Abrupt Temporal Fluctuations in the Chicken Fecal Microbiota Are Explained by Its Gastrointestinal Origin

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One of the main challenges in understanding the composition of fecal microbiota is that it can consist of microbial mixtures originating from different gastrointestinal (GI) segments. Here, we addressed this challenge for broiler chicken feces using a direct 16S rRNA gene-sequencing approach combined with multivariate statistical analyses. Broiler feces were chosen because of easy sampling and the importance for pathogen transmission to the human food chain. Feces were sampled daily for 16 days from chickens with and without a feed structure-induced stimulation of the gastric barrier function. Overall, we found four dominant microbial phylogroups in the feces. Two of the phylogroups were related to clostridia, one to lactobacilli, and one to *Escherichia/Shigella*. The relative composition of these phylogroups showed apparent stochastic temporal fluctuations in feces. Analyses of dissected chickens at the end of the experiment, however, showed that the two clostridial phylogroups were correlated to the microbiota in the cecum/colon and the small intestine, while the upper gut (crop and gizzard) microbiota was correlated to the lactobacillus phylogroup. In addition, chickens with a stimulated gizzard also showed less of the proximate GI dominating bacterial group in the feces, supporting the importance of the gastric barrier function. In conclusion, our results suggest that GI origin is a main determinant for the chicken fecal microbiota composition. This knowledge will be important for future understanding of factors affecting shedding of both harmful and beneficial gastrointestinal bacteria through feces.

The main transmission route for both malign and benign gut bacteria is through feces. Most of our current knowledge on the intestinal microbiota derives from studies of feces (10–12, 20, 25, 39, 40). A major challenge in understanding the ecology of the fecal microbiota, however, is that it can be composed of bacteria from different segments within the gastrointestinal (GI) tract (3, 7, 13). It is therefore highly important to understand what determines the composition of the fecal microbiota.

The aim of this work was to investigate the underlying mechanisms for temporal fluctuations of the fecal microbiota using chicken as a model system. We addressed two opposing hypotheses. The first is that the temporal fluctuations in the fecal microbiota reflect fluctuations in the gut microbiota, while the second is that fluctuations reflect variable discharge from different gut segments. Chicken gut comprises three upper gut segments, the crop, and the gizzard, in addition to the stomach. The crop is a food storage and fermentation organ, while the gizzard is used for grinding the food. The lower gut consists of the small intestine, the colon, and two large cecal fermentation chambers. The rationale for choosing chickens is the easy sampling and importance for pathogen transmission to humans (8). The shedding of gut bacteria is also important for understanding the transmission of both beneficial and harmful bacteria among individual chickens (41). Furthermore, it is also well known that it is possible to modify the gut physiology through feed structure, enabling the study of physiological effects on the gut microbiota composition (32). On the practical side, the relevance of understanding the composition of the fecal microbiota is related to the use of feces for diagnostic purposes (17).

Our analytical approach comprised direct sequencing of mixed 16S rRNA gene sequences combined with multivariate statistical analyses.

The direct sequencing of mixed 16S rRNA gene pools is a method recently developed (37, 42). The principle of the method is to generate mixed-sequence spectra based on a sequencing primer in a conserved region of the gene. These mixed sequences represent signatures for the dominant bacteria in the sample, which can be used for the subsequent downstream multivariate statistical analyses.

To identify the number of main fecal bacterial phylogroups, we utilized calibration-free approaches. We have previously demonstrated the use of a calibration-free multivariate technique, multivariate curve resolution with alternating least squares (MCR-ALS), on defined bacterial mixes where we obtained good resolution of 7 sequences in a simplex-lattice mixture design (42). Here, we applied this approach on fecal samples. By utilizing MCR-ALS, we were able to decompose a complex ecological system into a small number of important bacterial phylogroups (components) that vary among the samples. To investigate the origin of the MCR-ALS components derived from fecal samples, we compared these with sample spectra from different intestinal segments. A schematic outline of the analytical setup is given in Fig. 1.

