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Colonization of chickens with competitive exclusion products results in extensive differences in metabolite composition in cecal digesta

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ABSTRACT The concept of competitive exclusion is well established in poultry and different products are used to suppress the multiplication of enteric pathogens in the chicken intestinal tract. While the effect has been repeatedly confirmed, the specific principles of competitive exclusion are less clear. The aim of the study was to compare metabolites in the cecal digesta of differently colonized chickens. Metabolites in the cecal contents of chickens treated with a commercial competitive exclusion product or with an experimental product consisting of 23 gut anaerobes or in control untreated chickens were determined by mass spectrometry. Extensive differences in metabolite composition among the digesta of all 3 groups of chickens were recorded. Out of 1,706 detected compounds, 495 and 279 were differently abundant in the chicks treated with a commercial or experimental competitive exclusion product in comparison to the control group, respectively. Soyasaponins, betaine, carnitine, glutamate, tyramine, phenylacetaldehyde, or 3-methyladenine were more abundant in the digesta of control chicks while 4-oxododecanedioic acid, nucleotides, dipeptides, amino acids (except for glutamate), and vitamins were enriched in the digesta of chickens colonized by competitive exclusion products. Metabolites enriched in the digesta of control chicks can be classified as of plant feed origin released in the digesta by degradative activities of the chicken. Some of these molecules disappeared from the digesta of chicks colonized by complex microbiota due to them being metabolized. Instead, nucleotides, amino acids, and vitamins increased in the digesta of colonized chicks as a consequence of the additional digestive potential brought to the cecum by microbiota from competitive exclusion products. It is therefore possible to affect metabolite profiles in the chicken cecum by its colonization with selected bacterial species.

Key words: chicken, cecum, metabolome, microbiota, competitive exclusion

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INTRODUCTION

Probiotic treatment represents one of the alternatives for replacement of antibiotic growth promoters in commercial poultry production. Many beneficial effects of probiotics, that is, products consisting of a single or a few defined bacterial species, or competitive exclusion products, that is, products consisting of more usually undefined bacterial species, have been reported including immunomodulatory effects, competitive exclusion of pathogenic bacteria, production of antimicrobial substances and vitamins, fermentation of feed components which cannot be digested by the host, decrease in pH due to short-chain fatty acid production and improved energy extraction from the feed (Methner et al., 1997; Gantois et al., 2006; Stanley et al., 2013a; Wang et al., 2018; Wickramasuriya et al., 2022). Complex bacterial mixtures derived from the intestinal contents of donor hens and commercial products containing undefined chicken microbiota also induce protection against *Salmonella* colonization (Rantala and Nurmi, 1973; Methner et al., 1997; Varmuzova et al., 2016). On the other hand, individual cultures of gut anaerobes are usually ineffective in protection against enteric pathogens (Kubasova et al., 2019b) and complex mixtures have to be used to see protective effects (Papouskova et al., 2023). Rather unexpectedly, there is minimal immune response of chickens to the colonization with individual strains (Kubasova et al., 2019b) or to the colonization with complex microbiota (Volf et al., 2016). It is therefore unclear, how the protective effect of complex gut microbiota is exerted but one of possible explanations can be that metabolites released by gut microbiota may

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affect the behavior of other microbiota members. It has been shown that the expression of *Salmonella* virulence is suppressed by butyrate, a common end product of anaerobic fermentation (Gantois et al., 2006) and that soya-derived isoflavones improve chicken performance after infection with infectious bursal disease virus (Azzam et al., 2019). Metabolites produced by complex microbiota may therefore act directly against pathogenic microorganisms.

There are several reports on the chicken cecal metabolome. Interestingly, although nearly all papers correlated the metabolome profile with microbiota composition, none of them used experimental administration of microbiota members which are usually absent in the cecum of chickens from hatcheries. Instead, the majority of the papers used feed interventions, for example, feed supplementation with flavonoids or antibiotics. Several papers used probiotic strains from genera *Lactobacillus* or *Bacillus* (Chen et al., 2020; Park et al., 2020; Wu et al., 2021; Zhang et al., 2022; Wang et al., 2023). Unfortunately, *Bacillus* does not represent a common gut microbiota member in chickens and *Lactobacillus* strains are present in gut microbiota of chickens from the first days of their life (Videnska et al., 2014). Their additional administration is therefore of questionable value, especially when it is known that lactobacilli colonize the chicken intestinal tract poorly (Kubasova et al., 2019b; Juricova et al., 2022), which makes linking their administration with the metabolic profile rather complicated. Previous studies used broilers, that is, chickens with underdeveloped gut microbiota. Though such studies can bring new information, these do not address the key point of what is the effect of underdeveloped and mature microbiota on the cecal metabolome.

Microbiota of young chickens is quite variable, mostly formed by Firmicutes, but different from the microbiota of adult hens which consist of similar representation by Firmicutes and Bacteroidetes (Stanley et al., 2013b; Videnska et al., 2014; Gao et al., 2017; Xi et al., 2019). However, it is possible to colonize newly hatched chicks with adult type microbiota from d 1 of life (Varmuzova et al., 2016; Kubasova et al., 2019a). Such colonization is rapid and makes chicks immediately resistant to enteric pathogens (Rantala and Nurmi, 1973; Methner et al., 1997; Varmuzova et al., 2016; Kubasova et al., 2019a) though as mentioned above, changes in chicken gene expression in response to colonization are rather minor. The contradiction of a minimal chicken response but immediate resistance to pathogens led us to the current study in which we aimed to identify the differences in metabolite composition between the digesta of control and probiotic-treated chickens.

