 0.05$). [4] Pooled standard error of means.
NS, $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

3.4 Cecal AI-2 relative activity

A significant interaction ($P_{D\times P} \leq 0.01$) of diet specifications × phytogenic was noted for cecal AI-2 relative activity. In particular, treatment D95+ had higher AI-2 relative activity than the other three treatments (Figure 3). Broilers with a diet formulated with 95% specifications had significantly higher ($P_D \leq 0.01$) AI-2 relative activity. Phytogenic addition significantly increased ($P_P \leq 0.05$) AI-2 relative activity in the cecal digesta.

4 Discussion

It has been shown that dietary metabolizable energy (ME) and crude protein (CP) reduction negatively affected broiler performance and that phytogenics inclusion tended to ameliorate this effect (Paraskeuas et al., 2016; Griela et al., 2021). Several mechanisms for the growth enhancement of phytogenics have been proposed, some of which include antioxidant activity, antimicrobial and anti-quorum-sensing activity, and anti-inflammatory and transcription-modulating effects on gut health biomarkers (Pandey et al., 2019; Kikusato, 2021). Although phytogenics are mainly absorbed in the proximal gut, there is evidence that phytogenic addition can also modulate the cecal microbiota and the overall cecal ecosystem (Paraskeuas et al., 2016). The ceca have long been a target of research due to the wide range of microorganisms it harbors, which continuously signal mainly through metabolites to the gut epithelium, affecting bird health and productivity (Shang et al., 2018; Rodrigues et al., 2020). In turn, host receptors play a critical role in host–microbe communication through the recognition of bacteria and the subsequent immune response to preserve homoeostasis (Pan and Yu, 2014). The present study provides new knowledge on the effects of dietary energy and protein levels with or without phytogenic addition on the modulation of microbiota composition and metabolic activity, bacterial communications and host signaling, inflammation, and defense.

Different dietary components can change the structure and function of the gut microbiome. In this study dietary ME and CP specifications in combination with phytogenic administration were shown to affect the mucosa-associated microbiota (*Lactobacillus* spp. and *Clostridium* cluster IV). Moreover, diet specification was the main factor to modulate mucosa-associated microbiota, whereas phytogenic administration modulated cecal digesta microbiota. Limitations of nutrients in the host diet are known to shape the structure of gut-associated microbial communities (Luo et al., 2015; Dong et al., 2017; Paraskeuas and Mountzouris, 2019b). In addition, it is already known that phytogenics based on carvacrol, anethol, and limonen (Mountzouris et al., 2011; Paraskeuas and Mountzouris, 2019a) can shape, in a beneficial way, the cecal microbiota profile. In this study phytogenic addition favored the growth of bacteria belonging to *Clostridium* cluster IV (*C. leptum* group), a cluster that contains numerous butyrate-producing and fibrolytic species, whose metabolic activities have a positive effect on host gut health (Jeraldo et al., 2016; Guo et al., 2020), whereas the supplementation of phytogenics acted in an antimicrobial manner and decreased the levels of microbiota species (i.e., *E. coli* and *Clostridium* cluster I) that could act as pathogens in broilers (Fancher et al., 2020; Murase and Ozaki, 2022).

Most known microbiota-derived metabolites are volatile fatty acids (VFAs; e.g., acetate, propionate, and butyrate),

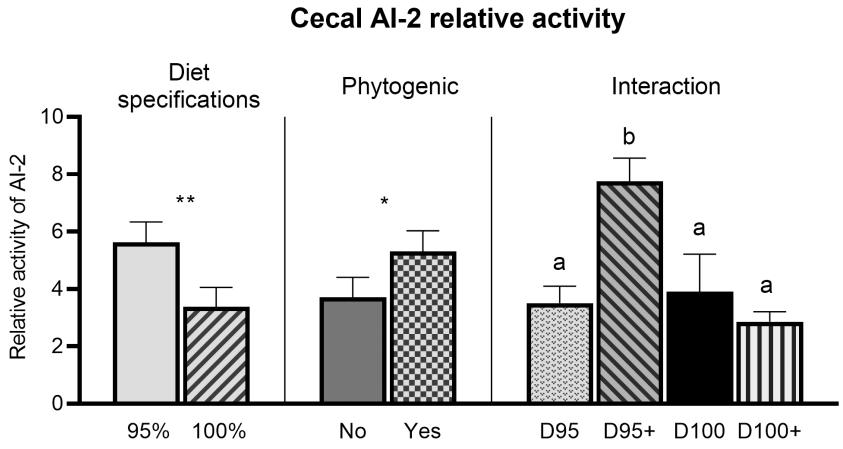

FIGURE 3
Effects of diet specifications %, phytogenic inclusion, and interaction effect of diet specifications × phytogenic inclusion on cecal AI-2 relative activity. Columns indicate means + SE and the asterisks denote level of statistical difference *P ≤ 0.05, **P ≤ 0.01. Treatment means with different superscripts **(A, B)** differ significantly (P < 0.05).

which have a multifaceted effect on the composition of the microbiota and on the host, beyond their contribution as an energy substrate to the intestinal epithelium (van der Hee and Wells, 2021). VFAs directly affect intestinal microbial composition by a cross-feeding mechanism, by their antimicrobial effects or by affecting bacterial gene expression, and indirectly affect intestinal microbial composition by maintaining the integrity of the gut barrier and modulating intestinal immunity (van Der Wielen et al., 2000; Sunkara et al., 2011; Lamas et al., 2019). Hence, dietary strategies leading to increased VFA production may benefit biomarkers of gut health.

