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Quantitative Genetic Background of the Host Influences Gut Microbiomes in Chickens

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Host genotype and gender are among the factors that influence the composition of gut microbiota. We studied the population structure of gut microbiota in two lines of chickens maintained under the same husbandry and dietary regimes. The lines, which originated from a common founder population, had undergone 54 generations of selection for high (HW) or low (LW) 56-day body weight, and now differ by more than 10-fold in body weight at selection age. Of 190 microbiome species, 68 were affected by genotype (line), gender, and genotype by gender interactions. Fifteen of the 68 species belong to *Lactobacillus*. Species affected by genotype, gender, and the genotype by gender interaction, were 29, 48, and 12, respectively. Species affected by gender were 30 and 17 in the HW and LW lines, respectively. Thus, under a common diet and husbandry host quantitative genotype and gender influenced gut microbiota composite.

T he gut microbiota is a complex ecosystem that has a symbiotic relationship with its host. Their interactions affect the physiological, immunological, and nutritional status of the host. A key question in understanding interactions between the host and gut microbiota is if the genetic background and gender of the former influences the population structure of gut microbiota when fed a common diet. The role of host genetics on shaping this vital 'microbial organ' is not clear[1]. Although environmental[2] and maternal effects[3–5] can affect the composition of the microbiota, results from human twin studies are equivocal[6,7]. Evidence from zebra fish and mice showed that the host influences the diversity and population of gut microbiota[8]. Moreover a single gene difference in the host can affect the population structure of gut microbiota[4,9–11]. The genotype of the host may affect its microbiota composition either directly through secretions into the gut, control of gut motility and modification of epithelial cell surfaces, or indirectly, through food and lifestyle preferences. These effects are likely to be small, and detecting them will require well controlled effects other than those of the host genotype. Thus, choosing a model organism maintained in essentially an identical environment with less maternal effects could enhance our understanding host genotype effects on gut microbiota.

Most host phenotypes involve complex traits that are controlled by multiple genes involving gene networks. There is a dearth of research related to quantitative genetic influences on gut microbiota. In order to address this issue and reduce noise, we conducted an experiment using two lines of chickens that had undergone long term divergent selection for the single trait, 56-day high (HW) or low (LW) body weight (Fig. 1). The lines originated from a common founder population and have complete pedigrees[12,13]. Throughout all generations, they have been maintained at the same location and reared on the same diets. Selection has resulted in more than a 10-fold difference between them for body weight at selection age. The maximum inbreeding coefficients (F) are 0.53 and 0.61, with a mean of 0.26 (SD 0.15) and 0.30 (SD 0.17) in LW and HW lines, respectively[13]. QTL mapping revealed 13 loci affecting growth in these two lines, however each locus explained only a small additive effect for this large phenotypic difference[14]. This moderate F combined with QTL results suggest that there is genetic diversity within
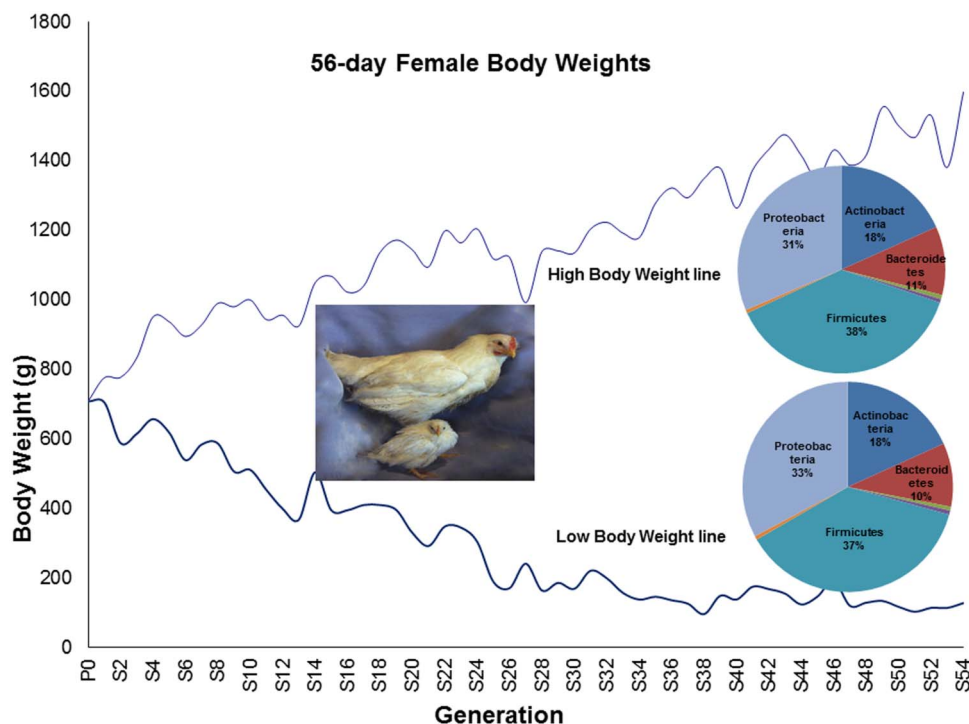
Figure 1 | Generation means at selection age (56-day body weight) for high (HW) and low (LW) line females and the distribution for the gut microbiomes composite for HW and LW lines at the phylum level.