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FIG 1 A schematic representation of data analysis. Spectra ($n = 625$) obtained by direct sequencing on samples were divided into data subsets depending on their origin. The dominating spectral profiles (components) from 492 samples (242 duplicated and 8 single fecal samples) were extracted using the MCR-ALS method. The number of available duplicates for each set of samples is given in parentheses. The 16S rRNA gene clone library of 1,903 clones and their spectra was downsized to the 101 most unique spectra. Correlation coefficients and coefficients of determination (r^2) were calculated between the extracted feces components and spectra from samples with different gut segment origins. The feces components were regressed to spectra from the most unique clones using PLSR. Sequences from unique clones that were significant in PLSR were identified using the closest match with the RDP10 database of 16S rRNA gene sequences.

We present results showing that apparent stochastic fluctuations in fecal microbiota composition can be explained by different sites of origin within the GI tract. Furthermore, we also present evidence supporting that the composition structure is affected by the diet-induced change in gut physiology (5).

MATERIALS AND METHODS

Sample collection and lab analysis. (i) Chicken and diets. Eighteen chickens were divided into 2 diet groups containing either coarse or fine Brewer's spent grain (BSG) and studied over a period of 16 days. Details regarding diets, bird management, and sampling are given elsewhere (5). Feces samples from each chicken were collected once a day. Droppings were fixed directly into isopropanol (85%) in clean trays placed under the cages. At day 17, the birds were killed by carbon dioxide asphyxiation, and the entire gastrointestinal (GI) tract was dissected out. A small sample (0.5 to 1 g) from each GI segment (crop, gizzard, cecum, and colon) was taken directly into 15-ml plastic tubes containing 10 ml isopropanol and kept at -20°C until analysis.

(ii) DNA extraction. DNA extraction of chicken feces and intestine contents was carried out according to a validated optimized automated protocol (30). Two DNA extractions were done for each fecal sample (technical replicate). Prior to extraction, the samples were vortexed for 10 s, and 0.5 ml of the contents was centrifuged for 10 min at 13,000 rpm. The supernatants were discarded and the pellets were further used in the protocol.

(iii) Direct 16S rRNA gene sequencing. The direct 16S rRNA gene-sequencing approach was performed on total DNA isolated from all samples (feces and intestinal contents), including on the technical replicates from the DNA extraction. PCR amplification of the 16S rRNA gene was

done using a primer set (5'-TCC TAC GGG AGG CAG CAG T-3' [Mangala Forward] and 5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3' [Mangala Reverse] [23]) binding to generally conserved regions of the 16S rRNA gene, providing a mixture of 16S rRNA genes. The relatively short amplicon (~ 470 nucleotides [nt]) is a tradeoff between robustness of the PCR and the phylogenetic information gained (26). The PCR mixture contained 1 U polymerase activity (DyNAzyme II Hot Start DNA polymerase; Finnzymes), 200 μM deoxynucleoside triphosphate (dNTP) mix, 0.2 μM each primer, 5 μl of total DNA in a total volume of 25 μl . The amplification profile was a 10-min activation step at 94°C followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The reaction was terminated with a 7-min elongation step at 72°C .

Prior to sequencing, the mixtures of 16S rRNA gene PCR products (5 μl) were cleaned for excess nucleotides and primers with 0.4 μl of Exo-Sap-IT (USB Corporation) at 37°C for 30 min followed by an inactivation step at 80°C for 15 min. The DNA sequencing reaction was carried out using the BigDye Terminator v1.1 cycle sequencing kit and universally conserved primer U515Fc30 (29) as a sequencing primer, in 25 cycles of 96°C for 15 s and 60°C for 4 min. Purification of the DNA sequencing products was performed using BigDye XTerminator purification kit (Applied Biosystems) according to the instructions supplied by the manufacturer. Sequencing was carried out using an ABI PRISM 3100 genetic analyzer (Applied Biosystems), revealing mixed-sequence spectra correlating to the complexity of the microbial diversity.