In the present study, both diet specifications and phytogenic supplementation individually or in combination modulated the cecal microbial metabolism, as significant changes were noted for the cecal VFA pattern. In particular, an interaction of diet specification × phytogenic supplementation was revealed for the acetic acid molar ratio. In addition, the 95% ME and CP specification diet decreased the molecular ratio of butyric acid, while phytogenic supplementation increased the butyric molar ratio and decreased branched VFAs. The changes in the VFA pattern observed in this work may be influenced by the cecal microbiota composition (Cao et al., 2010) or by the amount and type of feed substrates reaching the ceca (Svihus et al., 2013). The reduced branched-VFA molar ratios observed in this study by dietary phytogenic addition could be considered to be a beneficial feature for chicken gut ecology. Concentrations of branched VFAs may be used as an indicator for protein fermentation (Macfarlane et al., 1992). Excess protein fermentation in the gut is not desirable because harmful metabolites are produced (e.g., ammonia) which, in addition to burdening the environment, have been linked to negative effects on the epithelium (Qaisrani et al., 2015; Naseem and King, 2018)

The crosstalk among bacteria, the immune system, and dietary factors is able to modulate the mucosal barrier function (De Santis et al., 2015). The intestinal epithelium forms a dynamic physicochemical barrier to maintain immune homeostasis. Gut mucosae include physical barriers (i.e., mucus layer and the cell junction) and chemical barriers (i.e., antimicrobial peptides). In this sense, an additional purpose of this study was to assess the effect of the dietary factors examined in the relative quantification of gene transcripts that encode mucin 2, the major component of mucus in the large intestine (Duangnumsawang et al., 2021); zonula occludens-1 and -2; claudin-1 and occludin, tight junction proteins that regulate epithelial barriers (Lee, 2015); toll-like receptors 2 (type 2) and 4, which play a crucial role in the innate immune system by recognizing bacteria (Hug et al., 2018); interferon-γ, nuclear factor-κB, and inducible nitric oxide synthase, which are associated with inflammation (Aktan, 2004); and gallinacin-1 and -6, cathelicidin-2 and liver-expressed antimicrobial peptide 2, which are antimicrobial peptides and are part of the innate immune response (Cuperus et al., 2013). In addition, the gene expression of free fatty acid receptors 2 and 4, which are important biomarkers of intestinal microflora activity (Slawinska et al., 2019; Bartoszek et al., 2020), were also determined.

Tight junction proteins are the most significant feature of gut integrity and make up a barrier in the paracellular space. These proteins are subject to alteration and remodeling in response to external stimuli in the gut environment, such as nutrients and bacteria (commensals or pathogens) (Ulluwishewa et al., 2011). So far it has been shown that in non-challenging conditions the effect of reduced energy and protein levels has little impact on intestinal tight junction gene expression (Barekatain et al., 2019; Paraskeuas and Mountzouris, 2019a) and intestinal permeability (Barekatain et al., 2019) compared with standard full-specification diets. This work confirmed these effects since reduction of CP and ME by 5% did not impact expression of tight junction-related genes. Moreover, the fact that the inflammatory biomarkers studied (i.e., the expression of *NFKB1*, *NOS2*, *IFNG*) were not altered by the reduced ME and CP levels may also be explained by the optimal management and hygiene conditions followed in this study. In another study, reducing CP levels in combination with aflatoxin challenge altered tight junction-related gene expression, exacerbating the effect of aflatoxicosis on intestinal permeability, which was improved with a 10% increase in CP (Chen et al., 2016). Thus, the mucosa barrier can be affected by low-CP diets when a stress factor is present. Further research is needed to determine the effect of reduced ME and CP levels under commercial conditions where chicks are raised in environments with multiple stressors. On the other hand, phytogenic effects on the gene expression of tight junction proteins have been shown to vary from no effects (phytogenic based on menthol and anethole; Paraskeuas and Mountzouris, 2019a), as in this study and a previous study, to positive effects noted by TJ upregulation (phytogenic based on carvacrol, anethol, and limonen; Paraskeuas and Mountzouris, 2019b). It is understood that factors such as phytogenic composition and inclusion level may impact TJ expression, especially under challenging conditions (Ibrahim et al., 2020; Hashem et al., 2022).