The fact that chicks can be colonized by complex microbiota from the first days of their life provides a unique opportunity to compare the cecal metabolome of chickens with or without complex microbiota. In other words, it is possible to model and compare the metabolome of chickens from commercial settings and those representing the natural behavior of *Gallus gallus*, such as hatching in nests, remaining in contact with parents and being colonized by adult-type microbiota from the first days of life. The aim of this study therefore was to identify differently abundant metabolites in the cecal digesta of control chickens and those treated with commercial or experimental competitive exclusion products. Such knowledge can be used for targeted enrichment of digesta promoting the synthesis of biologically active molecules by administration of microbiota of specific composition.

MATERIALS AND METHODS

Chickens and Microbial Products

Three groups of newly hatched male ISA Brown chickens, each consisting of 5 chicks, were included in this study. Chickens in 2 experimental groups were treated either with 100 μL AVIGUARD prepared according to instructions of the manufacturer (Lallemand, Montreal, Canada) or with 100 μL of a mixture of 23 gut anaerobes (hereafter called anaerobe group) on d 1 of life. The main feed ingredients included wheat, maize, soya, pea, barley, and sunflower seeds. Analytically the feed consisted of protein (19%), crude ash (6.6%), fiber (4.3%), fat (3.6%), calcium (1.2%), phosphorus (0.73%), lysine (1.12%), and methionine (0.49%). The handling of animals in the study was performed in accordance with current Czech legislation Animal Protection and Welfare). The specific experiments were approved by the Ethics Committee of the Veterinary Research Institute followed by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic.

The anaerobe mixture contained *Bacteroides barnesiae*, *Bacteroides caecicola*, *Bacteroides caecigallinarum*, *Bacteroides coprophilus*, *Bacteroides gallinaceum*, *Bacteroides mediterraneensis*, *Bacteroides plebeius*, *Bacteroides salanitronis*, *Mediterranea massiliensis*, *Barnesiella viscericola*, *Bifidobacterium saeculare*, *Cloacibacillus porcorum*, *Desulfovibrio piger*, *Marseilla massiliensis*, *Megamonas funiformis*, *Megamonas hypermegale*, *Megasphaera hexanoica*, *Olsenella uli*, *Parasutterella secunda*, *Phascolarctobacterium faecium*, *Succinatimonas hippei*, *Sutterella faecalis*, and *Veillonella magna* (Medvecky et al., 2018). All these bacteria were selected as capable of colonization following a single dose administration (Kubasova et al., 2019b) Individual cultures were grown on Wilkins-Chalgren agar plates under anaerobic conditions (Kubasova et al., 2019b). After 48 h of growth, the cultures were washed from the agar surface with PBS and optical density was set to 1.0. In the next step all the cultures were pooled and used immediately for oral inoculation of newly hatched chicks. Chickens in the control group remained without any treatment. Chicken microbiota was given 1 wk to develop and establish, and on d 8 of life, all chickens were sacrificed under diethyl ether anesthesia, cecal contents were collected and frozen at

−20°C for microbiota characterization and at −80°C for metabolite extraction.

16S rRNA Gene Sequencing of Chicken Cecal Microbiota

Total DNA was purified using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). PCR with eubacterial primers amplifying the V3/V4 variable region of 16S rRNA genes, sequencing using the Illumina platform and all downstream sequence processing and analysis was performed exactly as described previously (Kubasova et al., 2022).

Extraction of Metabolites From Cecal Contents

Methanol extraction of fresh cecal digesta was performed as described earlier for human, rodent, pig, and ruminant fecal or feed samples (Matysik et al., 2016; Cesbron et al., 2017; Deda et al., 2017; Cheng et al., 2020). The cecal contents were weighed and mixed with extraction solution made of methanol and water at a 4:1 (v/v) ratio. Three hundred μL of extraction solution per 0.1 g of cecal content was used. The mixture was then agitated for 30 min at 4°C in a thermo-shaker followed by centrifugation at 16,000 × g for 15 min at 4°C. The supernatant was transferred to a fresh tube and stored at −20°C for 15 min followed by 15 min centrifugation at 4°C and 16,000 × g for protein precipitation and removal. The supernatant was dried in a SpeedVac, resuspended in 50 μL of methanol/water/acetonitrile (1:1:2), and centrifuged at 12,000 × g at 4°C for another 5 min to remove any residues that could lead to column clogging. The supernatant was transferred into a sample vial for liquid chromatography and 2 μL of supernatant was loaded to the chromatographic column.

UHPLC Separation

Chromatographic separation of the metabolites was performed on a Thermo Scientific Dionex Ultimate 3000 system equipped with Kinetex Core-Shell technology 1.7 μm HILIC 100 Å, 100×2.1 mm LC Column (Phenomenex, Torrance). The mobile phases consisted of acetonitrile:water (95:5, v/v, containing 0.05% formic acid and 100 mM ammonium formate, pH 3.5) (solvent A) and acetonitrile:water (1:1, v/v, containing 0.05% formic acid and 100 mM ammonium formate, pH 3.5) (solvent B). The solvent gradient changed according to the following conditions: from 0 to 2 min, 0% B; from 2 to 2.5 min, 0% B to 10% B; from 2.5 to 7.5 min, 10% B to 35% B; from 7.5 to 15 min, 35% B to 40% B; from 15 to 25 min, 40% B to 47% B; from 25 to 27 min, 47% B to 5% B; from 27 to 28 min, 5% B; from 28 to 29.5 min, 5% B to 0% B; from 29.5 to 40 min, 0% B. The column temperature was maintained at 40°C and the flow rate was set to 0.25 mL/min over a run time of 40 min.

