Differences in gut microbiota composition of laying hen lines divergently selected on feather pecking

Jerine A. J. van der Eijk,[*,†,1] Hugo de Vries,[‡] Joergen B. Kjaer,[§] Marc Naguib,[*] Bas Kemp,[†] Hauke Smidt,[‡] T. Bas Rodenburg,[*,†,#] and Aart Lammers[†]

[*]*Behavioural Ecology Group, Department of Animal Sciences, Wageningen University & Research, 6708 WD Wageningen, the Netherlands;* [†]*Adaptation Physiology Group, Department of Animal Sciences, Wageningen University & Research, 6708 WD Wageningen, the Netherlands;* [‡]*Laboratory of Microbiology, Wageningen University & Research, 6708 WE Wageningen, the Netherlands;* [§]*Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Animal Welfare and Animal Husbandry, 29223 Celle, Germany; and* [#]*Department of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, the Netherlands*

ABSTRACT Feather pecking (FP), a damaging behavior where laying hens peck and pull at feathers of conspecifics, is multifactorial and has been linked to numerous behavioral and physiological characteristics. The gut microbiota has been shown to influence host behavior and physiology in many species, and could therefore affect the development of damaging behaviors, such as FP. Yet, it is unknown whether FP genotypes (high FP [HFP] and low FP [LFP] lines) or FP phenotypes (i.e., individuals differing in FP, feather peckers and neutrals) differ in their gut microbiota composition. Therefore, we identified mucosa-associated microbiota composition of the ileum and cecum at 10 and 30 wk of age. At 30 wk of age, we further identified luminal microbiota composition from combined content of the ileum, ceca, and colon. FP phenotypes could not be distinguished from each other in mucosa-associated or luminal microbiota composition. However, HFP neutrals were characterized by a higher relative abundance of genera of Clostridiales, but lower relative abundance of *Lactobacillus* for the luminal microbiota composition compared to LFP phenotypes. Furthermore, HFP neutrals had a higher diversity and evenness for the luminal microbiota compared to LFP phenotypes. FP genotypes could not be distinguished from each other in mucosa-associated microbiota composition. Yet, FP genotypes could be distinguished from each other in luminal microbiota composition. HFP birds were characterized by a higher relative abundance of genera of Clostridiales, but lower relative abundance of *Staphylococcus* and *Lactobacillus* compared to LFP birds. Furthermore, HFP birds had a higher diversity and evenness for both cecal mucosa-associated and luminal microbiota compared to LFP birds at adult age. In conclusion, we here show that divergent selection on FP can (in)directly affect luminal microbiota composition. Whether differences in microbiota composition are causal to FP or a consequence of FP remains to be elucidated.

Key words: gut microbiota, composition, feather pecking, genotype, phenotype

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INTRODUCTION

The gut microbiota (i.e., the microorganisms in the gut) has been shown to influence both host behavior and physiology in numerous ways, where many studies compared conventional and germ-free (microbiota-deficient) rodents (see Cryan and Dinan, 2012; Sommer and Bäckhed, 2013 for reviews). Infection with enteric pathogens or altering microbiota composition, via, for example, anti-, pre- or probiotic treatment, has been shown to affect anxiety, activity, stress sensitivity, and the serotonergic, dopaminergic, and immune systems in rodents (Goehler et al., 2008; Esmaili et al., 2009; Bercik et al., 2010, 2011; Desbonnet et al., 2010; Bravo et al., 2011; Ait-Belgnaoui et al., 2014). The gut microbiota might have similar effects in birds. Germ-free quails showed reduced fearfulness compared to colonized quails (Kraimi et al., 2018). Probiotic treatment reduced fearfulness and improved memory in quails (Parois et al., 2017), reduced plasma serotonin

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[1]Corresponding author: jerine.vandereijk@wur.nl

levels in dominant hens, but not in subordinate hens (Hu et al., 2018), and enhanced serum and intestinal natural antibody titers (Haghighi et al., 2006). However, probiotic treatment did not affect corticosterone levels in laying hens (Lei et al., 2013), but broilers infected with *Clostridium perfringens* showed increased corticosterone levels compared to non-infected birds (Calefi et al., 2016). These findings suggest that gut microbiota influences behavior and physiology in poultry and could therefore influence an animal's ability to cope with environmental challenges, such as those encountered in animal husbandry.

Feather pecking (**FP**) is a damaging behavior in laying hens where they peck and pull at feathers of conspecifics. Feather pecking is multifactorial and has been linked to numerous behavioral characteristics, such as activity, fearfulness, stress sensitivity, but also to physiological characteristics, such as the serotonergic, dopaminergic, and immune systems (Rodenburg et al., 2013). Since similar behavioral and physiological systems affected by the gut microbiota have been linked to FP, the gut microbiota could affect the development of FP. For example, lines selected on high (**HFP**) and low FP (**LFP**) differ in stress response, locomotor activity, responses to various behavioral tests, central serotonergic and dopaminergic activity, immune competence, immune reactivity, natural antibody titers, and peripheral serotonin levels (Buitenhuis et al., 2006; Kjaer, 2009; Kjaer and Guémené, 2009; Kops et al., 2017; van der Eijk et al., 2018, 2019). Moreover, HFP and LFP birds differ in intestinal microbial metabolites and in microbiota composition determined from cecal droppings (Meyer et al., 2013; Birkl et al., 2018). Yet, it is unknown whether the FP selection lines (i.e., FP genotypes) differ in microbiota composition of specific gut sections. Furthermore, individuals within a line can become feather peckers, feather pecker-victims, victims, or neutrals (i.e., FP phenotypes), but it is unknown whether FP phenotypes differ in microbiota composition of specific gut sections. Identifying microbiota composition from different gut sections (i.e., ileum, cecum, or colon) is crucial as microbiota composition of fecal samples is variable because feces can originate from different gut sections (Sekelja et al., 2012), which differ in microbiota composition (Lu et al., 2003; Awad et al., 2016). It is further important to identify microbiota composition from different locations within gut sections (i.e., luminal and mucosa-associated microbial communities) as mucosa-associated microbiota composition has been shown to differ from luminal microbiota composition (Olsen et al., 2008; Awad et al., 2016). Furthermore, communication between the gut microbiota and the host occurs via several pathways (see Collins et al., 2012; Cryan and Dinan, 2012 for review), and the intestinal epithelium plays an important role in these communication pathways as it is the primary interface for host–microbiota crosstalk (Artis, 2008). Therefore, mucosa-associated microbiota might have more influence on the host compared to luminal microbiota as it is in closer proximity to the host (Ouwerkerk et al., 2013).