each line and the 10-fold body weight difference is because of accumulation of quantitative genes instead of single gene mutation. The detailed information of these two lines was provided in the supplemental section of description of high and low body weight lines of chickens. Aided by next generation sequencing technology, we investigated the population structure of the gut microbiota in adults of these two lines to address if the quantitative genetic background of the host influences gut microbiota.

Results

Fecal samples from 56 chickens which were comprised of 12 LW females, 14 LW males, 15 HW females, and 15 HW males were collected. In order to compare which 16S rRNA variable regions are more suitable to be used for identify taxonomy from chicken feces, a pilot experiment was performed. DNA from 5 fecal samples from each group of LW females, HW males, and HW females, totally 15 samples were used in the pilot experiment. Fragments encompassing V3, V4, V1–V3, and V4–V6 16S rRNA hypervariable regions were PCR amplified from each of those 15 DNA samples. PCR production of V3 and V4 were sequenced by Illumina miseq, and V1–V3 and V4–V6 were sequenced by Roche's GS FLX+. Taxonomy at species rank was classified by best hit classification function in MGRAST[15]. We identified 57, 190, 159, 97 species in at least three samples by V3, V4, V1–V3, V4–V6, respectively. These results are consistent with those showing that V3 underestimates species richness and V4 provides estimates similar to those obtained with the nearly full-length fragment[16]. The number of species identified by combination of these hypervariable regions are presented in Supplementary table S5. Thus, V4 of 16S rRNA were used for the rest of our study. A total of 1,141,781 of V4 16S rRNA sequences reads from the 56 samples with an average of 20,389 sequences reads for each sample (the minimum # of one sample was 7,590 and the maximum was 32,877) were used for this project. The average length of sequence reads was 228 bp, and they were classified into different taxonomy using MGRAST[15] (http://metagenomics.anl.gov/). The taxon abundance of each sample was generated into phylum, class, order, family, genera, and species levels using mainly database of

RDP, aided by Greengene, and SSU databases. Taxonomies present in at least 14 samples were considered as common and their abundance count of samples were used for further analysis. At the phylum level, the % distribution of microbiome community of HW and LW chickens, respectively was 38 and 37 for Firmicutes, 31 and 33 for Proteobacteria, 18 and 18 for Actinobacteria, 11 and 10 for Bacteroidetes, and less than 1 for Deinococcus-Thermus, Euryarchaeota, and Fusobacteria (Fig. 1). At the phylum level, the lines were similar ($p > 0.05$) for fecal microbiome microbial community structure; a pattern that was consistent until the family level (Fig. 1, and Supplementary Fig. S1). Because both lines originated from the same founder population with the major difference being that they were selected 54 generation for high or low body weight at 56 days of age, a trait controlled by quantitative genes, suggests the limitation of host quantitative genetic background influences on the population structure of gut microbiome from the phylum to family levels.

