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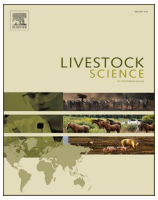

Factors Influencing the Succession of the Fecal Microbiome in Broilers

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HIGHLIGHTS

- Differences in performance parameters due to age and feed (and Sex).
- Age differences in fecal microbiota composition, i.e. succession of the microbiota, alpha- and beta-diversity.
- Two genera significantly different in feces of broilers between diets (EU vs USA), i.e. *Streptococcus* on day 7 and *bilophila* on day 21.
- Surprisingly, environment was significant for fecal microbiota composition, which represents two different compartments within the same stable.

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ABSTRACT

Health and performance are important aspects in the broiler industry. Underlying complex traits like total mean weight and feed efficiency are polygenic and related to genetic background and an association of the microbiota with these traits has been identified. Whether this association is also reflected in the fecal samples of broilers is not extensively investigated. The objective of this study was to investigate to what extend diet, genetics, and environment influence the fecal microbiome composition during the life time of broilers.

Two experiments were performed, in the first experiment the focus was on investigating if a European (EU) or United States (USA) diet effects the fecal microbiota in a commercial line (Cobb500). Whereas in the second diet (EU/USA) and lines with a genetic background (EU/USA) were investigated in relation to the fecal microbiota.

In the first experiment we observed a significant effect in commercial broiler line (Cobb500) of the 3-way interaction for *age* by *feed* by *sex* on Total Mean Weight (TMW), and the 2-way interaction of *age* by *feed* for Feed Conversion Ratio (FCR). For the microbiota data, we observed differences in alpha-diversity for Age. When comparing the diets on a time-point, this resulted in significant differences for Observed species at day 21 and for Observed species, Shannon index, and Pielou's evenness at day 35. In the beta-diversity, a significant effect of *age* by *feed* interaction was observed. Two genera were significantly different in feces of broilers between diets, i.e. *Streptococcus* on day 7 and *Bilophila* on day 21.

In the second experiment we observed only a significant effect for the main effect *age* on TMW. Alpha-diversity showed a significant increase for all three measures for *age*. Furthermore, a significant effect of *environment* was observed in the Observed species. This effect of *environment* was also observed in the beta-diversity, where a significant effect for *age* and *environment* was observed. This environmental effect was not expected, because here environment represents two different compartments within the same stable, unfortunately it was not possible to perform further down-stream analyses.

This research shows the different aspects (feed, sex, genetics, and environment) influence complex traits, like TMW and FCR and are affecting the fecal microbiome. We have shown that interventions, like feed and the effect on microbiome, are reproducible between experiments. Moreover, these results with these two genetic divers chicken lines suggest that the succession of the fecal microbiota was independent of genetic background.

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1. Introduction

Performance and health are important aspects of broiler production and has an economic impact on the broiler industry. Complex traits, like body weight (or total mean weight) (Tarsani et al., 2019) and feed efficiency (Reyer et al., 2015), are polygenic of nature and are of biological interest. Traditionally these traits have been studied solely from the host perspective (genetic markers / SNPs), however, in the last decade microbiome profile typing became more easily available. Recent studies have shown that different aspects can influence the microbiome composition in the small intestine (Feye et al., 2020), including age (Ballou et al., 2016a), sex (Lee et al., 2017), genetics (Schokker et al., 2015), feed (Engberg et al., 2002; Oakley et al., 2014), management (Wang et al., 2016), and environment, i.e. location (Siegerstetter et al., 2017) and housing system (Kers et al., 2018). Additionally, research has shown this association regarding the fecal microbiome composition and the influence of genetics (Diaz-Sanchez et al., 2019) and environment (Roto et al., 2016), as well as association to the Feed Conversion Ratio (Singh et al., 2012, 2014). Age plays a major role in shaping the microbiome composition. Immediately after hatch, the microbiome is being established and succession of different bacterial species will occur (Apajalahti et al., 2004; Schokker et al., 2015; Ballou et al., 2016b, 2017; Jurburg et al., 2019).

Supplements in diet and the administration route of feed (ingredients) can modulate the microbiome as is (Timmerman et al., 2006), as well as administrating a specific caecal inoculum (Yin et al., 2010). Such a caecal inoculum has shown to modulate the small intestinal microbiome composition and concurrently gene expression patterns in the small intestine, including cell cycle and ion transport processes (Yin et al., 2010). In conjunction, a detrimental effect can be observed when administering a therapeutic dosage of antibiotics in the first day of life, where up to two weeks the small intestinal microbiome composition is changed as well as a putative negative impact on immune system programming (Schokker et al., 2017). Collectively, these indicate that the microbiome composition of poultry are subject to modulation due to different aspects and are associated with immune development (health) and feed conversion (productivity) both (economic) important traits.

Modulating the microbiome with diet could affect intestinal functionality, i.e. immune function, morbidity through mechanisms that can alter nutrient absorption, intestinal health, and pathogen load. Meaning that a change of ingredients in diet due to as example a more circular economy will lead to a changed microbiome. The objective of this study was to investigate the effect on performance parameters and the fecal microbiome composition during the life time of broilers fed a typical European or United States diet in different genetic backgrounds. One advantage of investigating fecal microbiomes is noninvasive sampling methodology without sacrificing the birds. Another advantage could be that the fecal microbiome can serve as a proxies of known and unknown complex traits for (new) nutritional and breeding goals. However, controversy is still there whether or not the fecal microbiome is representative of the gastrointestinal tract microbiomes, it has been found that the fecal microbiome has a comparable diversity to that of the caecal microbiome (Oakley and Kogut, 2016).

2. Material and methods

2.1. Experimental animals

Two different experiments were conducted sequentially. The first experiment was to determine the effect of the chosen diets, i.e. European (EU) or United States of America (USA), on the fecal microbiome composition. The second experiment was to investigate to what extend genetics and environment (including diet and housing) influence the fecal microbiome composition of broilers.