Tandem Mass Spectrometry Analysis

Q-Exactive Focus Mass Spectrometer (Thermo-Fisher Scientific, Waltham) was used for analysis of cecal metabolites. Heated electrospray ionization source was used with spray voltage 3.8 kV. The capillary temperature was 350°C, the sheath gas flow rate was 35 arbitrary units and the auxiliary gas flow rate was 10 arbitrary units. Mass spectrometry detection was performed in the positive ion mode. Parameters of full scan acquisition were a scan range of 70 to 1,050 m/z, an automatic gain control target 1×10^6 charges, 70,000 resolution ($m/z = 400$) and a maximum injection time of 100 ms. Tandem mass (**MS/MS**) scan parameters used were a scan range of 70 to 1,050 m/z, an automatic gain control target 2×10^6 charges, 17,500 resolution ($m/z = 400$), and a maximum injection time of 50 ms. The sample injection volume was 2 μL and samples were run in full scan triplicates followed by 1 data-dependent run used only for identification. Quality control samples (**QC**) were prepared by mixing all samples at equal volumes and were run after every 10 samples. The clustering of QC samples in PCA analysis validated the stability of the analysis in time.

UHPLC-HRMS Data Processing

Raw data were preprocessed using the Thermo Scientific Compound Discoverer 3.1 software including background filtering, peak picking, integration, retention time (**RT**) alignment and peak alignment. The in-house library was prepared for the metabolite identification using a set of standards from IROA MSML Library (Sigma Aldrich, St. Louis, MO). The remaining compounds were identified based on exact molecular mass and MS/MS spectrum compared with on-line databases available at GNPS website (https://gnps.ucsd.edu/ release 30) (Wang et al., 2016). Spectral peaks present in blank samples and those without MS/MS were discarded from the analysis.

Statistical Analysis

Sequencing data were analyzed and visualized using STAMP software 2.1.3. Comparison of changes among groups was analyzed using Kruskal-Wallis H test, combined with Tukey post hoc test with Benjamine-Hochberg false discovery rate (**FDR**) correction ($P < 0.05$) and effect size (over 0.5).

Data from mass spectrometry were imported into MetaboAnalyst 5.0 (Pang et al., 2021). Unsupervised principal component analysis (**PCA**) was used to visualize variations and general clustering among all groups. Supervised partial least squares-discriminant analysis

(**PLS-DA**) was used to identify differential metabolites between the control and experimental groups and variable importance in projection (**VIP**) scores in the PLS-DA model were calculated. ANOVA followed by Tukey post hoc test was then used to determine P values for all compounds. The $\log_2$ fold change represented the ratio of the abundance of the average ion intensities of the individual compound in the cecum of inoculated chickens compared to the control chickens. Metabolites with a VIP value >1 and FDR <0.05 and $\log_2$ fold change >1 and <−1 were considered as discriminating between the groups. The heatmaps were prepared using R-studio.

RESULTS

Cecal Microbiota Composition in Control and Experimental Chickens

Out of 23 strains present in the anaerobe mixture, 18 were detected in the cecum of at least one of the treated chickens. AVIGUARD consisted of 110 Amplicon Sequence Variant (**ASVs**), of which *Bacteroides barnesiae, Bacteroides mediterraneensis, Bifidobacterium saeculare, Megamonas hypermegale, Megasphaera stantonii, Olsenella uli, Sutterella faecalis*, and *Marseilla massiliensis* were present also in the anaerobe mixture. List of all ASV present in the used batch of Aviguard,