(iv) Cloning and sequencing of 16S rRNA gene libraries. Eleven samples of both feces and GI origin were selected for further in-depth analysis (selection criteria are shown in the supplemental material, in the section entitled "Acquisition of pure bacterial spectra"). Clone libraries of 16S rRNA genes were generated and sequenced, obtaining one DNA spectrum

for each bacterial clone. Amplification of the 16S rRNA gene libraries was done using the PCR protocol used in a direct-sequencing approach. The resulting PCR mixtures were cloned into the plasmid vectors supplied with the TOPO TA cloning kit (Invitrogen), and TOP 10 One Shot chemically competent cells (Invitrogen) were subsequently transformed with the TOPO cloning reactions, all according to manufacturer's recommendations. Positive colonies were resuspended in 50 μ l of TE buffer and heated to 99°C for 10 min, and the cell debris were removed by centrifugation at 13,000 rpm for 3 min. Cell extracts (30 μ l) were transferred to new tubes containing the plasmid. The inserts of 16S rRNA in the clone libraries were PCR amplified with vector-specific primers HU 5'-CGC CAG GGT TTT CCC AGT CAC GAC G-3' and HR 5'-GCT TCC GGC TCG TAT GTT GTG TGG-3'. The PCR mixture contained 2 U DNA polymerase (DyNAzyme II; Finnzymes), 200 μ M dNTP mix, 0.2 μ M each primer, 1 μ l of cell extract in a total volume of 25 μ l. The amplification profile was 94°C for 4 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s. The reaction was terminated with a 7-min elongation step at 72°C.

(v) Sequencing of the 16S rRNA gene PCR products. Sequencing of the pure 16S rRNA gene PCR products was conducted in a manner similar to that used in the direct 16S rRNA gene-sequencing approach, but with Mangala Forward primer as the sequencing primer.

Data preprocessing. Each 16S rRNA gene sequence (both mixed and pure) was pairwise aligned to the 16S rRNA gene sequence of *Escherichia coli* using the Smith-Waterman algorithm (31). Common start and end positions were found for all sequences. The sequences and spectra were trimmed to comprise this common region, e.g., between positions 604 and 785 in the *E. coli* 16S rRNA gene. The spectra were corrected for shifts using correlation-optimized warping (36) and normalized by closure. A detailed description of data acquisition, normalization, and alignment/warping is described elsewhere (42).

Data analysis. A schematic outline of the data analytical setup is given in Fig. 1.

(i) PCA and MCR-ALS on mixed 16S rRNA gene spectra. The mixed and undefined bacterial sequences were matched to sequences in the Ribosomal Database Project (RDP10) (4) and the closest match was used to define the signature sequence of the dominant bacteria. PCA and MCR-ALS (see the supplemental material for a detailed explanation) were applied on the mixed-sequence spectra from crop, gizzard, ileum, cecum, colon, and feces samples to reduce the complexity and to explore the variation and dynamics of bacteria in the data set. We used the Matlab version 7.5.0 (MathWorks, Inc., Natick, MA) programming environment together with MCR-ALS implementation described here (15). The MCR-ALS constraints we used were nonnegativity (nnls) for both concentration and spectral profiles and closure (equal to 1) for concentration profiles. Other parameters were kept as default. The differences in the estimated bacterial composition between samples obtained from chicken fed with fine/coarse BSG were investigated by comparing their distributions and verified by the permutation test. The main components from feces samples were derived using MCR-ALS. In particular, we were interested in investigating how the different MCR-ALS components extracted from feces spectra correspond to the spectra obtained from analysis of the five inner organ samples. The coefficients of determination (r^2) between fecal components and samples from the five GI segments were used to indicate similarity. Then multiple comparison tests were performed using multi-compare and anova2 (two-way analysis of variance) functions in Matlab to investigate whether the highest mean r^2 (to a certain feces component) is significantly higher than mean r^2 to any other feces components at a 0.05 significance level.