2.2. Experiment 1

In the first experiment 5760 1-day-old chickens (Cobb500, both males and females) were housed in a facility with a closed system in one room, with a floor pen system with litter (wood shavings). Sexing was performed on one-day-old chicks by experts checking the feathers (primary and covert). The facility had 96 pens that each housed 60 birds. Where birds in 48 pens received the 'EU' diet and 48 pens received the 'USA' diet (see Supplementary File S1). A further subdivision in the pens was made based on sex, within each of the 48 pens, 24 were housed with females and the other 6 with males. Chickens had ad libitum access to feed and water.

Performance parameters, i.e. total mean weight (TMW; this is the weight of all chickens in a pen) and cumulative feed conversion ratio (FCR), for all 96 pens, were recorded at day 7, 21, 28, and 34, unfortunately day 14 was not measured because this was not feasible. For microbiome sampling, 24 pens were selected; six pens for EU female, six pens for EU male, six pens for US female, and six pens for US male. Per pen ten birds were randomly selected for microbiome sampling and these birds were followed in time. Fecal (cloacal) swabs (Swab Easy Swab Single from Brunschwig Chemie B.V., The Netherlands) were taken from these selected birds at day 1, 4, 7, 14, 21, 28, and 34. The first week was sampled more intensively because of the so called critical transition period. Because the performance parameter read-outs are per pen, the fecal swabs from the ten birds were pooled before sequencing. In this way it was possible to link the performance read-out with the microbiome compositions.

2.3. Experiment 2

The second experiment was performed later in time, in a different season than the first experiment. In the second experiment 2240 1-day-old chickens of either European or American genetic background (both males and females) were housed in a floor pen system with litter (wood shavings). Sexing was performed on one-day-old chicks by experts checking the feathers (primary and covert). A specific (Cobb) breed, was separately reared for 3–4 generations at each remote geographical location, i.e. US or EU. In our experiment we have used the fourth generation of either the US site (US genetics) or EU site (EU genetics). The same facility was used as in Experiment 1, however in this experiment two environments (i.e. two rooms in the same barn) within the facility were used. Both rooms used in this experiment are closed systems and with its own temperature and light regulation. The full experiment comprised of 112 pens, where 48 pens in environment (room) 1 housing 20 birds, and 64 pens in environment (room) 2 housing 20 birds (replicates). Furthermore, in experiment 2 both environments (rooms) had similar settings for temperature, light, shavings, and pen size, and each pen housed 20 birds. For microbiome sampling we have selected eight pens from both environments (total 16 pens). In each environment, two pens where involved a European genetic background and European feed, two pens with a European genetic background and USA feed, two pens with a USA genetic background and European feed, and two pens with a USA genetic background and USA feed. Chickens had ad libitum access to feed and water.

Performance parameters, i.e. TMW and FCR, were recorded at day 7, 21, 28, and 34. In addition, per pen ten birds were randomly selected for microbiome sampling and these birds were followed in time. Fecal (cloacal) swabs (Swab Easy Swab Single from Brunschwig Chemie B.V., The Netherlands) were taken from these selected birds at day 1, 7, 14, 21, 28, and 35. Because the performance parameter read-outs are per pen, the fecal swabs from the ten birds were pooled before sequencing. In this way we could link the performance read-out with the microbiome compositions.

2.4. Microbiome sequencing

Microbiota composition was determined for fecal swabs. Samples were frozen on dry-ice and stored at −80 °C until analysis. To isolate DNA, samples were mixed in a 1:1 ratio with PBS and centrifuged for 5 min at 4 °C at 300*xg*. Supernatant was collected and centrifuged for 10 min at 4 °C at 9000*xg*. DNA was extracted from the pellet using the "QIAamp DNA stool minikit" (Qiagen, Valencia, CA, USA) according to manufacturers' instructions, after mechanical shearing of the bacteria in Lysing Matrix B tubes (MP Biomedicals, Solon, OH, USA) using the FastPrep-24 for 3 times 30 s at a speed of 30 Hz (MP Biomedicals, Solon, OH, USA). Quality and quantity of DNA were checked using the NANOdrop (ND1000, Agilent Technologies, Santa Clara, CA, USA). PCR was used to amplify (20 cycles) the 16S rRNA gene variable-region (V3) fragment using forward primer V3_F (CCTACGGGAGGCAGCAG) and reverse primer V3_R (ATTACCGCGGCTGCTGG) (Schokker et al., 2018). PCR efficiency was checked on agarose gel. Amplicons were sequenced using paired-end sequencing, 2 × 150 bp technology on a MiSeq sequencer (Illumina, San Diego, CA, USA) at a sequencing depth with a median of 197 K read-pairs per sample and at least 50k read-pairs. One sample that did not pass the quality control was excluded.

2.5. Statistical analysis

2.5.1. Processing of performance data

For the performance data from study 1, i.e. TMW and FCR, we have performed the following linear model; $y \sim Age * Feed * Sex$, where *y* is [TMW or FCR], *Age* is [7, 21, 28, 34], *Feed* is [EU or US], and *Sex* is [Female or Male]. Thereafter an ANOVA was performed to test for statistical significance.

For the performance data from study 1, i.e. TMW and FCR, we have performed the following linear model; $y \sim age * genetics * feed * environment$, where *y* is [TMW or FCR], *age* is [7, 21, 28, 34], *genetics* is [EU or US], *feed* is [EU or US], and *environment* is [Room 1 or Room 2]. Thereafter an ANOVA was performed to test for statistical significance. All these analyses were performed within R (v3.6.1), using the *lm* and *anova* function from the *stats* package.

2.5.2. Processing of microbiome data

Sequence processing and statistical analyses were performed in R 3.6.1 (R Core Team, 2014). The amplicon sequences were filtered, trimmed, error-corrected, dereplicated, chimera-checked, and merged using the dada2 package (v.1.4.0, (Callahan et al., 2016)). By using the standard parameters except for *TruncLength*=140,100 and minOverlap=10, and reads were classified with the SILVA v.132 classifier.