Diversity of the fecal microbiome microbial community at the genera and species level. To further characterize the changes in microbiota imposed by the host genotype, 16S rRNA were classified taxonomically to the genera and species levels. For statistical analysis to detect effects by host quantitative genotype and gender, 103 genera were used. Of these 103 genera, 38 were affected by genotype, gender, and genotype by gender interactions ($p < 0.05$). Among these genera, 16 genera were affected by genotype, 26 by gender, and 8 by the genotype by gender interaction (Supplementary Table S3). Principle component analysis (PCA) of HW and LW samples of the 16 genera did not clearly separate the lines. Therefore, for further analysis, 190 species were used and 68 were affected by genotype, gender, or genotype by gender interactions ($p < 0.05$). Species affected by genotype ($p < 0.05$), gender, and genotype by gender interactions were 29, 48, and 12, respectively (Table 1). Using the 29 significantly affected species, it was possible to separate a majority of the LW from the HW samples (Fig. 2b) demonstrating a host quantitative genotype influence on the microbiome population structure.

Table 1 | ANOVA for species abundance

| Genus	species	p value (* p < 0.05, ** p < 0.01)			heritability
		genotype	sex	genotype*sex	
Lactobacillus	*acidophilus*	0.267	*0.000**	0.296	0.00
	agilis	0.013*	0.022*	0.012*	0.00
	coleohominis	0.038*	*0.001**	0.218	
	crispatus	0.492	0.039*	0.417	
	delbrueckii	*0.000**	0.010**	*0.000**	0.00
	gallinarum	0.859	*0.000**	0.049*	0.30
	hilgardii	0.029*	0.894	0.275	0.08
	intestinalis	0.615	0.305	*0.003**	0.37
	johnsonii	0.740	*0.002**	0.969	0.30
	kitasatonis	0.871	*0.000**	0.628	0.73
	pontis	*0.000**	*0.000**	*0.001**	0.07
	reuteri	0.182	*0.001**	0.087	0.89
	saerimneri	*0.000**	0.398	0.421	0.13
	salivarius	0.036*	0.430	0.507	0.00
	vaginalis	0.022*	*0.001**	0.916	0.00
Lactococcus	*lactis*	*0.001**	*0.005**	0.510	0.61
Clostridium	*botulinum*	0.033*	0.135	0.136	0.09
	glycolicum	0.043*	*0.001**	0.129	
	hylemonae	0.032*	0.041*	0.966	
	josui	0.880	*0.006**	0.710	
	tertium	0.039*	0.850	0.044*	0.00
Streptococcus	*dysgalactiae*	*0.003**	0.027*	0.321	0.66
	mitis	*0.005**	0.959	0.491	
	parauberis	0.338	0.518	0.038*	
	suis	0.546	*0.007**	0.405	
Enterococcus	*aquimarinus*	0.667	*0.003**	0.376	
	casseliflavus	0.121	0.049*	0.450	
	italicus	0.261	0.031*	0.550	
Bacillus	*niacini*	0.147	*0.002**	0.133	
	psychrodurans	0.261	0.013*	0.245	
	thermoamylovorans	0.028*	0.739	0.909	