The gut is constantly exposed to antigens that can trigger an immune response, which is characterized by activation of the NF-κb pathway. One of the negative regulatory mechanisms of this pathway is the downregulation of the transcription of TLRs and related genes (Villena and Kitazawa, 2014). In the present study, the 95% ME and CP specification diet resulted in upregulation of *TLR4*, whereas the 100% ME and CP specification diet with phytogenic downregulated both *TLR4* and *TLR2B* expression in the cecal epithelium. In healthy conditions, in intestinal epithelial cells, TLR expression is repressed to maintain homeostasis of the intestine and allow for commensal microbiome development (Villena and Kitazawa, 2014; Bruning et al., 2021). This effect could be attributed to either a bacteria-mediated mechanism due to the changes observed in the cecal microbiota or to the blocking of the binding of TLR ligands to the corresponding receptors, thus interfering with the intracellular signaling pathways (Paraskeuas and Mountzouris, 2019b; Paraskeuas and Mountzouris, 2019a; Rehman et al., 2021), or a combination of both.

Host defense peptides (HDPs), also known as antimicrobial peptides, are a critical component of the animal innate immune system, with direct antimicrobial (against bacteria, fungi, and viruses) and immunomodulatory activities (Hilchie et al., 2013). Avian HDPs have been shown to be modulated by dietary factors (Shao et al., 2016; Tian et al., 2016; Robinson et al., 2018) or by pathogens (Shao et al., 2016; Tian et al., 2016; Su et al., 2017). In this study, the inclusion of phytogenic in the 100% ME and CP specification diet did not affect the expression of *AvBD6*. However, phytogenic inclusion in the 95% ME and CP specification diet reduced the expression of *AvBD6* compared with phytogenic exclusion in this diet. In addition, phytogenic inclusion downregulated the expression of *LEAP2* in the 100% ME and CP specification diet. These results highlight the role of diet in the efficacy of phytogenics. Chicken AvBD6 and LEAP2 play an important role in chicken innate host defense (Van Dijk et al., 2007; Su et al., 2017) in ceca. However, a strong immune response is detrimental to the host and performance of animals in a chronic inflammation site such as the gut. In the case of healthy broilers, downregulation of HDPs could point to phytogenics' potential to contribute towards an improved intestinal environment *via* an anti-inflammatory mechanism. Downregulation of *AvBD6* and *LEAP2* will have to be considered in context, considering overall zootechnical performance, which has been shown to tend to be improved by phytogenic addition (Griela et al., 2021).

In the "noisy" environment of the gut, bacteria rely on quorum sensing to regulate survival and to compete with others for spatial dominance (Wu and Luo, 2021). In this study, the inclusion of phytogenic in the 100% ME and CP specifications diet (D100+) did not affect the relative activity of AI-2; however, phytogenic inclusion in the 95% ME and CP specifications diet (D95+) increased the relative expression of AI-2 compared with the non-supplementation of phytogenic. This result indicated that the combination of reduced ME and CP and phytogenic inclusion affected interspecies communication and may determine bacterial group behaviors (Fu et al., 2020). Thompson et al. (2015) showed that, in the mammalian gastrointestinal tract, AI-2 mediated communication among bacteria, thereby shaping the structure of the microbial community. *In vitro* studies that used pathogens isolated from broilers reported that plant extracts, α-pinene or carvacrol, act as quorum-sensing inhibitors (Šimunović et al., 2020; Wagle et al., 2020). The latter may be through an antagonistic action due to the similarity of their chemical structure to those of quorum-sensing signals or their ability to degrade quorum-sensing receptors such as autoinducer binding domain-containing protein (LuxR)/transcriptional regulator LasR (Kalia, 2013). In the present study, AI-2 relative expression was positively regulated on supplementation of phytogenic and this could be due to the presence of a multitude of AI-2-producing bacteria that could have been differentially affected by phytogenics. Consequently, it could be postulated that, except their bacterial growth-inhibiting ability, phytogenics, through diverse mechanisms of AI-2 regulation, can affect the communication of microbiota and, as a result, further modulate microbiota composition. As there are no other studies investigating the effect of phytogenic inclusion on the role of AI-2 in broiler guts, further metatranscriptomic and metagenomic research will be required to reveal and associate the changes of the bacterial behavior with the microbiota structure (composition) at a detailed level.

5 Conclusion

In conclusion, it has been shown that ME and CP dietary specifications, combined or not with phytogenic, modulate multilevel gut biomarkers, from microbiota composition and metabolic activity to microbial communications and host signaling, inflammation, and defense. Further exploration using metatranscriptomic analysis combined with metagenomic analysis of microbiota will provide important details about the changes in bacterial behavior in the complex gut environment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Department of Agriculture and Veterinary Policy, General Directorate of Agriculture, Economy, Veterinary and Fisheries.

Author contributions

KM and IP conceived the original idea. KM and IP conceived and planned the experiments. IP performed the analytical assays. IP wrote the manuscript with support from KM and VP. KM supervised the project. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2022.1094314/full#supplementary-material

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