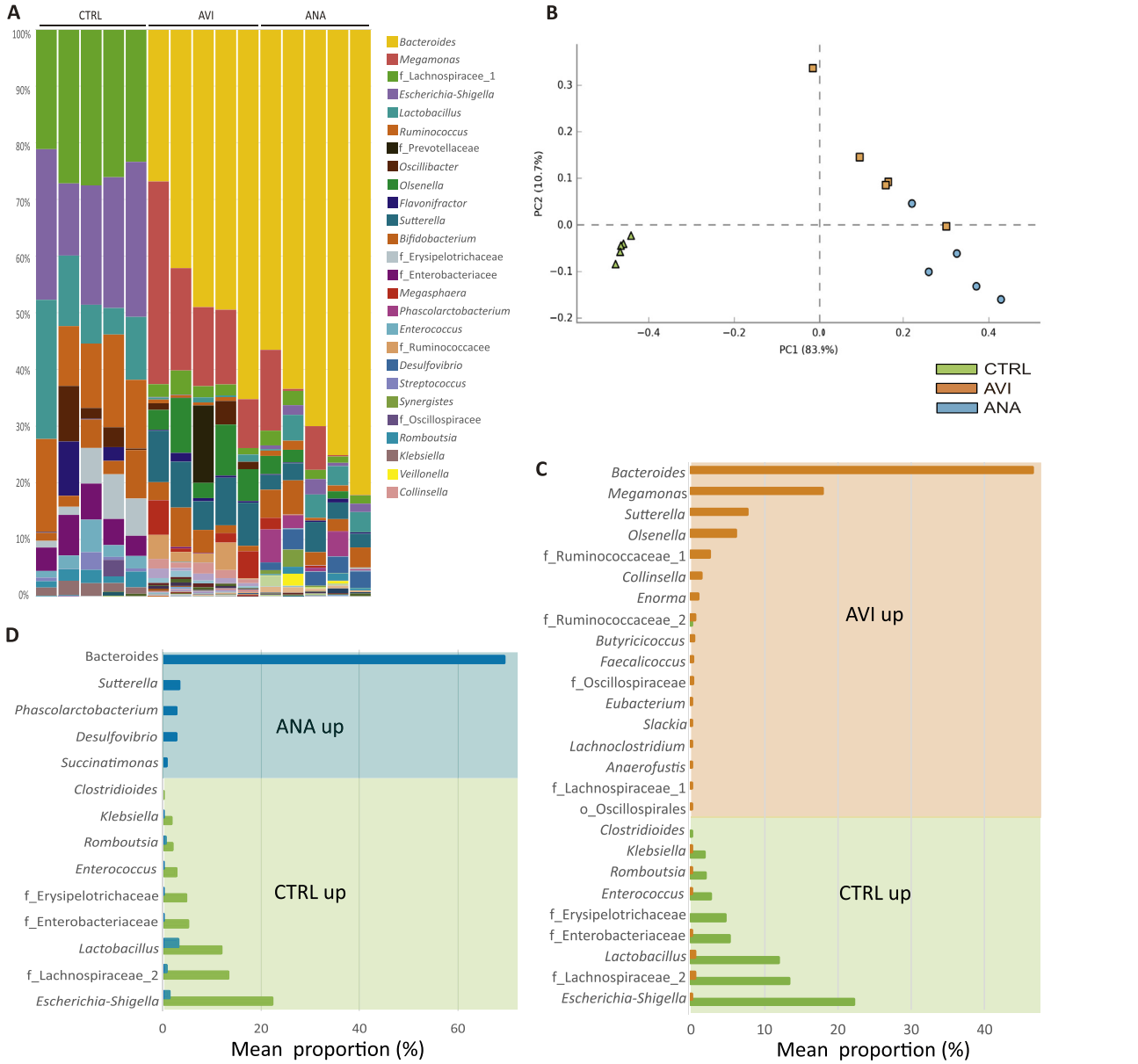

Figure 1. Cecal microbiota of control chickens and chickens treated with AVIGUARD or anaerobe mixture. (A) Cecal microbiota composition in individual 8-day-old chicks at genus level. (B) Principal component analysis (PCA) based on microbial composition determined by sequencing of 16S rRNA genes. (C) Differently abundant genera in AVIGUARD and control chickens ($P < 0.05$). (D) Differently abundant genera in anaerobe and control chickens ($P < 0.05$). CTRL, control chickens; AVI, AVIGUARD-treated chickens; ANA, anaerobe mixture-treated chickens. ANA up—genera more abundant in anaerobe mixture-treated chickens, AVI up—genera more abundant in AVIGUARD-treated chickens, CTRL up—genera more abundant in control chickens. The same color coding is used in panels B, C, and D, green—control chickens, blue—anaerobe mixture-treated chickens, orange—AVIGUARD-treated chickens.

and colonization ability of bacteria from the Aviguard and anaerobe mixture are summarized in Supplementary Table 1.

The microbiota composition of the control and both experimental groups differed considerably (Figure 1A, B). A lower abundance of families Lachnospiraceae, Enterobacteriaceae, Erysipelotrichaceae and genera *Escherichia-Shigella, Lactobacillus, Klebsiella*, and *Romboutsia* was observed both in AVIGUARD and anaerobe groups in comparison to control chickens. These taxa were replaced with genera *Bacteroides* and *Sutterella* in both experimental groups. Microbiota of AVIGUARD chickens was also significantly enriched for *Collinsella, Olsenella, Slackia*, and *Enorma* (all Actinobacteria), and *Megamonas, Butyricicoccus, Faecalicoccus, Eubacterium, Lachnoclostridium*, and *Anaerofustis* (all from Firmicutes) in comparison to control chickens (Figure 1C). In addition to *Bacteroides* and *Sutterella*, microbiota of anaerobe chickens was enriched significantly also for *Succinatimonas, Desulfovibrio*, and *Phascolarctobacterium* in comparison to control chickens (Figure 1D).

Metabolites in Chicken Cecal Digesta

In a total, 1,706 compounds were detected across all samples. The presence, absence and differential abundance of these compounds resulted in clear separation all 3 groups of chickens (Figure 2A and Supplementary Table 2). Unfortunately, only 98 compounds could be identified by their chemical names and the majority of compounds therefore remained identified only by mass spectrometry parameters.

Altogether 609 molecules (35.7% of all 1,706 detected molecules) were differentially abundant in the digesta of chickens from experimental groups compared to control chickens. In AVIGUARD-treated chickens, 495 molecules were differently abundant compared to control chickens. Of these, 204 were more abundant and 291 were less abundant in the digesta of AVIGUARD chickens than in control chickens. Similarly, 279 molecules were differently abundant in the cecal digesta of chickens treated with the anaerobe mixture compared to control chickens. Of these, 157 were significantly more abundant and 122 were less abundant in the digesta of anaerobe chickens compared to controls.

Differentially Abundant Compounds With Assigned Trivial Names

Only 58 differently abundant compounds could be assigned chemical names. These included nucleotides, amino acids, lipids, vitamins, and certain molecules of plant origin.

3-Methyladenine was the most abundant in the digesta of control chickens. Other detected nucleotides such as guanine, xanthine, hypoxanthine, adenosine, cytidine, cytosine, adenine, deoxyadenosine, methyladenosine, and inosine were all numerically the most abundant in digesta of 1 of the 2 experimental groups. Differences were observed for guanine and xanthine in both experimental groups compared to control chickens, for hypoxanthine, adenosine, cytidine and cytosine in the AVIGUARD chickens and for adenine and deoxyadenosine in the anaerobe chickens (Figure 3A).

Glutamate, acetyl-lysine, tyramine, and phenylacetaldehyde dominated in the digesta of control chickens and were less abundant in experimental chickens. All the remaining amino acids and their degradative products were enriched in the digesta of experimental chickens. Proline, isoleucine, tyrosine, kynurenate, and methylimidazoleacetic acid were