The aim of this study was to identify differences in gut microbiota composition in relation to FP genotype (HFP and LFP) and FP phenotype (feather peckers and neutrals). We focused on feather peckers and neutrals to identify factors related to the performance of FP and to neither performing nor receiving FP. We identified mucosa-associated microbiota composition from intestinal scrapings of the ileum and cecum and luminal microbiota composition from combined content of the ileum, ceca, and colon. Based on findings from Birkl et al. (2018), we hypothesized that FP genotypes would differ in gut microbiota composition with regard to the relative abundance of genera of Clostridiales and of *Lactobacillus*. We further hypothesized that FP phenotypes would differ with regard to enteric pathogens and the abundance of beneficial commensal bacteria as these bacteria can alter fearfulness, serotonergic activity, and stress sensitivity and might therefore affect the development of FP. We further determined whether behavior (fearfulness) and physiology (serotonin, corticosterone, and natural antibodies) were related to gut microbiota composition. We focused on these variables as studies show that they can be affected by gut microbiota and have been linked to FP (Rodenburg et al., 2013; Sun et al., 2014; de Haas and van der Eijk, 2018).

MATERIALS AND METHODS

Animals and Housing

White Leghorn birds from the 18th generation of lines selected on high and low FP were used (see Kjaer et al., 2001 for selection procedure). The HFP and LFP lines were divergently selected on FP for 7 generations and were maintained in subsequent generations. The parent stock was between 38 and 43 wk of age at the time of egg collection. A total of 304 chicks were collected from 2 batches of eggs that were incubated at an average egg shell temperature of 37.3°C and average relative humidity of 55.6%. The 2 batches had the same housing conditions and experimental set-up with 4 pens per line, but with 2 wk between batches (see van der Eijk et al., 2018 for more details). The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

Feather Pecking Observations

Severe FP was observed at an individual level at 8 to 9 and 28 to 29 wk of age and was defined as follows: "A bird grips and pulls or tears vigorously at a feather of another bird with her beak, causing the feather to lift up, break or be pulled out. The recipient reacts to the peck by vocalizing, moving away or turning towards the pecking bird" (derived from Newberry et al., 2007). Severe FP was observed from video recordings, and each observation lasted 15 min,

either in the morning (10:40 to 10:55 am) or in the afternoon (2:40 to 2:55 pm). The number of severe feather pecks, either given or received, was summed over 2 subsequent weeks, thus including one morning and one afternoon observation with a total observation period of 30 min, and was used to identify FP phenotypes (adapted from Daigle et al., 2015). When a bird gave more than one and received zero or one severe feather peck it was defined as a feather pecker. When a bird gave and received zero or one severe feather peck it was defined as a neutral. We did not include victims or feather pecker-victims in this study.

Tonic Immobility Test

At 28 wk of age, birds were individually subjected to a tonic immobility test for a maximum duration of 5 min. The tonic immobility test is considered a validated test for fearfulness in poultry (Forkman et al., 2007). Tonic immobility duration was recorded until the bird returned to upright position (see van der Eijk et al., 2018 for more details).

Blood Collection and Analyses

Blood was collected from all birds at 4, 9, 14, 19, 24, and 29 wk of age. Blood was taken from the wing vein using a heparinized syringe. Blood samples were used for determination of plasma IgM and IgG natural antibody titers against keyhole limpet hemocyanin using an indirect ELISA (see van der Eijk et al., 2019 for more details). Blood samples taken at 24 wk of age were used for determination of plasma corticosterone concentrations via a radioimmunoassay kit (MP Biomedicals, LLC, Orangeburg, SC) as described previously (Buyse et al., 1987). Blood samples taken at 24 wk of age were further used for determination of whole blood serotonin concentration via a fluorescence assay as described previously (Bolhuis et al., 2009).

Microbiota Sampling

At 10 and 30 wk of age 20 birds per line (2 to 3 birds per pen) were sacrificed via cervical dislocation to collect gut microbiota from intestinal samples. We collected intestinal scrapings from a ± 2 cm midsection of the ileum (between Meckel's diverticulum and ceca) and from the ceca, as the ileum is mainly involved in nutrient absorption and immune modulation, while in the ceca microbial fermentation occurs (Moran, 1982; Svihus et al., 2013). We removed the gut content and scraped off the mucosa using sterile scalpel blades. Samples were stored in cryovials at −80°C until further analysis. At 30 wk of age, we collected combined luminal content of the ileum, ceca, and colon. Combined luminal content was further collected for transplantation purposes of our subsequent study. Five milliliters of sterile saline was added per gram of gut content, and this was mixed for 10 s on a Vortex. Samples were centrifuged at low speed (58 × g) for 3 min to remove large particles, and supernatant was collected in clean tubes. Sterile 85% glycerol was added to a final concentration of 15%. Samples were stored in cryovials at −80°C until further analysis.

Microbiota Analysis

Total bacterial DNA was extracted from intestinal samples using a customized Maxwell 16 Total RNA protocol (Promega Corp., Madison, WI) with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corp., Indianapolis, IN). Briefly, 100 mg of stool or digesta was homogenized with 0.25 g of sterilized 0.1 mm zirconia beads and three 2.5 mm glass beads in 300 μL STAR buffer for 3 × 1 min at 5.5 m s^{-1} using a bead beater (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France), with a waiting step of 15 s in between. Samples were incubated with shaking at 300 rpm for 15 min at 95°C and pelletized by 5 min centrifugation at 4°C and 16,100 × g. Supernatant was collected, and the pellets were processed again (from bead beating onwards) using 200 μL fresh STAR buffer. Samples were incubated at 95°C and centrifuged as before. Supernatant was collected, pooled with the first supernatant, and 250 μL of the combined supernatant was used for purification with Maxwell 16 Tissue LEV Total RNA Purification Kit, catalog no.AS1220 (Promega Corp.) customized for DNA extraction in combination with the STAR buffer. DNA was eluted with 50 μL of DNAse- and RNAse-free water (Qiagen, Hilden, Germany). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and adjusted to 20 ng μL^{-1} with DNAse- and RNAse-free water. PCR amplification was carried out with primers directed to the V5-V6 region of the bacterial 16S rRNA gene, namely BSF784F (5′-RGGATTAGATACCC) and 1064R (5′-CGACRRCCATGCANCACCT). PCR reactions were done in duplicate, each in a total volume of 50 μL and containing 20 ng of template DNA. Each sample was amplified with a uniquely barcoded primer pair (10 μM each per reaction), 1x HF buffer (Thermo Fisher Scientific, Waltham, MA), 1 μL dNTP Mix (10 mM each; Roche Diagnostics GmbH, Mannheim, Germany), 1 U Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Fisher Scientific), and 36.5 μL of DNAse- and RNAse-free water. The amplification program included 30 s initial denaturation at 98°C, followed by 25 cycles (with the exception of ileal mucosal DNA samples which were processed with 30 cycles to yield sufficient amplicon fragments) of denaturation at 98°C for 10 s, annealing at 42°C for 10 s, elongation at 72°C for 10 s, and a final extension at 72°C for 7 min. PCR product presence and size (approximately 280 bp) was confirmed by gel electrophoresis using a 1% agarose gel. A total of 70 unique barcode tags were used in each library, and 2 artificial (mock)