For experiment 1, prior to analyses, the data were rarefied to 4326 per sample (no samples removed; *rarefy_even_depth*) with *set.seed* (111). The final dataset contained 1803 amplicon sequence variant (ASV). For experiment 2 also prior to analyses data were rarefied, i.e. to 8814 per sample (*rarefy_even_depth*) with *set.seed* (111) prior to analyses. And the final dataset contained 1658 ASVs.

Downstream analyses were performed with the *phyloseq* (version 1.28.0, (McMurdie and Holmes, 2013)), *microbiome* (1.6.0 (Lahti et al., 2017)), and *vegan* (version 2.5–7, (Oksanen et al., 2019)) packages.

Alpha-diversity measures were performed by *estimate_richness* and *evenness* functions of the phyloseq package. And beta-diversity measures were performed by the *ordinate* function (PCoA; bray) of the phyloseq package, followed by the functions *adonis* (phyloseq), *adonis.pair* (EcolUtils 0.1), and *betadisper* (phyloseq) at default settings. Further statistical testing for compositional differences was performed by *DESeq2* (v1.24.0), for this the dataset of experiment 1 was first filtered on detection (count above 228) and prevalent in at least 5% of the samples. Subsequently the significant genera were further filtered on their average relative contribution, i.e. above 0.01%, on each time-point in either the EU diet or the USA diet.

3. Results

3.1. Diet effects on performance parameters and microbiome composition

The descriptive statistics, i.e. the mean and standard error of the mean (SEM), of TMW and cumulative FCR per combination for *age* (d7, 21, 28, and 34), *feed* (EU/USA), and *sex* (Female/Male) are depicted in Table 1. This already shows differences in *age*, an increase of TMW and FCR, as well as the interaction with feed and *sex* where for example at day 34 the USA fed birds had a lower TMW compared to the EU fed birds. To test for statistical significance we performed a linear model, results are shown in Table 2 ($n = 96$ pens). Here we observed a significant effect was observed for the three-way interaction of *age* by *feed* by *sex* for TMW ($P = 0.016$). Whereas for the cumulative feed conversion ratio (FCR) only the two-way interaction of *age* by *feed* was significant ($P = 0.01$).

We first performed analysis on alpha- and beta-diversity on the microbiota data. Alpha-diversity measures showed a significant increase of observed species, Shannon index, and Pielou's evenness over time (Table 3). Because our prime interest was in the effect of *feed*, we compared the diets, i.e. EU vs. USA, per time-point. And significant results were observed at day 21 for Observed species and at day 34 for all three alpha-diversity measures, see also Table 3. A clear succession of the microbiota in time, i.e. the community turn-over, was observed. This was also observed when performing a permutational multivariate analysis of variance using dissimilarities (beta-diversity) on the microbiota data, this resulted in a significant effect for the two-way interaction of *age* by *feed* ($P = 0.001$, Fig. 1, Figure S1 Unweighted Unifrac and S2 Weighted Unifrac). The permutation test for homogeneity of multivariate dispersions was not significant. Thereafter, we performed a pairwise analysis for the multivariate analysis of variance using dissimilarities, this resulted in differences on day 1, 7, 21, 28, and 34, when comparing the diets, i.e. EU vs. USA. The next analysis was visualize these compositional differences on different taxa levels, i.e. phyla (Fig. 2) and (top 10 abundant) genera (Fig. 3). When testing for significant differences on a time-point between the diets (EU vs. USA) on the genera level, we observed only two genera, that have an average relative contribution above 0.01%. On day 7 this was *Streptococcus* (P_{adj}=0.01) and on day 21 *Bilophila* (P_{adj}=0.01) (Table 4).

Table 1
Descriptive statistics of experiment 1.

			Total Mean Weight		Feed Conversion Ratio	
Age	Feed	Sex[a]	Mean[b]	SEM[c]	Mean	SEM[c]
7	EU	F	0.20	0.003	0.74	0.007
		M	0.20	0.002	0.73	0.006
	USA	F	0.20	0.002	0.74	0.005
		M	0.20	0.001	0.74	0.010
21	EU	F	1.01	0.006	1.22	0.002
		M	1.10	0.007	1.20	0.002
	USA	F	0.95	0.006	1.24	0.002
		M	1.01	0.014	1.24	0.007
28	EU	F	1.59	0.004	1.36	0.002
		M	1.80	0.013	1.32	0.004
	USA	F	1.41	0.017	1.40	0.007
		M	1.51	0.019	1.42	0.007
34	EU	F	2.04	0.011	1.49	0.006
		M	2.37	0.013	1.45	0.003
	USA	F	1.70	0.031	1.59	0.013
		M	1.84	0.035	1.59	0.012

[a] F is female and M is male.
[b] in kilograms.
[c] standard error of the mean.

Table 2
Results of linear model with performance of experiment 1.

	Total Mean Weight			Feed Conversion Ratio		
Predictors	*Estimates*	*CI*[a]	*p*	*Estimates*	*CI*[a]	*p*
(Intercept)	−0.32	−0.39 – −0.25	<0.001	0.57	0.53 – 0.61	<0.001
Age	0.07	0.07 – 0.07	<0.001	0.03	0.03 – 0.03	<0.001
Feed	0.12	0.02 – 0.22	0.014	−0.02	−0.08 – 0.03	0.39
Sex	−0.11	−0.21 – −0.01	0.027	0	−0.05 – 0.06	0.897
Age * Feed	−0.01	−0.02 – −0.01	<0.001	0	0.00 – 0.01	0.01
Age * Sex	0.01	0.01 – 0.02	<0.001	0	−0.00 – 0.00	0.237
Feed * Sex	0.07	−0.07 – 0.21	0.32	−0.02	−0.09 – 0.06	0.674
(Age * Feed) * Sex	−0.01	−0.01 – 0.00	0.016	0	0.00 – 0.00	0.219

Observations: 96.
[1] CI, Confidence interval.