Blautia	*schinkii*	0.966	0.030*	0.375	
	sp. Ser5	0.626	0.048*	0.616	
	sp. Ser8	0.941	0.042*	0.738	
Brachybacterium	*paraconglomeratum*	*0.001**	*0.009**	0.029*	0.72
Acinetobacter	*calcoaceticus*	0.960	0.043*	0.146	
Actinobacterium	*Aac-100*	0.994	0.044*	0.778	
Actinomyces	*urogenitalis*	0.430	0.011*	0.018*	
Aeriscardovia	*aeriphila*	0.047*	0.629	0.307	0.49
Aerococcus	*viridans*	0.736	0.030*	0.711	
Aeromonas	*media*	0.843	*0.004**	0.794	
Agrobacterium	*tumefaciens*	0.627	0.573	0.044*	
Arcobacter	*butzleri*	0.214	0.047*	0.759	
	cryaerophilus	0.010*	0.619	0.177	
Arthrobacter	*sp. NyZ415*	0.355	0.031*	0.774	
Brevundimonas	*diminuta*	0.047*	0.037*	0.345	
Carnobacterium	*sp. St2*	0.174	0.188	0.010*	
Cellulomonas	*fimi*	0.251	0.023*	0.933	
Coprococcus	*catus*	0.341	0.013*	0.053	
Desulfovibrio	*fairfieldensis*	0.335	0.015*	0.599	
Erwinia	*rhapontici*	0.411	0.041*	0.226	
Fusobacterium	*necrophorum*	*0.005**	0.036*	0.574	0.55
Listeria	*grayi*	0.764	0.010*	0.680	
Lysinibacillus	*sphaericus*	0.026*	0.445	0.154	
Marvinbryantia	*formatexigens*	0.154	0.030*	0.141	
Melissococcus	*plutonius*	0.110	*0.005**	0.317	
Micrococcus	*luteus*	*0.009**	0.089	0.540	
Pantoea	*agglomerans*	0.023*	0.086	0.263	
Pediococcus	*pentosaceus*	0.014*	0.979	0.611	0.00
Psychrobacter	*glacincola*	*0.009**	0.402	0.118	0.42
Psychrobacter	*sp. PRwf-1*	0.022*	0.542	0.660	0.10
Rikenella	*microfusus*	0.146	0.048*	0.166	
Rothia	*dentocariosa*	0.509	*0.007**	0.828	
Rothia	*mucilaginosa*	0.018*	*0.001**	0.449	
Sarcina	*ventriculi*	0.078	*0.005**	0.195	
Sporosarcina	*ureae*	0.986	0.045*	0.204	
Veillonella	*atypica*	0.956	*0.001**	0.728	
Yersinia	*intermedia*	0.043*	0.923	0.035*	
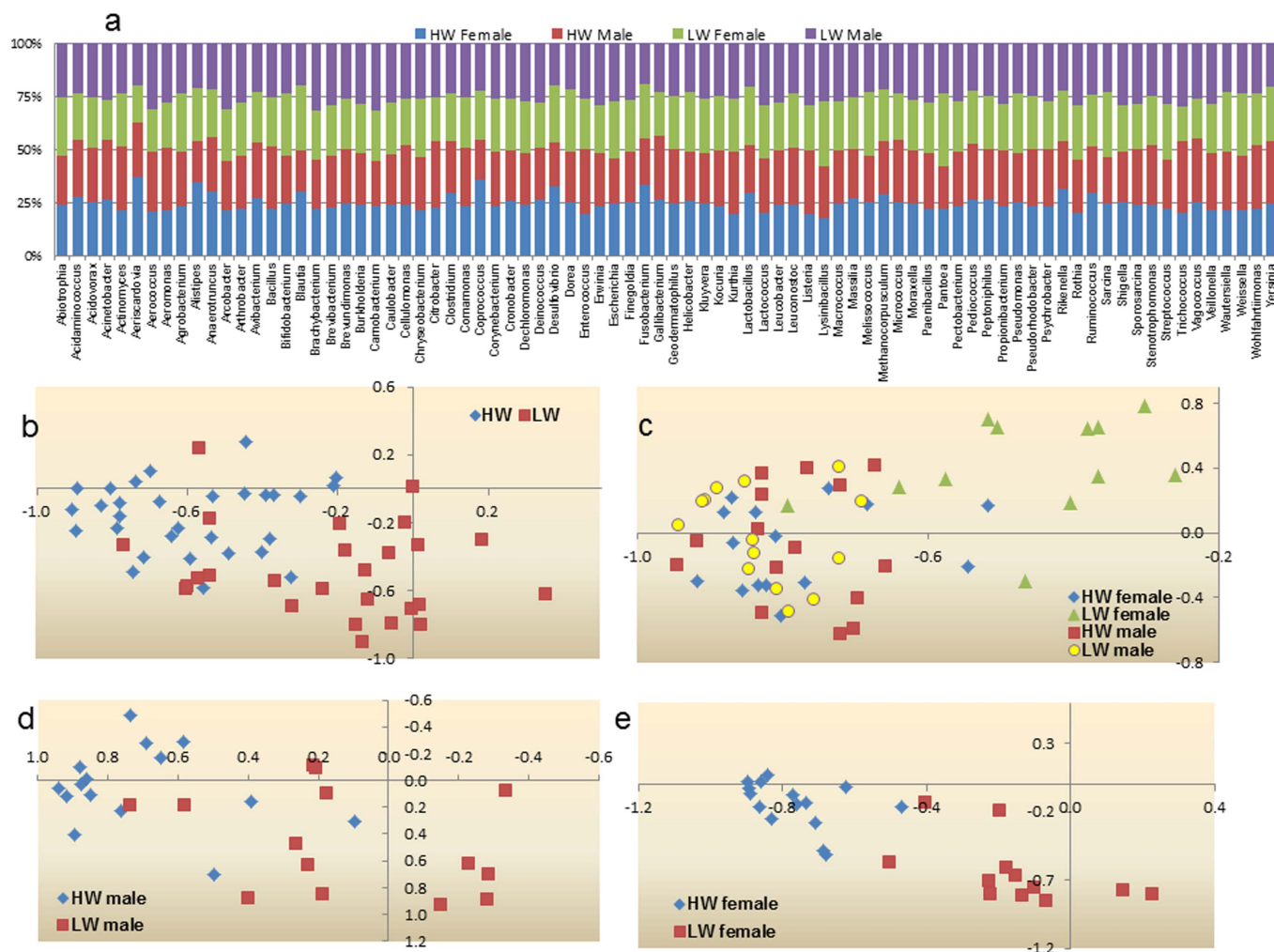
Figure 2 | Gut microbiota compositions for two lines (HW and LW) of chickens with different quantitative genotypes. (a) Distribution of gut microbiomes composite for HW females, HW males, LW females and LW males at the genera level. (b) The principal components analysis (PCA) plot of 56 samples from HW and LW lines using 29 gut microbiome species which were affected by quantitative genetics background. (c) PCA plot of 56 samples from HW and LW lines using 11 gut microbiome species belong to *Lactobacillus* which were affected by gender. (d) PCA plot of 29 samples from HW and LW males using 8 gut microbiome species which were affected by quantitative genetic background. (e) PCA plot of 27 samples from HW and LW females using 20 gut microbiome species which were affected by quantitative genetic background.