communities were included in addition to a water (no template) control. PCR products were purified using the HighPrep PCR kit (MagBio Genomics Inc., Gaithersburg, MD), and DNA concentrations were measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). A total of 100 ng of each barcoded PCR product was added to an amplicon pool that was subsequently concentrated using the HighPrep PCR kit to a volume of 20 μL. The DNA concentration of the amplicon pool was measured using the Qubit dsDNA BR Assay Kit, and the libraries were sent for Illumina HiSeq sequencing (Eurofins Genomics, Ebersberg, Germany). Data were processed and analyzed using NG-Tax, an in-house bioinformatics pipeline, as described by Ramiro-Garcia et al. (2016), which resulted in a minimum of 1,945 reads and a maximum of 948,018 reads per sample. Alpha diversity (i.e., richness, diversity, and evenness indices) was calculated with CANOCO 5 software package (Biometris, Wageningen, the Netherlands). Richness (S) was defined as the number of species in a sample. The Shannon diversity index (H) describes the diversity of the microbiota and was calculated using the formula below. Evenness estimates the similarity of species abundance (H/log(S)) (Finotello et al., 2018).

$$H = \sum_{i=1}^{S} (P_i * \ln P_i)$$

P_i = fraction of the entire population made up of species i
$\sum$ = sum from species 1 to species S

Statistical Analysis

Multivariate analysis was applied for data interpretation. In order to relate changes in total microbial composition to explanatory variables, redundancy analysis (**RDA**) was used as implemented in the CANOCO 5 software package (Biometris). FP genotype (HFP and LFP) and the interaction between FP genotype and FP phenotype (feather pecker and neutral) were introduced as nominal variables (see Table 1 for sample sizes). The relative contribution of 198 genus-level phylogenetic groups identified by 16S rRNA gene sequencing was used as response variables. Average natural antibody IgG and IgM titers were introduced as quantitative variables at 10 (averages from 4 and 9 wk of age) and 30 wk of age (averages from 4 to 29 wk of age). Tonic immobility duration (fearfulness), corticosterone, and serotonin levels were included as quantitative variables for the analyses of 30 wk of age. Analyses were performed for each age (10 or 30 wk of age) and gut section (ileum, cecum, or luminal content) separately. The Monte Carlo Permutation test (number of permutations 499) with forward selection was applied to test for significance of the effect of FP genotype, FP genotype * FP phenotype, tonic immobility, serotonin, corticosterone, and natural antibodies IgM and IgG on microbiota composition. Batch was included as covariate. *P*-values were corrected using Bonferroni correction. We performed Kruskal–Wallis tests in SAS Software version 9.3 (SAS Inst. Inc., Cary, NC) to identify differences between FP genotypes and FP genotype * FP phenotype in individual microbial groups, richness, diversity, and evenness indices. Post hoc comparisons were made with the Dwass, Steel, Chritchlow-Fligner method.

Table 1. The number of birds sampled for gut microbiota (and total number of birds in study) per phenotype (feather peckers (P) or neutrals (N)) within the high (HFP) and low feather pecking (LFP) lines for each gut section (ileum, cecum, or luminal content) at 10 and 30 wk of age.

Gut section	Ileum		Cecum		Luminal content	
Phenotype	P	N	P	N	P	N
Age (10 wk)						
HFP	4 (19)	2 (72)	5 (19)	5 (72)	–	–
LFP	3 (5)	4 (92)	4 (5)	4 (92)	–	–
Age (30 wk)						
HFP	6 (6)	10 (29)	6 (6)	10 (29)	5 (6)	10 (29)
LFP	4 (4)	10 (58)	4 (4)	10 (58)	4 (4)	10 (58)

RESULTS

Microbiota Composition

Multivariate redundancy analysis showed that FP genotype * FP phenotype groups could not be distinguished from each other in mucosa-associated or luminal microbiota composition. It is interesting to note that LFP feather peckers contributed to explaining the observed variation in mucosa-associated microbiota composition of the ileum at 10 wk of age ($P = 0.036$). Furthermore, LFP feather peckers and neutrals contributed to explaining the observed variation in luminal microbiota composition ($P = 0.040$ and $P = 0.036$, respectively). However, after Bonferroni correction these effects were no longer significant. FP genotypes could not be distinguished from each other in mucosa-associated microbiota composition. However, the RDA diagram for luminal microbiota composition showed that HFP and LFP birds could be distinguished from each other (Figure 1). Monte Carlo Permutation testing showed that the HFP and LFP lines significantly contributed to explaining the observed variation in microbiota composition ($P = 0.012$ and $P = 0.020$, respectively). The HFP line was correlated positively with the genera *Anaerotruncus*, *Butyricicoccus*, *Desulfovibrio*, *Eubacterium*, *Faecalibacterium*, *Sellimonas*, *Succinatimonas*, and *Sutterella* as well as unclassified genus-level groups within families Alcaligenaceae and Ruminococcaceae, and negatively with *Lactobacillus*.