The MCR-ALS components for feces were regressed onto unique pure bacterial spectra which were obtained by 16S rRNA gene cloning of a selection of samples and subsequent sequencing. This was done in order to identify bacteria that were dominant and responsible for the main variation in feces samples and consequently in the different GI segments.

(ii) Selection of the most unique clones from 16S rRNA gene clone libraries. We trimmed the data set of 1,903 spectra extracted from 16S rRNA clone libraries to 101 of the most distinctive clones. The selection of unique pure spectra, described in more detail in reference 24, was done by performing PCA on all 1,903 spectra and dividing the PCA plot in grid with intervals of size 0.05. One random spectrum was selected from each interval that contained over five sequences.

(iii) Regression between MCR-ALS-extracted spectral profiles and pure spectra from 16S rRNA clone libraries. In order to estimate the composition of MCR-ALS-extracted spectral profiles from feces samples, a regression analysis using the multivariate method called partial least-squares regression (PLSR) (see the supplemental material for an introduction) was performed between pure and MCR-ALS spectral profiles. The data were centered and leave-one-out cross-validation was used for the validation. Uncertainty and significance of regression coefficients were estimated using a jack-knifing technique (21). Only bacteria with significant regression coefficients above a certain threshold value were selected to describe the mixed bacterial spectral profile. The threshold was chosen by visual inspection of histograms of regression coefficient values.

(iv) Phylogenetic tree construction. Evolutionary relationships of selected bacterial sequences were inferred using the neighbor-joining method (27). The consensus tree was constructed after 500 replications (9) and taken to represent the evolutionary history of the bacterial species analyzed (9). The percentages of replicate trees in which the associated bacterial species clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method (34) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (33).

(v) Validation of results. To validate the results, leave-one-out cross-validation was used in PCA and PLSR. This procedure repeats the calibration/validation of a model several times, each time using one sample as a test set and the rest of the data as a training set. The robustness and stability of MCR-ALS results were investigated in a more explorative manner by using different initial values and different numbers of components. In addition, MCR-ALS was performed on 5 subsets constructed by removing 10% randomly selected samples from the original data set. The corresponding extracted components were compared to each other by calculating Pearson's correlation. To test hypotheses concerning the difference in bacterial abundance between chickens fed with fine BSG and coarse BSG, we used the permutation test. Ten thousand new data sets were created by randomly assigning the two feed structure types (fine/coarse) to each sample, and the results were compared to the experimental values. The null hypothesis is rejected at a P value of <0.01 , i.e., if the experimental values are observed in the simulated data set <100 times ($0.01 \cdot 10,000$).

Nucleotide sequence accession numbers. A reference library of 101 of the most unique 16S rRNA gene clone sequences has been deposited into the NCBI nucleotide database under accession numbers [HQ629960](#) to [HQ630060](#).

RESULTS

Identification of the dominant bacterial components (phylogroups) in the fecal microbiota. The spectra obtained from sequencing directly on 16S rRNA gene mixtures from feces were resolved in five main components (Fig. 2) covering most of the variation (cumulative fit of 66.23%) among the samples. MCR-ALS results for four and six components did not differ considerably from results using five (results not shown). We chose to use the five-component model, since most of the biologically nonrelevant information was collected in the fifth component, seen as a noisy spectrum and a nonsense DNA sequence. To validate the model, MCR-ALS was performed on 5 different data subsets where 10% randomly selected samples were removed each time.

FIG 2 Five components extracted from 492 feces samples using MCR-ALS. The four nucleotide spectra are plotted on top of each other marked with different colors. The overlapping peaks indicate the presence of different bacteria. x axis, migration time (ms); y axis, signal intensity (relative fluorescence units [rfu]).

The components from each submodel showed high reproducibility and stability compared to the components from the main model ($r^2 > 0.99$).