3.2. Environment, genetic, and diet effects on performance parameters and microbiome composition

The performance results, i.e. TMW and FCR, of the second experiment are shown in Table 5, where the mean and standard error of the mean are depicted for each *age* (d7, 21, 28, and 35), *genetics* (EU/USA), and *feed* (EU/USA). The performance results of the linear model of the second experiment are shown in Table 6 ($n = 64$ pens), only one significant effect was observed, i.e. in TMW for *age* ($P = 0.01$), independent of *feed* and *genetics*.

We first performed analysis on alpha- and beta-diversity on the microbiota data. Alpha-diversity measures showed a significant increase of observed species (P=<0.001), Shannon index (P=<0.001), and Pielou's evenness (P=<0.001) for Age. Furthermore, a significant effect of Environment was observed in observed species ($P = 0.01$). This effect of *environment* was also observed when performing a permutational multivariate analysis of variance using dissimilarities (beta-diversity) on the relative microbiota data (Fig. 4, Figure S3 Unweighted Unifrac and S4 Weighted Unifrac), this resulted in a significant effect for *age* ($P = 0.001$) and *environment* ($P = 0.027$), and a trend for the interaction *age* by *environment* ($P = 0.72$), and *feed* ($P = 0.073$). This latter effect of *environment* was not expected, because here environment represents two different compartments within the same stable, unfortunately this also affected the ability to perform further down-stream analyses, i.e. not having sufficient power. Nevertheless, to generate a generic picture of the microbiota composition, only the phyla level was visualized (Figure S5).

4. Discussion

In this study, we have conducted two experiments to investigate the effects of different aspects, i.e. feed and genetics that influence the fecal microbiota succession of broilers. It has already been established that the fecal microbiota is highly variable (Oakley and Kogut, 2016), however, this is still the most efficient and noninvasive way to sample birds without having to sacrifice them. Moreover, the fecal microbiota is a combination of microbiota that are resident in ileum and ceca (Sekelja et al., 2012). One study reported different taxonomic and functional changes in the (fecal) microbiota when comparing birds with either a high or a low FCR, they observed 33 different genera (Singh et al., 2014) whereas another study identified fourteen microbiota Quantitative Trait Loci (mQTL) (Mignon-Grasteau et al., 2015). These studies show the microbiome effect when comparing two extreme phenotypes, in our study we investigated the effect of different aspects, i.e. two different feeds in composition and genetic background separated for four generations, on such phenotypes and their corresponding fecal microbiota.

The first experiment focused on the effect of diet, i.e. EU vs. USA based diets, in a commercial broiler line (Cobb500), where significant changes were observed for the three-way interaction *age* by *feed*, by *sex* for the total mean weight (TMW) and a significant two-way interaction *age* by *feed* for the FCR. The cumulative FCR for the period 0 to 7 days was below 1, this is normal (Cobb-Vantress, 2018) and due to the fact that the broilers also get nutrition of the yolk sac. These obtained results were as expected, as earlier studies have already shown differences in feed conversion In chickens fed with corn- or wheat-based diets (Kiarie et al., 2014; Munyaka et al., 2016). In our study, also significant differences in the microbiota composition were observed for *age* and *feed*. These observations corroborate with earlier published work on the microbiota succession (Lu et al., 2003) and impact of diet (Pan and Yu, 2014) in chickens. Both alpha- and beta-diversity showed significant differences in time (succession of the fecal microbiota), as well as between the diets (EU vs. USA) on days 21 and 34 for alpha-diversity measures. Whereas for beta-diversity measures differences due to diet were observed for days 1, 7, 21, 28, and 34. Focusing on specific genera

Table 3
Microbiota diversity results per age-diet groups of experiment 1.

Age	Diet	Alpha diversity									Beta- diversity	
		Observed	SD[a]	p-val[b]	Shannon	SD	p-val[b]	Evenness[c]	SD	p-val[b]	adonis[d]	betadisper[e]
1	EU	14	3	0.14	1.35	0.19	0.18	0.52	0.08	0.67	0.028	0.47
	USA	12	4		1.23	0.17		0.51	0.06			
4	EU	61	17	0.58	2.62	0.4	0.38	0.64	0.07	0.24	0.359	0.26
	USA	68	26		2.78	0.56		0.67	0.09			
7	EU	109	29	0.95	3.56	0.37	0.41	0.76	0.05	0.11	0.01	0.5
	USA	114	35		3.27	0.7		0.69	0.12			
14	EU	205	38	0.22	3.68	0.46	0.98	0.69	0.08	0.59	0.161	0.2
	USA	225	69		3.51	0.76		0.65	0.12			
21	EU	371	53	0.02	4.25	0.29	0.44	0.72	0.04	0.84	0.001	0.48
	USA	424	71		4.34	0.34		0.72	0.06			
28	EU	482	77	0.51	4.31	0.36	0.38	0.7	0.05	0.35	0.001	0.18
	USA	472	55		4.42	0.39		0.72	0.06			
34	EU	503	54	0.04	4.53	0.22	5E-04	0.73	0.03	0.009	0.001	0.52
	USA	558	56		4.89	0.18		0.77	0.03			

[a] SD; standard deviation.
[b] Wilcoxon-test on the Shannon index.
[c] Pielou's evenness index.
[d] Bray-Curtis as dissimilarities, pairwise-adonis (adjusted P-value).
[e] Bray-Curtis as dissimilarities, betadisper (permuted P-value).