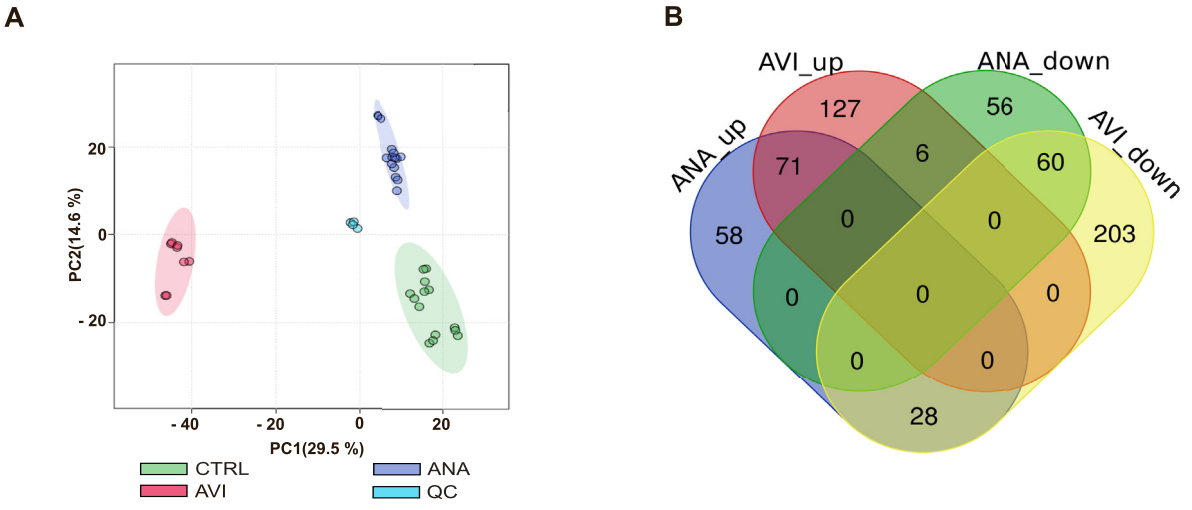

Figure 2. Discrimination of differently treated chickens based on the presence of low molecular weight metabolites in cecal contents. Cecal metabolomic profiles separated chickens in individual groups as shown by the unsupervised principal component analysis (PCA) (A). Treatment with AVIGUARD (AVI) or anaerobe mixture (ANA) affected the abundance of hundreds of molecules (B). Discriminators "up" or "down" relates to the comparison with control chickens (CTRL). Metabolites differently abundant in cecal digesta of anaerobe mixture and AVIGUARD-treated chicks can be seen in the Supplementary Table 3. Quality controls (QC), mixtures of all samples ran after every 10th sample as a control of stability of liquid chromatography tandem mass spectrometry system.