Because there are significant physiological and biological functions between adults, within line comparisons were made between genders. There were 20 and 8 species that differed for HW and LW females and males, respectively (Table 2) and the samples could be separated into categories which matched their host quantitative genotype (Fig. 2d, e). The separation was clearer for females than males suggesting that while the host quantitative genotype influenced the population structure of gut microbiota, within a line the target species were not necessarily the same for females and males.

Gender has a significant effect on gut microbiota composition. Forty-eight species were affected ($p < 0.05$) by gender with the pattern being line dependent (Table 1, and Supplementary Fig. S2). That is, 30 species were different ($p < 0.05$) between males and females in the HW line (Supplementary Table S1 and Supplementary Fig. S2) and 17 species in the LW line according to T-test (Supplementary Table S2 and Supplementary Fig. S2). Our data are from adult chickens where the females have a large demand for egg production. This major biological difference between adult males and females could easily contribute to gender differences in gut microbiota composition. The large difference between lines for body weight and their maintenances reflect a situation in which the daily food intake is much less for LW than HW individuals. However, their

mean percent egg production is similar (59 ± 11 in HW and 48 ± 13 in LW) (Supplementary Table S4) which could explain the line by gender interaction.

***Lactobacillus* is a major diversity genera.** Of the 68 significantly different abundance of species, 15 belong to *Lactobacillus*. From these 15 species via PCA we could identify LW females from all samples (Fig. 2c). Of the 20 species that were different ($p < 0.05$) between HW and LW females (Table 2), 6 belong to *Lactobacillus*. Tissue growth and reproduction are energy-dependent processes and *Lactobacillus* is involved in metabolites of bile acids which are related obesity and the metabolic syndrome[17–22], which also plays a key role in metabolites of phenolic, benzoyl, and phenyl derivatives which are related to weight loss[23,24]. Moreover, *Lactobacillus* could affect lipid metabolites which impact intestinal permeability and activate the intestine-brain-liver neural axis to regulate glucose homeostasis[25,26]. Thus *Lactobacillus* may impact LW females in food digestion, energy homeostasis, and energy-related metabolites in order to achieve the similar egg production even though their food intake is relatively less on a body weight basis than that for the HW line.