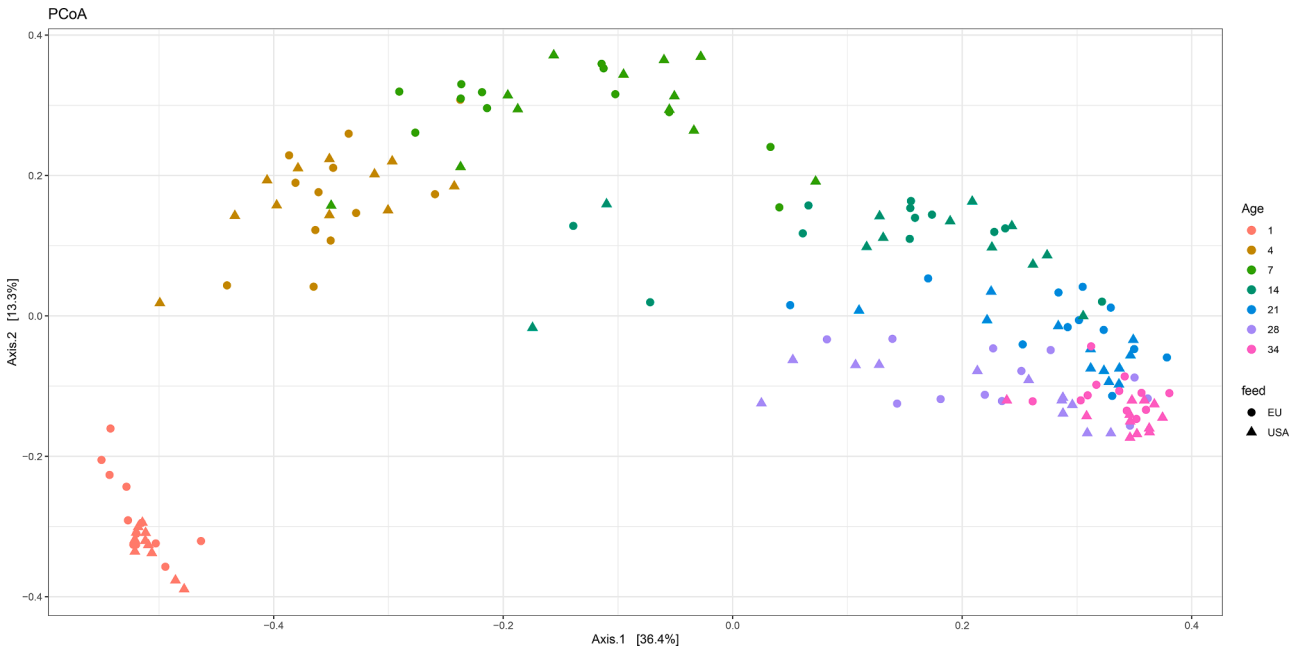

Fig. 1. Principal Coordinate Analysis using the Bray Curtis dissimilarities of all samples of experiment 1. Each symbol represents a (pooled) sample, where the shape represents the feed, i.e. circle is EU and the triangle is USA. The color indicates the age of the birds at sampling, i.e. red is d1, brown is d4, dark green is d7, blue-green is d14, light blue is d21, lilac is d28, and magenta is d34. The first axis explains 36.4% of the variation and the second axis represents 13.3% of the variation (on the rarefied data). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