In order to decipher the extracted MCR-ALS components, we utilized pure bacterial spectra from 16S rRNA gene clone libraries of 11 samples (see the supplemental material for details). We downsized the clone libraries from 1,903 to the 101 most distinctive clones (DNA spectra), i.e., one representative for each closely related bacterial phylogroup. The results from PLS regression analysis between 101 pure spectra and components 1, 2, 3, and 4 extracted from feces revealed that only a few highly abundant distinctive pure spectra were important for each component (Table 1). The PLS regression result, however, was rather poor for component 5 ($r^2 < 0.6$), suggesting that this component includes other biological information that is unresolved due to technical limitations of the approach. The nucleotide sequences of bacterial clones that were found to be important for components 1 to 4 were matched in the RDP10 database, and the results are shown in Table 1. A phylogenetic tree of 16S rRNA gene sequences from Table 1 is shown in Fig. 3. Four distinct clusters were formed, one for each MCR-ALS component-specific phylogenetic group. The clusters for components 2 and 4 represented more diverse phylogenetic groups containing sequences with the longest evolutionary distances. *Escherichia coli* and *Shigella* were the most distantly related to the other bacterial clones in the phylogenetic tree.

Finally, we tested the reproducibility of the component (phylogroup) quantification. The estimated proportions of the components were compared between 242 replicated DNA purifications from the samples. The concentrations of all components except 5 showed good reproducibility ($r^2 \geq 0.94$) (Table 2).

Comparisons between samples from feces and gastrointestinal segments. The differences in bacterial composition between crop, gizzard, ileum, cecum, and colon were investigated by performing PCA on the mixed spectra (Fig. 4). We can see a clear

separation between ileum, cecum/colon, and gizzard/crop samples. The two latter groups were difficult to separate further. Based on classification results of consensus sequences from mixed samples using the RDP10 database, we found that the cecum/colon group harbored diverse microbiota, most of them having the closest match to unclassified *Clostridiales* and unclassified *Lachnospiraceae* in the RDP10 database, while gizzard/crop and ileum samples were dominated by lactobacilli and *Clostridiaceae*, respectively.

In order to investigate whether fecal microbiota is a distinctive group or contains similar groups related to other segments, we mapped spectra from feces samples onto the PCA model of samples from other segments. We did not find any distinct clustering of feces samples (results not shown). The five components from the fecal samples were therefore compared to DNA spectra measured on samples collected directly from crop, gizzard, ileum, cecum, and colon at the day of dissection. Figure S5 in the supplemental material summarizes the comparisons. Component 2 derived from fecal samples correlated significantly higher than other components with the samples from cecum and colon samples. Component 4 correlated highest with crop and gizzard samples. Component 3 was highly correlated to the ileum samples from one chicken ($r^2 = 0.98$ and 0.94), while the ileum sample from the other chicken showed relatively low correlation ($r^2 < 0.76$) to the fecal components. The first and the fifth components had poor similarity with samples originating from the aforementioned chicken organs.

Impact of feeding structure on microbial composition. From the estimated concentrations of MCR-ALS components 1 and 4, we observed that the chickens fed with fine BSG exhibited higher abundance of these components than chickens given coarse BSG (see Fig. S3 in the supplemental material). The results were validated by permuting the data set 10,000 times and calculating the abundance of the components. The permutation test showed that