Host genetic factors shape individual microbiome species diversity. We agree that abundance of each species in gut micro-

Table 2 | Gender comparisons (T-test) between HW and LW lines for species abundance

| Genus | species | Relative fold change | p value (* p < 0.05, ** p < 0.01) |
		HW females/LW females	
Lactobacillus	*agilis*	2.51	*0.002***
	coleohominis	−1.38	0.029*
	delbrueckii	2.63	*0.000***
	intestinalis	1.91	0.019*
	pontis	−3.08	*0.000***
	saerimneri	1.57	0.049*
Lactococcus	*lactis*	−1.57	*0.002***
Clostridium	*botulinum*	1.80	0.024*
	glycolicum	1.35	0.032*
	tertium	2.50	0.011*
Aeriscardovia	*aeriphila*	2.10	0.021*
Enterococcus	*faecium*	−2.38	0.024*
Bacillus	*thermoamylovorans*	−1.13	0.045*
Chryseobacterium	*haifense*	−1.61	0.015*
Lysinibacillus	*sphaericus*	−1.64	*0.008***
Pediococcus	*pentosaceus*	1.28	0.045*
Psychrobacter	*glacincola*	−1.41	*0.005***
Rothia	*mucilaginosa*	−1.54	0.013*
Streptococcus	*dysgalactiae*	−1.32	0.016*
Weissella	*cibaria*	−1.31	0.028*

| Genus | species | Relative fold change | P value (* p < 0.05, ** p < 0.01) |
		HW males/LW males	
Lactobacillus	*saerimneri*	2.03	*0.003***
	vaginalis	−1.50	0.026*
Arcobacter	*cryaerophilus*	−1.57	0.018*
Brachybacterium	*paraconglomeratum*	−1.36	*0.001***
Carnobacterium	*sp. St2*	−1.85	0.025*
Enterococcus	*cecorum*	1.53	0.030*
Fusobacterium	*necrophorum*	1.43	0.038*
Streptococcus	*mitis*	−1.38	0.030*

+: HW/LW.
−: LW/HW.

biota can be treated as quantitative trait which is influenced by both environmental and host genetic factors[5]. In an effort to distinguish the proportion of host genetics factors in shaping individual microbiome species diversity, and because the chickens used in this experiment were pedigreed, we calculated the heritability for 23 species (13 belong to *Lactobacillus*) using REML algorithm by wombat[27] (Table 1). Although some of the heritabilities were high, none was significantly different from 0. This may be because the size of population was not large.

Discussion

In individual vertebrates, the species composition and their relative proportion in gut microbial communities vary and are influenced by both the environmental and genetic background of the host. Because external factors such as diet, husbandry, maternal, and litter effects can influence this complex ecosystem, they could dilute or mask the impact of the host's genetic background. Thus, in order to study the host quantitative genetic background that may influence the composition of the gut microbiota, we needed to control for these environmental factors. In this paper, the HW and LW lines of chickens are used as a model. These lines originated from a common founder population and were maintained in a similar environment for the 50+ generations with the variable being selection for either high or low 56-day body weight. Thus, they address the impact of host quantitative genetic background to the population structure of the gut microbial community.

We treated abundance of species in the gut microbial community as a quantitative trait to address how host background influenced the composition of gut microbiota. Using this strategy, it is obvious that variation in some species is influenced by the quantitative genetic background of the host. The pattern of host genetic influence is different in adult males and females demonstrating gender as a factor that impacts the composition of gut microbiota. Of host-microbe interactions, *Lactobacillus* is a major genera suggesting that *Lactobacillus* plays a key role related to phenotype of body weight[28]. *Lactobacillus* has a long history as an exogenous probiotic. In our study, we also found that LW females had differing levels of *Lactobacillus* compared to HW females. Recently, it was reported that feeding laying hens with 0.6% metabolite combinations of *Lactobacillus* in the diet improved egg production[29]. Also, some species of *Lactobacillus* accelerated gonadal differentiation in zebrafish[30]. Although lacking of direct evidence showing how specific species in *Lactobacillus* contribute to egg production in LW, it is possible that there is an association.