To further identify differences in mucosa-associated microbiota composition, we determined whether FP genotypes or FP genotype * FP phenotype groups differed in relative abundance of microbial groups. At

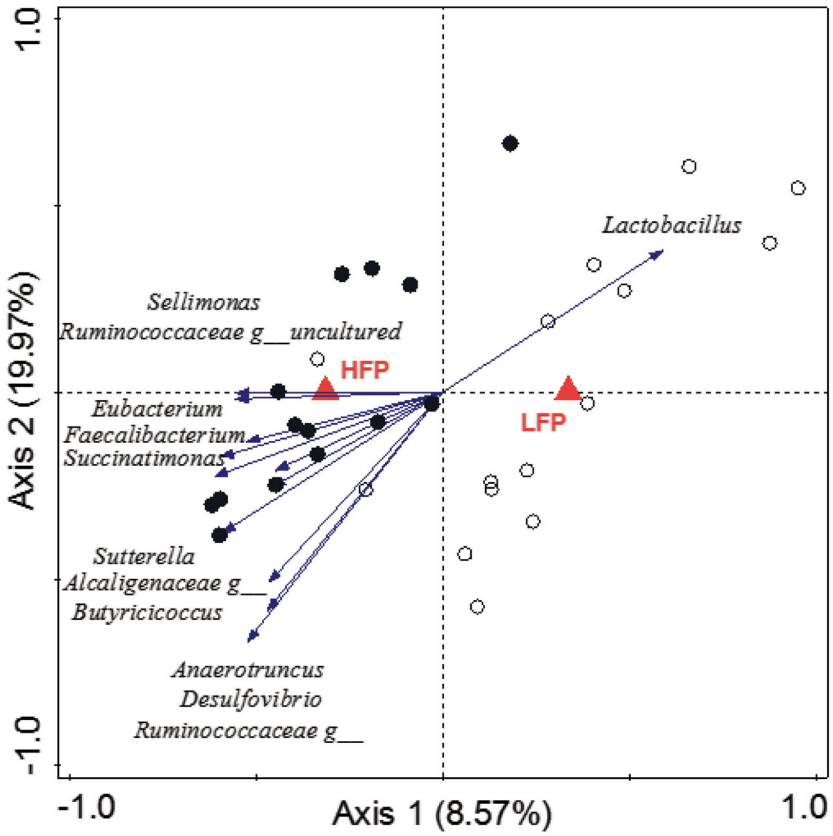

Figure 1. Triplot for partial RDA of luminal microbiota composition (combined content of the ileum, ceca, and colon) at 30 wk of age. Nominal environmental variables high feather pecking (HFP) line and low feather pecking (LFP) line are represented by red triangles. Samples are grouped by line: HFP (black filled circles) and LFP (black open circles). Microbial groups contributing at least 20% to the horizontal explanatory axis are represented as vectors. The horizontal axis explains 8.57% of the total variance in the dataset. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

10 wk of age only one microbial group differed significantly between FP genotypes for the cecum and none for the ileum (for details, see Table 2). At 30 wk of age the relative abundance of one microbial group differed significantly between FP genotypes for the ileum and multiple microbial groups differed significantly for the cecum. For the luminal content (combined content of the ileum, ceca, and colon), the relative abundance of multiple microbial groups differed significantly between FP genotypes (for details, see Table 3). Of these, the genera *Anaerotruncus, Butyricicoccus*, *Desulfovibrio, Eubacterium*, *Faecalibacterium*, *Lactobacillus*, *Sellimonas*, *Sutterella,* and genus-level taxa assigned to families Alcaligenaceae and Ruminococcaceae were identified as microbial groups contributing at least 20% to the horizontal explanatory axis in the RDA (see Figure 1). No differences were found between FP genotype ∗ FP phenotype groups, except for 1 microbial group for the cecum and 3 microbial groups for the luminal content at 30 wk of age (for details, see Table 4).

None of the quantitative environmental variables included in the RDA contributed to explaining the observed variation in gut microbiota composition at 10 or 30 wk of age, except for serotonin and natural antibody IgG. Serotonin level showed a tendency for contributing to explaining the observed variation in

Table 2. Average relative contribution (%) of genus-level groups different between the high feather pecking (HFP) and low feather pecking (LFP) lines in mucosa-associated microbiota of the ileum and cecum at 10 and 30 wk of age.

	P-value		
	HFP vs. LFP	HFP	LFP
Age (10 wk)			
Ileum		n = 6	n = 7
–	–	–	–
Cecum		n = 10	n = 8
Subdoligranulum	0.013 ↑	6.91 ± 1.42	2.50 ± 0.85
Age (30 wk)			
Ileum		n = 16	n = 14
Aliidiomarina	0.049 ↑	0.15 ± 0.09	0.0 ± 0.0
Bacillaceae;g__	0.016 ↑	0.79 ± 0.17	0.32 ± 0.07
Caldalkalibacillus	0.013 ↑	2.21 ± 0.50	0.74 ± 0.15
Idiomarina	0.009 ↑	0.12 ± 0.06	0.01 ± 0.01
Nesterenkonia	0.018 ↑	3.81 ± 0.72	1.46 ± 0.25
Tetragenococcus	0.043 ↑	0.14 ± 0.05	0.02 ± 0.01
Trichococcus	0.049 ↑	0.14 ± 0.07	0.0 ± 0.0
Turicibacter	0.018 ↓	0.06 ± 0.03	0.44 ± 0.18
Cecum		n = 16	n = 14
Anaerotruncus	0.038 ↑	0.79 ± 0.08	0.53 ± 0.08
Butyricicoccus	0.020 ↑	2.31 ± 0.66	1.29 ± 0.51
Flavonifractor	0.023 ↑	1.88 ± 0.90	0.25 ± 0.07

P-values are from the Kruskal–Wallis test. Values represent means ± SEM. ↑ and ↓ respectively indicate higher and lower relative abundance when comparing the HFP line to the LFP line. The genus-level groups with a relative abundance lower than 0.1% in both lines are not shown. Groups that could not be assigned to a specific genus are by classified by the family name appended with "; g_".

Table 3. Average relative contribution (%) of genus-level groups different between the high feather pecking (HFP) and low feather pecking (LFP) lines in luminal microbiota at 30 wk of age.

	P-value		
	HFP vs. LFP	HFP	LFP
Luminal		n = 15	n = 14
Alcaligenaceae;g__	0.029 ↑	0.18 ± 0.07	0.01 ± 0.01
Anaerotruncus	0.005 ↑	0.37 ± 0.05	0.16 ± 0.04
Butyricicoccus	0.001 ↑	1.09 ± 0.19	0.40 ± 0.15
Clostridiales;g__	0.019 ↑	0.46 ± 0.10	0.14 ± 0.05
Deferribacteraceae;g__	0.033 ↑	0.34 ± 0.14	0.03 ± 0.02
Desulfovibrio	0.026 ↑	0.39 ± 0.08	0.17 ± 0.06
Erysipelatoclostridium	0.016 ↑	1.07 ± 0.20	0.42 ± 0.09
Eubacterium	0.010 ↑	0.17 ± 0.07	0.0 ± 0.0
Faecalibacterium	0.001 ↑	5.31 ± 0.76	2.21 ± 0.58
Fusicatenibacter	0.023 ↑	1.62 ± 0.25	0.88 ± 0.27
Lactobacillus	0.001 ↓	20.0 ± 3.09	41.84 ± 4.84
Olsenella	0.038 ↑	0.76 ± 0.23	0.29 ± 0.11
Peptococcus	0.026 ↑	0.44 ± 0.06	0.28 ± 0.08
Ruminiclostridium	0.015 ↑	1.19 ± 0.13	0.66 ± 0.13
Ruminococcaceae;g__	0.008 ↑	1.88 ± 039	0.64 ± 0.18
Sellimonas	0.005 ↑	0.20 ± 0.05	0.02 ± 0.02
Staphylococcus	0.028 ↓	0.30 ± 0.18	1.50 ± 0.92
Sutterella	0.010 ↑	0.89 ± 0.12	0.48 ± 0.08
Ruminococcaceae;g__ uncultured	0.003 ↑	2.26 ± 0.40	0.91 ± 0.15