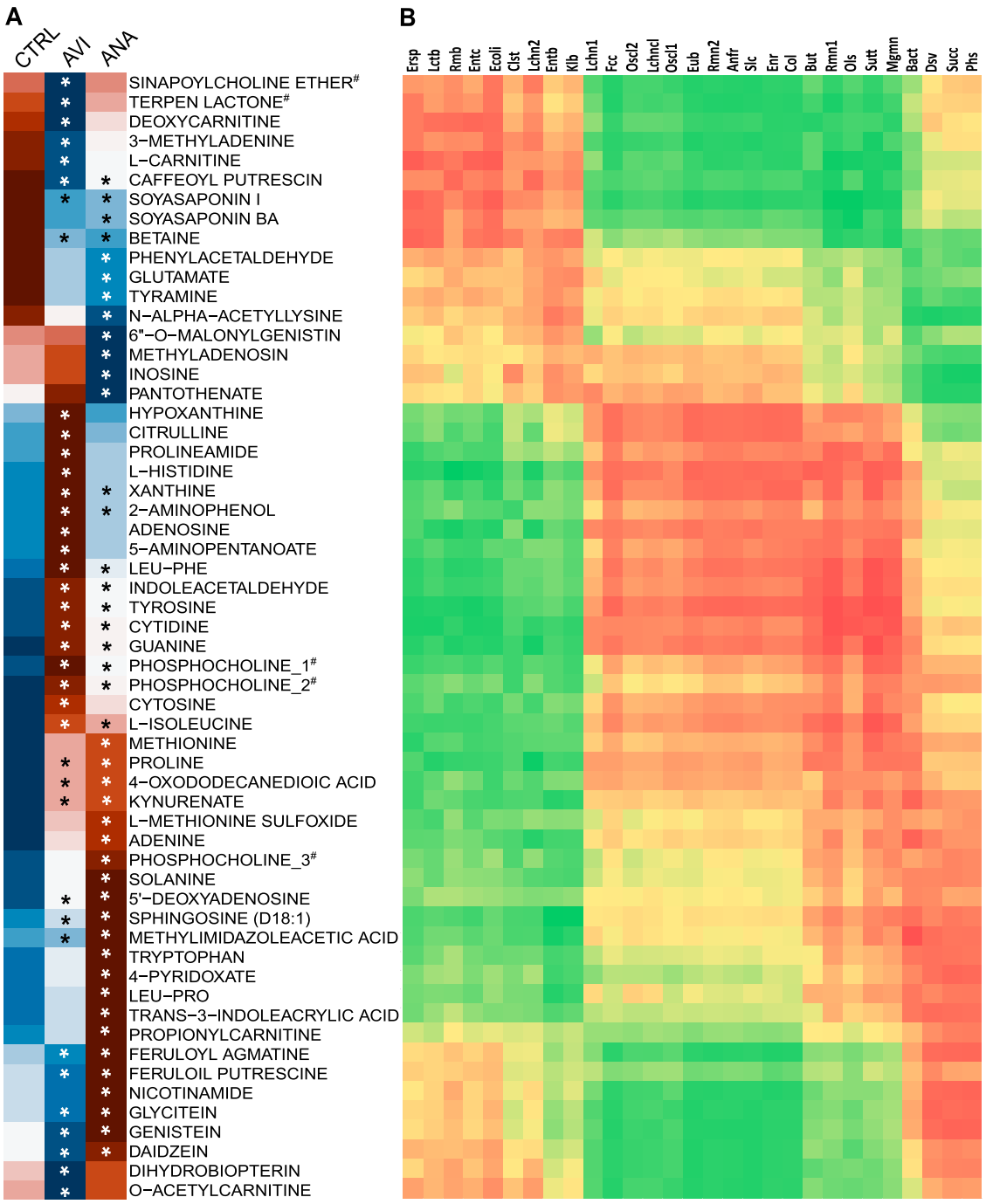

Figure 3. Distribution of molecules with assigned chemical name among groups. Panel A, abundance of molecules with assigned names in digesta of control (CTRL), AVIGUARD (AVI), or anaerobe (ANA)-treated chickens. # marks compounds with a shortened name, sinapoylcholine ether: Guaiasylglycerol-beta-sinapoylcholine ether, TERPEN LACTONE: (4r)-3-methylidene-4-[(E)-3-methyl-4-(4-methyl-5-oxooxolan-2-Yl)But-2-enyl]oxolan-2-one, phosphocholine_1: 1-(9z-octadecenoyl)-sn-glycero-3-phosphocholine, phosphocholine_2: 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine, phosphocholine_3: 1-hexadecanoyl-2-octadecadienoyl-Sn-glycero-3-phosphocholine. Blue color represents abundance lower than average, the red color represents abundance higher than average, and white color represents the average abundance. Panel B, correlations of the molecules with bacterial genera different ($P < 0.05$) among experimental groups. Red color indicates positive correlation, green color represents negative correlation, and yellow corresponds to no correlation. The names of compounds are common for both panels. Phs, *Phascolarctobacterium*; Succ, *Succinatimonas*; Dsv, *Desulfovibrio*; Bact, *Bacteroides*; Mgmn, Megamonas; Sutt, Sutterella; Ols, Olsenella; Rum1, f_Ruminococcaceae_1; But, *Butyricicoccus*; Col, *Collinsella*; Enr, *Enorma*; Slc, *Slackia*; Anfr, *Anaerofustis*; Rmn2, f_Ruminococcaceae_1; Eub, *Eubacterium*; Oscl2, o_Oscillospirales; Lchncl, *Lachnoclostridium*; Oscl1, f_Oscillospiraceae; Fcc, *Faecalicoccus*; Lchn1, f_Lachnospiraceae_1; Klb, *Klebsiella*; Entb, f_Enterobacteriaceae; Lchn2, f_Lachnospiraceae_2; Clst, *Clostridioides*; Ecoli, *Escherichia*; Entc, *Enterococcus*; Rmb, *Romboutsia*; Lctb, Lactobacillus; Ersp, f_Erysipelotrichaceae. *Different from control chicks at $P < 0.05$.

increased in the digesta of both experimental groups. Histidine, prolineamide, and citrulline dominated in the AVIGUARD-treated chickens and tryptophan, methionine, and methionine sulfoxide in the anaerobe mixture-treated chickens (Figure 3A).

Three different phospholipids, that is, 1-hexadecanoyl-2-octadecadienoyl-sn-glycero-3-phosphocholine, 1-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine dominated in the digesta of both experimental groups. In

addition, sphingosine (d18:1) was also significantly more abundant in the digesta of both experimental groups compared to control chickens. 4-Oxododecanedioic acid was another lipid molecule of significantly higher abundance in the digesta of both experimental groups (Figure 3A).

Vitamins and cofactors were also affected by microbial colonization. Pyridoxate and nicotinamide were significantly more abundant in the digesta of anaerobe chickens compared to control chickens. On the other hand, pantothenate was less abundant in the digesta of anaerobe chickens and dihydrobiopterin was less abundant in the digesta of AVIGUARD-treated chickens compared to control chickens (Figure 3A).

Betaine, caffeoyl putrescine, and soyasaponin I were the most abundant in the digesta of control chickens. Guaiasylglycerol-β-sinapoylcholine ether, carnitine, deoxycarnitine, and soyasaponin Ba dominated in the digesta of control chickens and were least abundant in AVIGUARD-treated chickens. Feruloyl agmatine, feruloyl putrescine, genistein, glycitein, and daidzein were more abundant in the digesta of anaerobe chickens than in control chickens. However, these molecules were more abundant in the digesta of control chickens than in AVIGUARD-treated chickens. Solanine and propionyl carnitine were more abundant in the digesta of anaerobe chickens than in control chickens with an intermediate level in the digesta of AVIGUARD chickens (Figure 3A).

Metabolome and Microbiota Composition

In the next analysis we tested potential associations between discriminative gut microbiota members and differently abundant metabolites with assigned trivial names. The highest positive correlation for 3-methyladenine common in the digesta of control chickens was observed for *E. coli*, unclassified *Lachnospiraceae* and unclassified *Erysipelotrichaeceae*. The presence of all other nucleotides correlated positively with *Bacteroides, Megamonas*, and *Sutterella* (Figure 3B).

Glutamate, tyramine, phenylacetaldehyde, and acetyl-lysine correlated the most with microbiota of control chickens, *Klebsiella* and *Enterococcus* in particular. Dipeptides, histidine, tyrosine, isoleucine, proline, methionine, and indolacetaldehyde positively correlated with *Megamonas* and *Sutterella*, and methylimidazoleacetic acid and kynurenate positively correlated with *Bacteroides* and *Sutterella*.

Vitamins like nicotinamide, pyridoxate, and dihydrobiopterin positively correlated with bacteria specific to the anaerobe mixture, that is, *Phascolarctobacterium, Succinatimonas*, and *Desulfovibrio*. On the other hand, *Phascolarctobacterium, Succinatimonas*, and *Desulfovibrio* negatively correlated with pantothenate in digesta which was common in the digesta of control chickens and exhibited the highest positive correlation with *Enterococcus*.

Out of the molecules of plant feed origin, glycitein, genistein, feruloyl agmatine, solanine, and propionylcarnitine positively correlated with *Phascolarctobacterium, Succinatimonas*, and *Desulfovibrio*. Caffeoyl putrescin, carnitine, deoxycarnitine, soyasaponin I, soyasaponin Ba and betaine positively correlated with *E. coli, Enterococcus*, and *Lactobacillus* (Figure 3B).

Mutual Correlation of Metabolites

The majority of molecules detected in this study remained characterized only by mass spectrometry parameters, which did not allow identification of their origins. Such molecules were only characterized based on correlation analysis with the expectation that clustering of these molecules with molecules of known chemical names will allow for prediction of their basic characteristics.