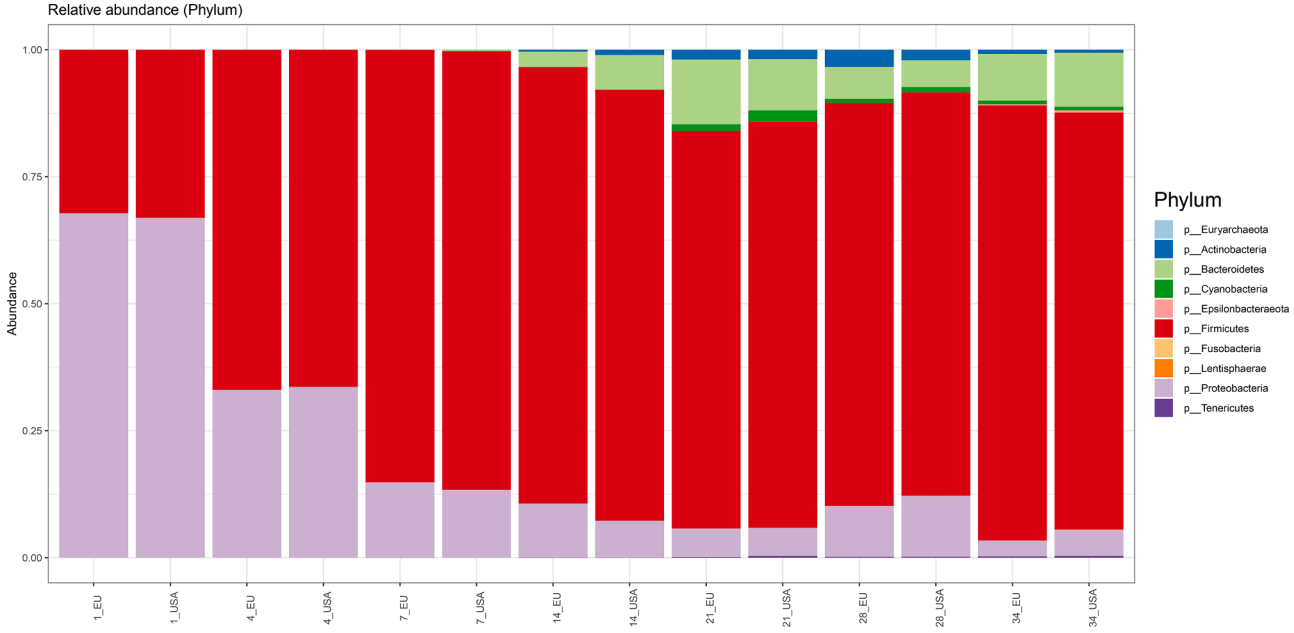

Fig. 2. Diet-grouped compositional data on phylum level of experiment 1. The x-axis depicts the diet-groups per day of age (e.g. 1_EU represents day 1 EU diet). The y-axis depicts the relative abundance. Each color represents a different taxa; light blue, Euryarchaeota; blue, Actinobacteria, light green, Bacteroidetes; green, Cyanobacteria; pink, Epsilonbacteraeota; red, Firmicutes; light orange, Fusobacteria; orange, Lentisphaerae; lilac, Proteobacteria, and purple, Tenericutes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that were significantly different between groups receiving different diets on a specific day, we observed two genera, i.e. *Streptococcus* on day 7 and *Bilophila* on day 21. The ARC of *Streptococcus* in EU diet was 0.003% (±0.009) and in the USA diet 0.073% (±0.064) ARC. In our performance data at day 7 no differences were observed between the diets with respect to the TMW or FCR, contrary to our study it has been observed that birds with a high FCR also showed higher *Streptococcus* (Singh et al., 2014). Moreover, another study showed a negative correlation of *Streptococcus* (in ileum) with body weight (Han et al., 2016). *Streptococcus*, as being a member of the lactic acid producing bacteria, have already been applied as probiotics in chicken (Rodriguez-Lecompte et al., 2012). In our study, we observed a higher ARC of *Streptococcus* in chickens with USA genetic background, independent of the diet, but no differences in the performance at day 7. Also later in life the performance parameters of the USA-fed chickens compared to the EU-fed showed lower values in the TMW, this suggests no major involvement

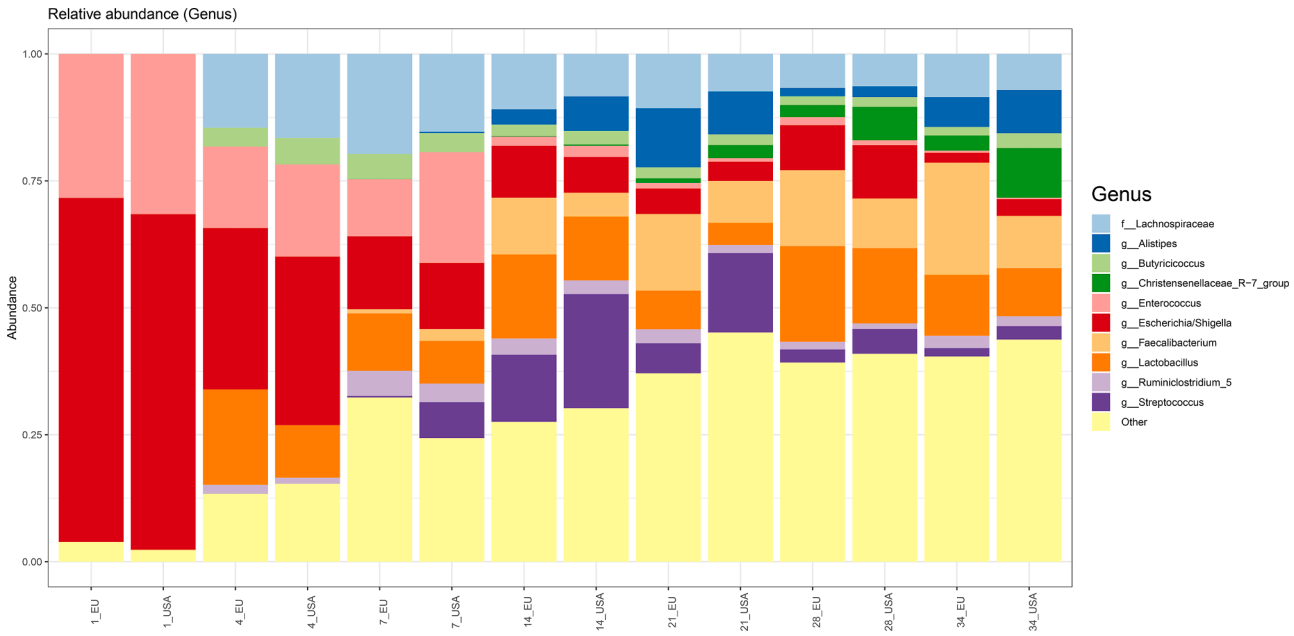

Fig. 3. Diet-grouped compositional data on top 10 genera level of experiment 1. The x-axis depicts the diet-groups per day of age (e.g. 1_EU represents day 1 EU diet). The y-axis depicts the relative abundance. Each color represents a different taxa; Each color represents a different taxa; light blue, Lachnospiraceae; blue, Alistipes, light green, Butyricicoccus; green, Christensenellaceae_*R* − 7_group; pink, Enterococcus; red, Escherichia/Shigella; light orange, Faecalibacterium; orange, Lactobacillus; lilac, Ruminiclostridium_5, and purple, Streptococcus; and yellow, Other (this encompasses all non-top 10 bacterial genera). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Significant genera[a] in experiment 1.

Genus	Age	Feed	mean	SD[b]	P_{adj}[c]
Streptococcus	7	EU	0.003	0.009	0.001
		USA	0.073	0.064	0.001
Bilophila	21	EU	0.004	0.004	0.001
		USA	0.015	0.008	0.001

[a] prevalent in 5% of the samples and average relative contribution above 0.01%.
[b] SD = standard deviation.
[c] P_{adj}, corrected by Benjamini and Hochberg method.

for *Streptococcus* shift due to diet, environment or genetic background, for the performance parameters. We observed higher *Bilophila* in broilers fed the USA diet compared to the EU diet on day 21, i.e. 0.015% (±0.008) and 0.004% (±0.004), respectively. *Bilophila* is known to be involved in bile acid metabolism (Baron et al., 1989; Ridlon et al., 2014), and in a study with resistant starch and corn starch, birds from the corn starch had higher abundances of *Bilophila* (approximately 1% ARC) compared to the (4%) resistant starch (approximately 0.5% ARC) (Zhang et al., 2020). Furthermore, in the cecum *Bilophila* was also positively correlated with body weight at day 21 and 28 (Johnson et al., 2018). Although in our data we observed a higher ARC of *Bilophila* in the corn-rich diets (USA), the associated performance data did not show significant differences. Taken together, the results of this first experiment showed that performance parameters and fecal microbiota are subject to change based on age and diet.