P-values are from the Kruskal–Wallis test. Values represent means ± SEM. ↑ and ↓ respectively indicate higher and lower relative abundance when comparing the HFP line to the LFP line. The genus-level groups with a relative abundance lower than 0.1% in both lines are not shown. Groups that could not be assigned to a specific genus are by classified by the family name appended with "; g-"

mucosa-associated microbiota composition of the ileum at 30 wk of age ($P = 0.068$). *Akkermansia*, *Bifidobacterium*, *Blautia*, *Dorea*, *Enterobacter*, *Faecalibacterium*, *Granulicatella*, *Klebsiella*, *Eubacterium*, *Lactococcus*, *Micrococcus*, *Parabacteroides*, *Prevotella*, *Rhodococcus*, *Roseburia*, *Serratia*, *Veillonella* and *Victivallis,* and unclassified genus-level taxa within the Enterobacteriaceae and Porphyromonadaceae were positively correlated and *Helicobacter* was negatively correlated with serotonin level (Figure 2). Natural antibody IgG titer showed a tendency for contributing to explaining the observed variation in mucosa-associated microbiota composition of the cecum at 30 wk of age ($P = 0.066$). *Alistipes*, *Butyricicoccus*, *Desulfovibrio,* and an unclassified genus-level group within the Rikenellaceae were positively correlated to natural antibody IgG titer (Figure 3).

Richness, Diversity, and Evenness Indices

FP genotype effects were found on all indices, where the HFP line had higher diversity ($\chi^2 = 4.85$, df = 1, $P = 0.028$) and evenness ($\chi^2 = 3.98$, df = 1, $P = 0.046$) indices for the mucosa-associated microbiota of the cecum compared to the LFP line at 30 wk of age (see Table 5). Furthermore, the HFP line had higher richness ($\chi^2 = 6.88$, df = 1, $P = 0.009$), diversity ($\chi^2 = 10.43$, df = 1, $P = 0.001$), and evenness ($\chi^2 = 10.43$, df = 1, $P = 0.001$) indices for the luminal microbiota compared to the LFP line at 30 wk of age. FP genotype ∗ FP phenotype groups did not differ in richness, diversity, or evenness indices, except for the diversity ($\chi^2 = 12.69$, df = 3, $P = 0.005$) and evenness ($\chi^2 = 13.93$, df = 3, $P = 0.003$) indices for the luminal microbiota (see Table 6). HFP neutrals had higher diversity and evenness indices compared to LFP feather peckers and neutrals ($P < 0.05$).

DISCUSSION

The aim of this study was to identify differences in gut microbiota composition in relation to FP genotype and FP phenotype. We further determined whether or not behavioral or physiological parameters were related to gut microbiota composition. We identified mucosa-associated microbiota composition from the ileum and cecum of feather peckers and neutrals from the HFP and LFP lines at young and adult ages. We further identified luminal microbiota composition (combined content of the ileum, ceca, and colon) at adult age.

The interaction between FP genotype and FP phenotype did not contribute to explaining the observed variation in mucosa-associated or luminal microbiota composition, and we only found several differences in relative abundance of genus-level microbial groups. This suggests that FP phenotypes from the HFP and LFP lines did not differ in microbiota composition. Microbiota composition might thus not explain the

Table 4. Average relative contribution (%) of genus-level groups differing between feather peckers (P) and neutrals (N) of the high feather pecking (HFP) and low feather pecking (LFP) lines in mucosa-associated microbiota of the cecum and luminal microbiota at 30 wk of age.

		HFP		LFP	
	P-value	P	N	P	N
Cecum		n = 6	n = 10	n = 4	n = 10
Anaerostipes	0.017	0.0 ± 0.0^{a}	$0.11 \pm 0.07^{a,b}$	0.47 ± 0.18^{b}	$0.29 \pm 0.25^{a,b}$
Luminal		n = 5	n = 10	n = 4	n = 10
Lactobacillus	0.003	$28.33 \pm 5.08^{a,b}$	15.87 ± 3.29^{a}	47.13 ± 3.33^{b}	39.72 ± 6.65^{b}
Butyricicoccus	0.006	$0.84 \pm 0.16^{a,b}$	1.22 ± 0.28^{a}	0.08 ± 0.08^{b}	$0.53 \pm 0.20^{a,b}$
Faecalibacterium	0.003	$4.20 \pm 1.00^{a,b}$	5.87 ± 1.01^{a}	1.02 ± 0.52^{b}	2.69 ± 0.74^{b}

P-values are from the Kruskal–Wallis test. Values represent means ± SEM. The genus-level groups with a relative abundance lower than 0.1% in both lines and phenotypes are not shown. Means within a row lacking a common superscript differ significantly ($P < 0.05$).