Of the top 100 most abundant molecules without chemical names, cecum_1 and cecum_8 molecules correlated positively with abundances of daidzein, genistein, glycitein, feruloyl agmatine, or feruloyl putrescine, that is, compounds of soya origin (Mebrahtu et al., 2004). Cecum_4, cecum_7 or cecum_9 molecules positively correlated with a cluster of molecules including betaine or schaftoside, that is, molecules of wheat origin (Likes et al., 2007; Filipcev et al., 2018; Balli et al., 2019; Zivkovic et al., 2023). Cecum_6 molecule correlated with 2-aminophenol, tyrosine and n-benzylformamide which were common in the ceca of AVIGUARD-treated chickens (Figure 4 and Supplementary Table 4 for full list of correlations).

Correlation analysis also showed that 2 clusters without extensive mutual correlation formed within molecules characteristic for cecal digesta of control chickens. Tyramine, phenylacetaldehyde, glutamate, glutamine, betaine, N-acetyl-L-carnosine or schaftoside formed the first cluster while guaiasylglycerol-beta-sinapoylcholine ether, caffeoyl putrescin, soyasaponin I, soyasaponin Ba, L-carnitine, deoxycarnitine, and 3-methyladenine formed the second one. The former cluster can be linked with molecules present in wheat while the latter with those present in soya.

Finally, we checked for the compounds with the highest positive or negative correlations. There were 11 groups of compounds (mostly pairs) exhibiting higher than 0.95 positive correlation. These included tyramine and phenylacetaldehyde; N-acetylputrescine and cecum_120; cecum_4 and cecum_102; cecum_7, cecum_47, cecum_68, cecum_98 and cecum_107; cecum_16, cecum_17 and cecum_18; cecum_12, cecum_27, cecum_41, cecum_71 and cecum_90; feruloyl agmatine, feruloyl putrescine and cecum_129; trans-3-indoleacrylic acid and tryptophan; cecum_85, Leu-Phe, isoleucine, indoleacetaldehyde, tyrosine and N-benzylformamide; N-benzyl-N-isopropyl-5-methyl-3-phenyl-4-isoxazolecarboxamide and cecum_59; thiamine and 4-methyl-5-thiazoleethanol.

There were also 6 groups of compounds exhibiting lower than −0.9 negative correlations. These included cecum_7 and tryptophan; cecum_106 and citrulline; cecum_21 and cecum_92; xanthine and cecum_121; Val-Pro with negative correlation toward compounds cecum_12,

Figure 4. Correlation of compounds with assigned chemical names and top compounds with mass spectrometry characteristics only. Two clusters without extensive mutual correlation were formed within compounds present in cecal digesta of control chickens (all within blue box). Tyramine, phenylacetaldehyde, glutamate, glutamine, betaine, or schaftoside formed the first cluster while caffeoyl putrescin, soyasaponin I, soyasaponin Ba, carnitine, and 3-methyladenine formed the second one. Inosine, tyramine, phenylacetaldehyde, glutamate, pantothenate, and methyladenosin negatively correlated with compounds characteristic for digesta of anaerobe chickens but positively correlated with compounds characteristic for AVIGUARD-treated chickens (black box) indicating that bacteria specific for anaerobe mixture, that is, *Phascolarctobacterium, Succinatimonas*, and *Desulfovibrio*, could be associated with their degradation. Daidzein, glycitein, genistein, feruloyl agmatine, feruloyl putrescine, O-acetylcarnitine, and dihydrobiopterin negatively correlated with compounds characteristic for digesta of AVIGUARD-treated chickens suggesting that their degradation was dependent on AVIGUARD-specific microbiota, that is, gram-positive Actinobacteria or Firmicutes. Pink box, compounds characteristic for anaerobe-treated chickens. Gray box, compounds characteristic for digesta of AVIGUARD-treated chickens.

cecum_41 cecum_71 and cecum_90; cecum_15 with negative correlation toward compounds genistein, feruloyl agmatine, feruloyl putrescine, cecum_129, cecum_119 and cecum_117 (Supplementary Table S4).

DISCUSSION

More than 600 compounds discriminated between control and experimental groups. Such findings show that it is possible to modulate the profile of biologically active compounds in the gut by a combination of microbiota and feed composition. Perhaps surprisingly, it is not simple to conclude why particular molecules increased in their abundance. In most of the cases, additional digestion capacity followed by a release of new feed ingredients into digesta associated with chicken colonization with additional bacterial species can explain increased abundance. However, newly introduced bacterial species can also degrade particular molecules which then decrease in abundance. Finally, chicken host may release its molecules in cecum lumen and resorption of small molecules by a chicken may also considerable affect abundance of low molecular weight compounds in digesta.

The abundance of amino acids, nucleotides, lipids, and derivates of these compounds differed among the groups. Glutamate, glutamine, tyramine, 3-methyladenine, and phenylacetaldehyde dominated in the digesta of control chickens and were less abundant in the digesta of AVIGUARD or anaerobe mixture colonized chickens. The most likely explanation is that i) the digestive activity of chickens is enough for their release from feed, ii) these compounds are present in the feed in an amount exceeding the requirements of the chicken and iii) bacterial species present in the AVIGUARD or anaerobe mixture degrade these compounds further.