TABLE 1 Overview of the composition of the MCR-ALS-extracted components^a

Component	Accession no.	Best match in RDP10 (order/family)	Seq ^b count	% of total (n = 1,903) sequences	Regression coefficient
Component 1 ($r^2 \approx 0.85$)	HQ630060	<i>Escherichia coli/Shigella</i>	6	0.3	0.1889
Component 2 ($r^2 \approx 0.92$)	HQ630057	<i>Blautia</i>	27	1.4	0.018
	HQ630039	<i>Coprococcus</i>	6	0.3	0.0182
	HQ630020	<i>Coprococcus</i>	8	0.4	0.0186
	HQ629999	<i>Coprococcus</i>	6	0.3	0.019
	HQ630021	<i>Coprococcus</i>	10	0.5	0.0202
	HQ630048	Unclassified <i>Clostridiales</i>	20	1.1	0.018
	HQ630054	Unclassified <i>Clostridiales</i>	44	2.3	0.0181
	HQ630006	Unclassified <i>Clostridiales</i>	10	0.5	0.0187
	HQ630029	Unclassified <i>Clostridiales</i>	14	0.7	0.0189
	HQ630053	Unclassified <i>Clostridiales</i>	9	0.5	0.0189
	HQ630026	Unclassified <i>Clostridiales</i>	15	0.8	0.0204
	HQ630033	Unclassified <i>Clostridiales</i>	6	0.3	0.0211
	HQ630034	Unclassified <i>Clostridiales</i>	9	0.5	0.0214
	HQ630007	Unclassified <i>Lachnospiraceae</i>	7	0.4	0.0192
	HQ630001	Unclassified <i>Lachnospiraceae</i>	10	0.5	0.0208
Component 3 ($r^2 \approx 0.9$)	HQ629966	<i>Clostridiaceae</i> 1	6	0.3	0.0473
	HQ629967	<i>Clostridiaceae</i> 1	6	0.3	0.0508
	HQ629968	<i>Clostridiaceae</i> 1	6	0.3	0.0515
	HQ629972	<i>Clostridiaceae</i> 1	11	0.6	0.055
	HQ629986	<i>Clostridiaceae</i> 1	10	0.5	0.0569
	HQ629974	<i>Clostridiaceae</i> 1	9	0.5	0.0576
	HQ629981	<i>Clostridiaceae</i> 1	17	0.9	0.0635
	HQ629982	<i>Clostridiaceae</i> 1	37	1.9	0.064
	HQ629987	<i>Clostridiaceae</i> 1	26	1.4	0.0646
	HQ630034	Unclassified <i>Lachnospiraceae</i>	9	0.5	0.0527
Component 4 ($r^2 \approx 0.91$)	HQ629962	<i>Lactobacillus</i>	11	0.6	0.0402
	HQ629965	<i>Lactobacillus</i>	10	0.5	0.0408
	HQ629979	<i>Lactobacillus</i>	6	0.3	0.0427
	HQ629963	<i>Lactobacillus</i>	6	0.3	0.0433
	HQ629964	<i>Lactobacillus</i>	10	0.5	0.0434
	HQ629978	<i>Lactobacillus</i>	6	0.3	0.0448
	HQ629980	<i>Lactobacillus</i>	8	0.4	0.0475
	HQ629977	<i>Lactobacillus</i>	14	0.7	0.0481
	HQ629970	<i>Lactobacillus</i>	8	0.4	0.0483
	HQ629976	<i>Lactobacillus</i>	19	1.0	0.0492
	HQ629971	<i>Lactobacillus</i>	10	0.5	0.0515
	HQ629975	<i>Lactobacillus</i>	6	0.3	0.0516
	HQ629973	<i>Lactobacillus</i>	8	0.4	0.0531

^a Phylogroups were determined according to the closest match in RDP10 database. Bacterial names in boldface are the bacteria with highest regression coefficient values for the specific component.

^b Seq, sequence.

there are significantly ($P < 0.01$) more samples containing lactobacilli (MCR-ALS component 4) and *Escherichia coli* and *Shigella* (MCR-ALS component 1) from chickens fed with fine BSG.

Temporal fluctuations. The investigation of temporal development of relative abundance of the five MCR-ALS components isolated from the feces samples showed rather intricate fluctuations within each chicken (see Fig. S4 in the supplemental material), and the overall temporal trends were quite weak (see Table S1). An interesting observation occurred at day 3, where feces samples from two chickens showed excess of *E. coli/Shigella* (see Fig. S2), but already by the next day the abundance of this group had decreased markedly. Even though it emerged and dominated again on day 12 in chicken 6-18, it almost completely disappeared the following day.