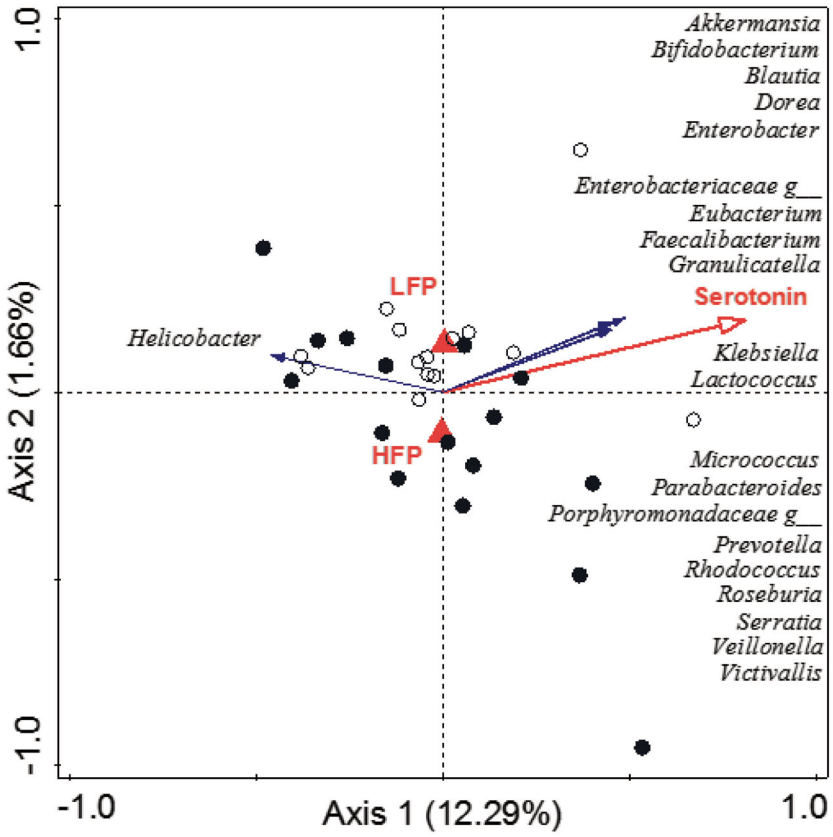

Figure 2. Triplot for RDA of mucosa-associated microbiota composition of the ileum at 30 wk of age. Nominal environmental variables high feather pecking (HFP) line and low feather pecking (LFP) line are represented by red triangles, and quantitative environmental variable whole blood serotonin level is represented by the open red arrow. Samples are grouped by line: HFP (black filled circles) and LFP (black open circles). Microbial groups contributing at least 20% to the horizontal explanatory axis are represented as vectors (closed arrows). The horizontal axis explains 12.29% of the total variance in the dataset. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

behavioral and physiological differences previously found between feather peckers and neutrals (Jensen et al., 2005; Kops et al., 2013; Daigle et al., 2015) and between feather peckers and neutrals of the present study (van der Eijk et al., 2018, 2019). Furthermore, HFP neutrals had higher diversity and evenness indices for the luminal microbiota compared to LFP phenotypes, suggesting that the microbiota in HFP neutrals was characterized by a more even distribution of relative abundances and higher diversity compared to LFP phenotypes. However, caution is needed when interpreting these results as we had relatively low sample sizes for FP phenotypes within FP genotypes. Furthermore, we observed FP behavior for a limited amount of time which might have led to FP behavior not being observed. However, continuous observation is impractical, and the strength of our study is that we identified phenotypes based on observations just prior to microbiota sampling.

FP genotype did not contribute to explaining the observed variation in mucosa-associated microbiota composition of the ileum or cecum at young or adult age, suggesting that divergent selection on FP did not affect mucosa-associated microbiota composition. Therefore, mucosa-associated microbiota composition might not explain the behavioral and physiological differences previously found in the FP selection lines (Kjaer, 2009; Kjaer and Guémené, 2009; Kops et al., 2017) and in HFP and LFP birds of the present study (van der Eijk et al., 2018, 2019). However, the relative abundances of genus-level taxa within the Alteromonadales, Bacillales and Lactobacillales were higher and that of a genus-level group within the Erysipelotrichales was lower in the mucosa-associated microbiota of the ileum from HFP birds compared to LFP birds at adult age. For the mucosa-associated microbiota of the ceca, HFP birds had a higher relative abundance of genus-level taxa within the Clostridiales compared to LFP birds at both young and adult ages.

FP genotype did contribute to explaining the observed variation in luminal microbiota composition at adult age with genera of Clostridiales contributing more to the luminal microbiota of the HFP line, while *Lactobacillus* contributed more to that of the LFP line. However, it should be noted that FP genotype explained less than 9% of the total variation in luminal microbiota composition. In addition, we identified luminal microbiota composition from combined content of the ileum, ceca, and colon, while gut sections have been shown

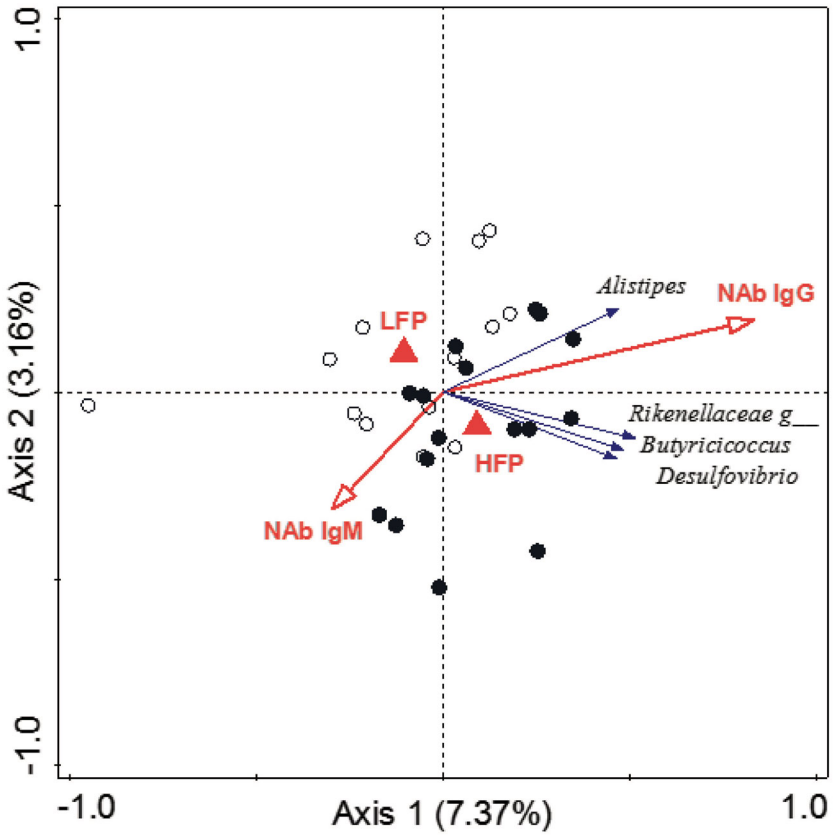

Figure 3. Triplot for RDA of mucosa-associated microbiota composition of the cecum at 30 wk of age. Nominal environmental variables high feather pecking (HFP) line and low feather pecking (LFP) line are represented by red triangles, and quantitative environmental variables natural antibody (NAb) IgG and IgM titers are represented by open red arrows. Samples are grouped by line: HFP (black filled circles) and LFP (black open circles). Microbial groups contributing at least 20% to the horizontal explanatory axis are represented as vectors (closed arrows). The horizontal axis explains 7.37% of the total variance in the dataset. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