The second experiment, performed sequentially to the first experiment, focused on investigating the interaction between feed, genetics, the impact on performance parameters and fecal microbiota during the lifetime of broilers. The effect of the EU vs USA based diet on the microbiome is as compared to the first experiment with the commercial broilers, this was independent of the genetic lines used, which was studied in experiment 2. Thus diet affect microbiome in both genetic lines used. Broilers were divided into two different compartments within the same stable (of the same farm), due to the number of repeats per

Table 5
Descriptive statistics of experiment 2[a].

				Total Mean Weight		FCR[b]	
Age	Environment	Genetics	Feed	Mean	SEM[c]	Mean	SEM[c]
7	1	EU	EU	0.18	0	0.84	0.02
			US	0.18	0	0.78	0.01
		US	EU	0.18	0.01	0.89	0.03
			US	0.18	0.01	0.86	0.09
	2	EU	EU	0.18	0.01	0.95	0.04
			US	0.18	0	1.12	0.3
		US	EU	0.18	0	0.9	0.08
			US	0.18	0	0.93	0.02
21	1	EU	EU	1.02	0	1.25	0.04
			US	0.99	0.01	1.27	0.04
		US	EU	1.04	0.03	1.34	0.04
			US	1.01	0.04	1.4	0.09
	2	EU	EU	1.06	0.01	1.39	0.11
			US	1.05	0.03	1.72	0.42
		US	EU	1.08	0.01	1.36	0.13
			US	1.1	0.01	1.44	0.06
28	1	EU	EU	1.79	0.07	1.43	0.03
			US	1.78	0.01	1.45	0.04
		US	EU	1.81	0.04	1.44	0.04
			US	1.75	0.03	1.52	0.10
	2	EU	EU	1.86	0	1.49	0.11
			US	1.78	0.02	1.87	0.45
		US	EU	1.85	0	1.51	0.10
			US	1.8	0.05	1.58	0.07
35	1	EU	EU	2.64	0.07	1.56	0.09
			US	2.6	0.03	1.81	0.33
		US	EU	2.57	0.09	1.58	0.03
			US	2.52	0	1.74	0.17
	2	EU	EU	2.73	0.01	1.63	0.18
			US	2.57	0	2	0.47
		US	EU	2.77	0	1.79	0.33
			US	2.57	0	1.76	0.01

[a] *n*=8 replicates per group.
[b] FCR, feed conversion ratio.
[c] standard error of the mean.

Table 6
Results of linear model with performance of experiment 2.

	Total Mean Weight			Feed Conversion Ratio		
Predictors	*Estimates*	*CI*[a]	*p*	*Estimates*	*CI*[a]	*P*
(Intercept)	−8.41	−22.90 – 6.07	0.25	0.55	−0.29 – 1.38	0.20
Age	1.51	0.93 – 2.09	<0.001	0.03	−0.01 – 0.06	0.10
Environment	−0.79	−9.95 – 8.37	0.86	0.13	−0.40 – 0.66	0.62
Genetics	−0.5	−20.98 – 19.99	0.96	0.26	−0.92 – 1.45	0.66
Feed	1.76	−18.73 – 22.24	0.86	−0.48	−1.66 – 0.71	0.42
Age * Environment	0.03	−0.33 – 0.40	0.86	0	−0.02 – 0.02	0.88
Age * Genetics	−0.01	−0.83 – 0.81	0.98	−0.01	−0.06 – 0.04	0.70
Environment * Genetics	0.55	−12.41 – 13.50	0.93	−0.19	−0.94 – 0.56	0.61
Age * Feed	−0.07	−0.89 – 0.75	0.87	0.01	−0.04 – 0.06	0.61
Environment * Feed	−0.17	−13.13 – 12.79	0.98	0.31	−0.43 – 1.06	0.40
Genetics * Feed	−0.98	−29.95 – 27.99	0.95	0.27	−1.41 – 1.94	0.75
(Age * Environment) * Genetics	−0.01	−0.52 – 0.51	0.98	0.01	−0.02 – 0.04	0.60
(Age * Environment) * Feed	−0.06	−0.58 – 0.46	0.82	0	−0.03 – 0.03	0.87
(Age * Genetics) * Feed	−0.04	−1.20 – 1.12	0.94	0	−0.07 – 0.07	0.96
(Environment * Genetics) * Feed	0.34	−17.98 – 18.66	0.97	−0.18	−1.24 – 0.88	0.74
(Age * Environment * Genetics) * Feed	0.06	−0.68 – 0.79	0.88	−0.01	−0.05 – 0.04	0.80
Observations: 64						

[a] CI, confidence interval.