DISCUSSION

There was little continuity in microbial development in feces over time in each chicken. This observation contrasted the microbiota components from the different GI segments, which were relatively consistent among the individual chickens analyzed. The mapping of fecal samples onto the PCA model of samples from different GI segments did not give any clear distinction of fecal samples. Hence, fecal samples were the direct result of varying contributions of microbiota from different GI segments. Similar results were observed by calculating correlations between microbiota in fecal samples and the microbiota in the different GI segments. Thus, to explain the underlying mechanisms for temporal variation in fecal microbiota, we propose that the temporal shifts in the

FIG 3 Phylogenetic tree illustrating the relationship of selected bacterial sequences with significant and high regression coefficients in regression between pure bacterial spectra and MCR-ALS extracted spectral profiles (components) from mixed feces. The best match to RDP10 regarding the order/family level is indicated in the parentheses. The number of bootstrap replicates (>0.5) that support each branch (500 replicates in total) is shown on nodes.

fecal microbiota were caused by the discharge of microbiota from different GI segments. It is known that chicken can excrete the cecum content two or three times each day, but still very little is known about the physiology of excretion from other GI segments.

The above interpretation that fluctuations in the feces microbiota in chicken are varying mixtures of otherwise relatively stable microbiota from different GI segments could be important with respect to the ongoing search for the presence of a universal core microbiota in different organisms, since most studies uses feces as a proxy for the gut microbiota (19, 28, 35, 38). To our knowledge, no other studies so far have investigated the correlation between the dominant luminal bacteria in various GI segments and the temporal fluctuations in the fecal microbiota composition, although there have been several studies showing momentary differences (6, 7). Given variable discharge from different GI segments in other metazoans as proposed for chickens, microbial

core phylogroups could have been overlooked in analyses of feces. In humans and other mammals, however, the variable discharge is probably not as pronounced as for chickens. A study of the gut microbial composition and its stability over time in elderly people, however, also indicates temporal variability of the human fecal microbiota (2). Whether this represents discharge from different gut segments remains to be determined.

Lactobacilli along with *Proteobacteria* subgroup *E. coli* and *Shigella* were most abundant in the feces of chickens fed with the fine BSG. In addition, according to our analysis lactobacilli were most dominant in crop and gizzard. This suggests that chickens fed with coarse BSG have a stronger barrier for releasing the lactic acid bacteria through the digestive system than for those fed with fine BSG. This interpretation is in accordance with previous studies showing that feed structure stimulates the gizzard barrier function (1, 18).

Component 1 (*Shigella/E. coli*) is of particular interest due to pathogen transmission (14, 16). This group is particularly important with the emergence of pathogenic *E. coli* strains with the ability to colonize both humans and chickens (22).

The microbial compositions of the two chickens having high contents of *Shigella/E. coli* at day 3 seemed to undergo a remarkable change the day after (see Fig. S2 in the supplemental material). Although we could not link this component to a specific GI segment, it is possible that it also has a reservoir within the GI tract. The abrupt changes in discharge of these bacteria pose a particular challenge with respect to food safety and monitoring schemes.

TABLE 2 Coefficient of determination determined on the bases of 225 replicated samples for all five MCR-ALS components extracted from feces samples

Component	Coefficient of determination (r^2)
Component 1	0.97
Component 2	0.94
Component 3	0.98
Component 4	0.96
Component 5	0.57

FIG 4 A PCA score plot showing separation of samples isolated from 5 different gut segments in chicken. The main MCR-ALS component extracted from samples in each circle is highly correlated to one of the MCR-ALS components from feces samples. The latter component is indicated next to the circle.

In conclusion, we have demonstrated the application of a high-throughput multivariate approach for exploring and detecting GI segment-specific bacterial groups found in feces and identifying the predominant bacteria within these groups. These analyses have led to new insight for the underlying mechanisms directing the fecal microbiota composition in chicken.

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