to differ in luminal microbiota composition in chickens (Awad et al., 2016). HFP birds also had a higher relative abundance of genera of the order Clostridiales, but lower relative abundance of *Staphylococcus* and *Lactobacillus* in the luminal microbiota compared to LFP birds. This supports our findings from the multivariate redundancy analysis and is similar to our findings from the mucosa-associated microbiota of the cecum. HFP birds further had higher diversity and evenness indices in the cecum and luminal content and a higher richness index in the luminal content compared to LFP birds at adult age, suggesting that the gut microbiota of HFP birds had a higher number of different species, a more even distribution of species relative abundances (meaning that less dominant species are present), and a higher diversity compared to the gut microbiota of LFP birds. Overall, our findings give first indications of differences in luminal microbiota composition and alpha diversity between the FP selection lines.

Behavior (fearfulness) and physiology (serotonin, corticosterone, and natural antibodies) did not contribute to explaining the observed variation in gut microbiota composition except for serotonin and natural antibody IgG. Serotonin tended to contribute to explaining the observed variation in mucosa-associated microbiota composition of the ileum and natural antibody IgG to that of the cecum at adult age. However, it should be noted that serotonin explained less than 13% and natural antibody IgG less than 8% of the total variation in mucosa-associated microbiota composition. Genera of the order Clostridiales were positively related to high serotonin levels. In general, clostridia were found to modulate serotonin metabolism in the gut by, for example, activating serotonin synthesis (Yano et al., 2015). Some of the genera, specifically *Faecalibacterium*, *Eubacterium,* and *Roseburia*, are butyrate producers (Duncan et al., 2002a,b; van den Abbeele et al., 2013), and butyrate can stimulate serotonin secretion (Fukumoto et al., 2003). Other genera that were positively related to serotonin level were *Klebsiella* and *Lactococcus*, which are serotonin producers (Özogul, 2004; Özogul et al., 2012), *Akkermansia*, which was shown to modulate tryptophan metabolism (i.e., serotonin precursor) in germ-free mice (Derrien et al., 2011) and *Bifidobacterium*, increased tryptophan levels in the frontal cortex of rats (Desbonnet et al., 2008). Thus, serotonin levels might be higher due to the presence of these genera in the mucosa-associated microbiota of the ileum. *Alistipes* and unclassified members of the Rikenellaceae were positively related to natural

Table 5. Richness, diversity, and evenness indices of mucosa-associated microbiota of the ileum and cecum, and luminal microbiota from the high feather pecking (HFP) and low feather pecking (LFP) lines at 10 and 30 wk of age.

	HFP	LFP
Age (10 wk)		
Ileum	n = 6	n = 7
Richness	31.8 ± 3.33	31.4 ± 4.92
Diversity	2.09 ± 0.19	2.10 ± 0.30
Evenness	0.60 ± 0.04	0.61 ± 0.08
Cecum	n = 10	n = 8
Richness	35.0 ± 1.83	36.5 ± 0.78
Diversity	2.73 ± 0.07	2.74 ± 0.06
Evenness	0.77 ± 0.01	0.76 ± 0.01
Age (30 wk)		
Ileum	n = 16	n = 14
Richness	36.3 ± 3.26	37.7 ± 4.21
Diversity	1.93 ± 0.19	1.93 ± 0.19
Evenness	0.54 ± 0.05	0.53 ± 0.04
Cecum	n = 16	n = 14
Richness	59.9 ± 1.73	55.9 ± 2.68
Diversity	3.23 ± 0.09^{a}	3.02 ± 0.07^{b}
Evenness	0.79 ± 0.02^{a}	0.76 ± 0.02^{b}
Luminal	n = 15	n = 14
Richness	61.1 ± 1.84^{a}	50.8 ± 3.10^{b}
Diversity	3.22 ± 0.09^{a}	2.52 ± 0.18^{b}
Evenness	0.78 ± 0.02^{a}	0.64 ± 0.04^{b}

Values represent means ± SEM. Richness: the number of species in a sample (S). Diversity: the Shannon diversity index (H). Evenness: similarity of species abundance (H/log(S)). Means within a row lacking a common superscript differ significantly ($P < 0.05$).

antibody IgG titer. Species of the genera *Alistipes*, belonging to the family Rikenellaceae, are considered bacterial pathogens and were found to induce intestinal inflammation in mice (Moschen et al., 2016). Thus, natural antibody IgG titers might be higher due to the presence of these genera in the mucosa-associated microbiota of the cecum.

Many genera within the order Clostridiales, which were higher or contributed more to the microbiota composition of HFP birds, are butyrate producers, such as *Anaerotruncus*, *Butyricicoccus*, *Eubacterium*, *Faecalibacterium*, *Flavonifractor*, and *Subdoligranulum* (Duncan et al., 2002b; Eeckhaut et al., 2011; Li and Li, 2014). The relative abundance of *Butyricicoccus* and *Faecalibacterium* was also higher in luminal microbiota from HFP neutrals compared to LFP phenotypes. Butyrate has immunomodulatory potential (Wang et al., 2008; Vinolo et al., 2009) and can stimulate serotonin secretion, thereby activating the vagus nerve (Fukumoto et al., 2003). Many studies have identified effects of butyrate or butyrate-producing bacteria on brain function and behavior (see Stilling et al., 2016 for review). Although we did not measure butyrate concentrations, a previous study showed that HFP birds had higher n-butyrate but lower i-butyrate in cecal digesta compared to LFP birds (Meyer et al., 2013). Thus, the behavioral and physiological differences seen between the FP selection lines might partly be caused by increased production of butyrate. Increased abundance of Clostridiales has further been related to autism spectrum disorders (Parracho et al., 2005; Williams et al., 2011) and major depressive disorder (Zheng et al., 2016) in humans. Patients with autism further had increased abundance of *Desulfovibrio* and *Sutterella* (Finegold, 2011; Williams et al., 2012), but reduced abundance of

Table 6. Richness, diversity, and evenness indices of mucosa-associated microbiota of the ileum and cecum, and luminal microbiota from feather peckers (P) and neutrals (N) of the high feather pecking (HFP) and low feather pecking (LFP) lines at 10 and 30 wk of age.