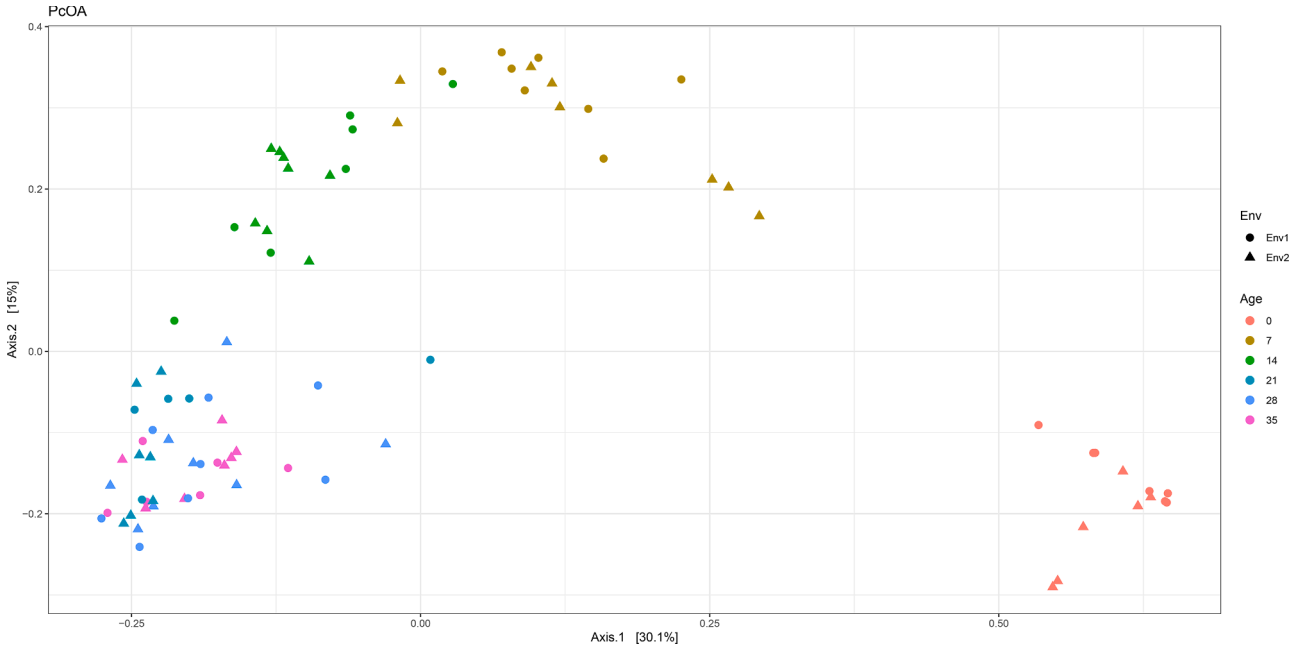

Fig. 4. Principal Coordinate Analysis using the Bray Curtis dissimilarities of all samples of experiment 2. Each symbol represents a (pooled) sample, where the shape represents the environment, i.e. circle is environment 1 (Env1) and the triangle is environment 2 (Env2). The color indicates the age of the birds at sampling, i.e. red is d0, brown is d7, dark green is d14, blue-green is d21, light blue is d28, and lilac is d35. The first axis explains 30.1% of the variation and the second axis represents 15.0% of the variation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

group to measure the performance parameters. When we took this into account for the statistics as environment the microbiome differences were most pronounced in the two environments, i.e. also TMW, FCR differed between the two compartments, although the eggs were all incubated in the same hatchery, and chicks were randomly divided in the pens and compartments nevertheless the changes observed in the performance parameters and fecal microbiota were striking, significance was observed for TMW, FCR, and the microbiota composition. Similar observations of influences of the environment and the microbiota diversity were already observed for ileal, caecal, and fecal microbiota samples (Siegerstetter et al., 2017), however, the referred environment is on a geographical scale, while our experimental setting was dived in compartments. Additionally, batch to batch variation in (caecal) microbiota has also been observed (Stanley et al., 2013), meaning that different seasons and maybe even differences in hygiene protocols, when cleaning out the stable or compartment, for the next flock can influence their microbiota composition. This difference, either the different hygiene status or the starter microbiota composition could have affected the outcome in our study.

Another significant aspect was age, this was observed for both the performance parameters and microbiota diversity and composition. For performance parameters, like weight, a plethora of examples are already published often focusing on specific diets or dietary interventions (Qaisrani et al., 2014; van Krimpen et al., 2017), or genetic background (Marcato et al., 2008). For the gut microbiota it is also known that the succession is a dynamic and plastic process (Lu et al., 2003; Ranjitkar et al., 2016; Jurburg et al., 2019). Within both experiments of this study we observed an increase in TMW and FCR in time, as well as

compositional changes in the fecal microbiota. Consequently, it was not possible to disentangle the effect of age and TMW, when correlating these variables. When comparing the first and the second experiment in this study, a reproducibility of the community turn-over was observed, i.e. the succession patterns. Additionally, both experiments showed a dietary (*feed*) effect on the fecal microbiota which strengthens that diet can modulate this microbiota composition that in turn could be linked or associated to certain phenotypic traits, like intestinal immune development. Meaning that by changing diet, based on wheat or maze, and probably other diets, will affect the microbiome composition.

Genetics has a small effect on the microbiota succession in the gut, as has been established for fat deposition (Wen et al., 2019a). This phenomena of succession being independent of genetics was also observed in another chicken study (Wen et al., 2019b), as well as in humans (Rothschild et al., 2018). Contrary, another study showed that when you rear genetically different chicken lines in a similar environment and with the same diet, microbiota succession is different up to 14 days of age (Schokker et al., 2015). Here, we have used genetically lines that were only four generations apart, suggesting that these lines were still much alike and consequently the effect on the microbiota composition was not significant.

In our study, we also investigated which microbiota genera contributed the most to the phenotypes, TMW and FCR (data not shown), however due to confounding of age and performance parameters we could not analyze this. Moreover, the sample size of our experiments was on the low side, compared to other studies were the focus was on highly contrasting phenotypes and where bacterial groups were identified that were predictive for FCR (Diaz-Sanchez et al., 2019).

6. Conclusions

We observed differences in performance parameters and in the succession of the fecal microbiota of growing broilers, when fed either a EU or USA diet. When investigating several aspects simultaneously, i.e. genetics and feed, we observed that environment and age and feed are most predominantly impacting the microbiota composition. Meaning that when designing new diets this will not only affect FCR but also microbiome composition and thereby intestinal immune development.

Ethics statement

This study is considered not to be an animal experiment by the Wageningen Research Animal Welfare Body (Instantie voor Dierenwelzijn).

CRediT authorship contribution statement

Dirkjan Schokker: Conceptualization, Methodology, Software, Formal analysis, Writing – original draft, Writing – review & editing. **Britt de Klerk:** Conceptualization, Methodology, Resources, Investigation. **Randy Borg:** Conceptualization, Methodology, Resources. **Alex Bossers:** Software, Formal analysis, Writing – review & editing. **Johanna M.J. Rebel:** Supervision, Writing – review & editing.

Declaration of Competing Interest

Authors declare no conflict of interest.

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Supplementary materials

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