Glutamate and glutamine are the most common amino acids in soya and wheat proteins (Hou et al., 2019) explaining their high abundance in digesta. Glutamate significantly increased in cecal digesta also following depletion of cecal microbiota by antibiotic therapy during which *Bacteriodes, Olsenella, Desulfovibrio*, or *Megasphaera* decreased and *E. coli* increased (Zhang et al., 2021), that is, similar modifications in microbiota composition as we observed. Finally, glutamate dehydrogenase is highly expressed by *Bacteroides* in vivo (Polansky et al., 2015), all indicating that an increased abundance of *Bacteroides* results in a decrease of glutamate in cecal digesta. This is also in agreement with the highest negative correlation between glutamate and *Bacteroides* recorded in this study. Since glutamate is a neurotransmitter (Mortezaei et al., 2013), its higher abundance in digesta of control chickens may have consequence for host behavior. Sources of 3-methyladenine is less clear. Correlation analysis indicated that it may belong among metabolites released from soya but there are no reports on its presence in soya. Interestingly, 3-methyladenine is a biologically active compound inhibiting autophagy (Fronza and Gold, 2004; Li et al., 2023).

The remaining amino acids, nucleotides, lipids and their derivatives dominated in the digesta of AVIGUARD or anaerobe chickens. Their presence can be associated with the degradative function of microbiota itself. *Bacteroides* species are known to degrade complex polysaccharides by extracellular cellulosomes (Ponpium et al., 2000; Polansky et al., 2015; Medvecky et al., 2018). The degradation of the plant cell wall followed by spontaneous lysis of cell wall-deficient plant cells as documented by increased abundances of 1-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine, 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine, 1-hexadecanoyl-2-octadecadienoyl-sn-glycero-3-phosphocholine, and sphingosine (d18:1), all lipid compounds from the cytoplasmic membrane, may result also in the release of nucleotides and amino acids, followed by immediate fermentation of amino acids to metabolic by products as shown by the positive correlation for tyrosine and N-benzylformamide, or tryptophan and indolacrylic acid. Purines being commonly present in the chicken intestine was already observed (Chen et al., 2020; Park et al., 2020) and the fact that adenine was the most abundant compound of all nucleotides can be influenced by the fact that adenine can be released from DNA, RNA and also from free ATP. The presence of excess amino acids and nucleotides in AVIGUARD and anaerobe-colonized chickens may permit a decrease in the nutrient contents of chicken feed if the chicks are colonized by complex microbiota from the first days of life.

Soyasaponins, betaine, carnitine, deoxycarnitine, caffeoyl putrescin, or 6′-O-malonylgenistin, since common in digesta of control chickens, represent compounds that are likely released into the digesta by the degradative activity of chickens. Since these compounds were absent from experimental chickens, bacterial species present both in AVIGUARD and anaerobe mixture, that is, *Bacteroides, Megamonas, Megasphaera, Olsenella*, or *Sutterella*, could metabolize these compounds further.

Isoflavonoids daidzein, genistein and glycitein, and phenolamines feruloyl putrescine and feruloyl agmatine were the most abundant in anaerobe chickens and the least abundant in AVIGUARD-treated chickens. This is suggestive of their release from plant components of the feed by microbiota present both in anaerobe and AVIGUARD followed by additional degradation by microbiota present only in AVIGUARD-treated chickens. Interestingly, these compounds were negatively correlated with cecum_15 compound. Cecum_15 compound therefore might be of structural function and its degradation results in simultaneous deliberation of genistein, feruloyl putrescine, and feruloyl agmatine into the cecal digesta. Since all these compounds are biologically active (Azzam et al., 2019; Killiny and Nehela, 2020; Cheng et al., 2022), colonization of chickens by microbiota of specific composition may allow to control isoflavonids and phenolamines in digesta.

Different variants of carnitine were the least abundant in AVIGUARD chickens suggesting that microbiota members differentiating these chickens from the other 2 groups could metabolize carnitine. None of microbiota members present in control chickens could efficiently utilize carnitine and this remained highly represented in the digesta. Microbiota present in anaerobe chickens could not degrade carnitine but modified carnitine by acetylation or propionylation which resulted in an increase of acetyl- and propionylcarnitine with a concomitant decrease in carnitine. The benefits of carnitine in poultry production have been studied with contradictory results. Although there are reports on the positive role of carnitine for growth promotion, improving the immune system or antioxidant function (Bhatti et al., 2018; Jahanian and Ashnagar, 2018; Rouhanipour et al., 2022), others reported a minor effect (Yousefi et al., 2023).

Finally, there were 2 compounds, 4-oxododecanedioic acid dominating in the digesta of experimental chickens and phenylacetaldehyde dominating in control chickens, both having a relationship to insects. Phenylacetaldehyde is common in flowers where it acts as an insect attractant (Batallas and Evenden, 2023). Microbiota composition affecting phenylacetaldehyde levels in the digesta and fecal material can therefore affect transmission of gut microbiota by insects. Since *Salmonella* efficiently colonize young chickens with microbiota similar to that in control chickens in this study (Beal et al., 2004; Crhanova et

al., 2011), fecal phenylacetaldehyde may support the spread of *Salmonella* to new hosts by insects. On the other hand, 4-oxododecanedioic acid is toxic to *Spodoptera exigua* larvae (Rivero et al., 2021). If this compound is toxic also to larvae of coprophagic insect species, its presence may decrease the spread of microbiota from animals with well-established microbiota.

CONCLUSIONS

Rather unexpectedly, untargeted metabolomics was able to distinguish clearly between different probiotic treatments. A high number of molecules were differentially abundant in cecal digesta of differently colonized chicks. Although the majority of compounds present in the digesta remained identified only by their mass spectrometry characteristics, some of them will appear in the future as of importance for microbiota-chicken interactions and some of them can be used as markers of probiotic colonized chickens even now. In addition, the low number of compounds identified by their chemical names allowed us to conclude that microbiota composition affects the presence of biologically active compounds like glutamate, tyramine, kynurenate, isoflavonoids, phenolamines, nucleotides, amino acids, and vitamins. This permits the use of bacterial mixtures of defined composition to control the abundance of the desired compounds in the cecal digesta, which has a direct effect on chicken performance. There is also no reason why a similar approach cannot be applied to other animal species, including humans.

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DISCLOSURES

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

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.103217.

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