	HFP		LFP	
	P	N	P	N
Age (10 wk)				
Ileum	n = 4	n = 2	n = 3	n = 4
Richness	31.3 ± 5.22	33.0 ± 1.00	37.3 ± 2.85	27.0 ± 8.09
Diversity	2.05 ± 0.29	2.16 ± 0.08	2.33 ± 0.13	1.93 ± 0.53
Evenness	0.60 ± 0.06	0.62 ± 0.02	0.64 ± 0.02	0.59 ± 0.14
Cecum	n = 5	n = 5	n = 4	n = 4
Richness	35.4 ± 1.69	34.6 ± 3.49	35.5 ± 1.32	37.5 ± 0.65
Diversity	2.72 ± 0.06	2.75 ± 0.13	2.71 ± 0.09	2.77 ± 0.07
Evenness	0.76 ± 0.01	0.78 ± 0.02	0.76 ± 0.02	0.77 ± 0.02
Age (30 wk)				
Ileum	n = 6	n = 10	n = 4	n = 10
Richness	29.2 ± 5.22	40.5 ± 3.72	33.8 ± 7.81	39.3 ± 5.16
Diversity	1.62 ± 0.24	2.11 ± 0.25	1.65 ± 0.31	2.04 ± 0.24
Evenness	0.49 ± 0.07	0.57 ± 0.06	0.47 ± 0.07	0.56 ± 0.05
Cecum	n = 6	n = 10	n = 4	n = 10
Richness	58.8 ± 3.68	60.5 ± 1.84	56.5 ± 3.57	55.6 ± 3.57
Diversity	3.08 ± 0.21	3.32 ± 0.06	2.89 ± 0.10	3.07 ± 0.09
Evenness	0.75 ± 0.04	0.81 ± 0.01	0.72 ± 0.03	0.77 ± 0.02
Luminal	n = 5	n = 10	n = 4	n = 10
Richness	62.4 ± 3.37	60.5 ± 2.29	46.0 ± 4.22	52.7 ± 3.96
Diversity	3.08 ± 0.16^{a,b}	3.29 ± 0.10^{a}	2.29 ± 0.18^{b}	2.61 ± 0.24^{b}
Evenness	0.75 ± 0.03^{a,b}	0.80 ± 0.02^{a}	0.60 ± 0.03^{b}	0.66 ± 0.05^{b}

Values represent means ± SEM. Richness: the number of species in a sample (S). Diversity: the Shannon diversity index (H). Evenness: similarity of species abundance (H/log(S)). Means within a row lacking a common superscript differ significantly ($P < 0.05$).

Staphylococcus (De Angelis et al., 2013). This is similar to what we found for the luminal microbiota composition of HFP birds compared to LFP birds, suggesting that specific gut microbiota compositions might be related to the development of these disorders and maladaptive behaviors, such as FP. Interestingly, HFP birds had higher richness, diversity, and evenness indices compared to LFP birds. Autistic subjects were found to have higher fecal microbial richness and diversity compared to control subjects (Finegold et al., 2010; De Angelis et al., 2013), although the opposite or no relation has also been found (Kang et al., 2013; Kushak et al., 2017).

The relative abundance of *Lactobacillus* was lower in HFP birds and contributed more to the luminal microbiota composition of LFP birds. The relative abundance of *Lactobacillus* was also lower in luminal microbiota of HFP neutrals compared to LFP phenotypes. *Lactobacillus* can influence both the innate and adaptive immune systems in chickens (Brisbin et al., 2011, 2015) and behavior in rodents, for example increasing locomotor activity or reducing anxiety and depression-related behavior (Bravo et al., 2011; Liang et al., 2015; Liu et al., 2016). This is potentially done via stimulation of serotonin receptors (Horii et al., 2013) or by increasing serotonin and dopamine in the brain (Liu et al., 2016). It is interesting to note that we previously found HFP birds to respond more actively to behavioral tests, suggesting they are less fearful compared to LFP birds (van der Eijk et al., 2018), which is opposite to what would be expected based on the higher relative abundance of *Lactobacillus* in LFP birds. Thus, it remains to be determined whether differences in gut microbiota composition are causal to behavioral and physiological differences between the FP selection lines.

Gut microbiota composition is influenced by many factors, including endogenous and exogenous ones, such as genotype and diet (Spor et al., 2011). Thus, divergent selection on FP might cause the differences seen in gut microbiota composition. Our findings are supported by a previous study using the FP selection lines, where bacteria within the order Clostridiales showed increased relative abundance, but bacteria of the genus *Lactobacillus* showed reduced relative abundance in cecal droppings of HFP birds compared to LFP birds (Birkl et al., 2018). It is striking that these findings were consistent across studies, suggesting a strong influence of FP genotype on microbiota composition. It is further interesting to note that FP genotype contributed to explaining the observed variation in luminal microbiota composition, but not in mucosa-associated microbiota composition. Yet, luminal microbiota composition might be more determined by environmental effects such as diet, whereas mucosa-associated microbiota composition might be more determined by host genetics (van den Abbeele et al., 2011). A possible explanation for this discrepancy is that differences in luminal microbiota composition might arise because of feather eating in the HFP line. Previous studies have shown that HFP birds ingest more feathers compared to LFP birds (Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2009). Furthermore, the FP selection lines differed in ileal and cecal microbial metabolites and HFP birds had a higher number of feather particles in their gizzards compared to LFP birds (Meyer et al., 2013). Moreover, birds fed feathers in their diet differed from control birds in microbial metabolites and microbial composition, with a higher number of enterobacteria in the ileum and cecum, and a higher number of clostridia in the cecum (Meyer et al., 2012). Thus, feather eating might alter microbial composition. Interestingly, HFP birds had a higher relative abundance of *Nesterenkonia* in the ileum at adult age. *Nesterenkonia* species have been found to produce alkaline proteases which can hydrolyze keratinaceous substrates, such as feathers (Gessesse et al., 2003; Bakhtiar et al., 2005). Although we did not identify the level of feather eating or feather particles in the gut, HFP birds showed more FP compared to LFP birds throughout the experiment (van der Eijk et al., 2018). Further research is needed to identify whether differences between the FP selection lines in gut microbiota composition might be caused by feather eating.

In conclusion, FP phenotypes did not differ in luminal or mucosa-associated microbiota composition in the present study. However, birds from lines divergently selected for high respectively low FP differed in luminal, but not mucosa-associated, microbiota composition. Whether differences in microbiota composition are causal to FP or a consequence of FP (i.e., feather eating) remains to be elucidated.

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