Methods

Animals and sample collection. Protocols used for this experiment were consistent with those approved by the Institutional Animal Care and Use Committee at Virginia Tech. At 245 days of age fecal samples were obtained randomly from 15 males and 15 females from generation 54 of the HW and LW lines. These 60 individuals were random samples from the larger population of their generation which had been under similar husbandry conditions. All were housed in individual cages (dimensions of 48 × 28 × 46 cm) in the same room with at 14:10 light:dark photoperiod. By keeping each chicken in an individual cage with wire floors, we prevented uncontrolled particle intake as well as feathers which may influence microbiota[31]. Chickens were fed a corn-soybean non-pathogen free diet in mash form. Details are provided in supplement of breeder diet ingredients. Cages had sloping wire floors with papers beneath them to collect feces. At 10:00 am papers beneath the cages were replaced and

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during the next hour fresh fecal samples were collected and immediately stored at 4°C with long term storage at −70°C.

DNA Extraction, PCR Amplify 16S rRNA, amplicon sequence and sequence data processing. Microbial genome DNA was extracted from fecal samples using QIAamp DNA stool mini kit (QIAGEN, cat#51504) following the manufacture's recommendation. The V3, V4, V1–V3, and V4–V6 hypervariable regions of 16S rRNA were PCR amplified from microbial genome DNA which were harvested from fecal samples using barcoded fusion primers (forward primers: 5′ACTCCTACGGGAGGCAGCAG3′, 5′AYTGGGYDTAAAGNG 3′, 5′AGAGTTTGATCCTGGCTCAG3′, and 5′GTGCCAGCMGCNGCGG3′, reverse primers: 5′TTACCGCGGCTGCTGGCAC3′, 5′TACNVGGGTATCTAATCC 3′, 5′TTACCGCGGCTGCTGGCAC3′, 5′GGGTTNCGNTCGTTG3′ for V3, V4, V1–V3, and V4–V6, respectively). The annealing temperature and extend time are 63.8°C, 30 sec, 42.3°C, 30 sec, 55°C, 60 sec, and 55°C, 60 sec for V3, V4, V1–V3, and V4–V6, respectively. The PCR condition were 94°C for 5 min; 94°C for 30 sec, annealing temperature for 30 sec and 72°C for extend time, repeat for 30 cycles; 72°C 7 min. PCR product was excised from a 1.5% agarose gel and purified by QIAquick Gel Extraction Kit (QIAGEN, cat# 28706). Barcoded V4–V6 and V1–V3 amplicons were sequenced by Roche GS FLX+. Barcoded V3 and V4 amplicons were sequenced using pair-end method by Illumina Miseq with a 6 cycle index read. Sequences with an average phred score lower than 25, containing ambiguous bases, homopolymer run exceeds 6, having mismatches in primers, or sequence length shorter than 100 bp were removed. For V3 and V4 pair-end reads, only sequences that overlap longer than 10 bp and without any mismatch were assembled according to their overlap sequence. Reads which could not be assembled were discarded. Barcode and sequencing primers were trimmed from sequence (V1–V3, and V4–V6) or assembled sequence (V3, and V4). Trimmed sequences were uploaded to MGRAST for further study.

Taxonomy classification and statistical analysis. Each sample's trimmed sequence was compared to the RDP, Greengene, and SSU databases using the best hit classification option to classify the abundance count of each taxon. The classification parameters were 8 for maximum e-value cutoff, 98 for minimum % identity cutoff, and 120 bp for minimum alignment length cutoff. This process was archived by MGRAST[15]. The metagenome sequences used in this paper are publicly available from the MGRAST (http://metagenomics.anl.gov/).

The abundance count was transformed by log2, and then normalized as follows: From each log transformed measure, the arithmetic mean of all transformed values was subtracted and the difference divided by the standard deviation of all log transformed values for the given sample. After this procedure, the abundance profiles for all samples will exhibit a mean of 0 and a standard deviation of 1. ANOVA was performed by stats package in R following the model of Y = genotype + gender + genotype by gender + error. T-tests were perform by Microsoft excel and all p-values were adjusted by FDR using the BH method by mt.rawp2adjp function in R (http://faculty.mssm.edu/gey01/multtest/multtest-manual.pdf). Heritability and its estimation accuracy were calculated using AI-REML algorithm by wombat (didgeridoo.une.edu.au/km/wombat.php).

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Author contributions
W.C., H.M., P.S. and Y.Z. contributed to the initial design of this project. H.M., P.S., H.W. and Y.Z. collected samples. Z.S., F.T., G.W., H.W., H.Z., J.Z., L.Z., W.Z. and Z.Z. conducted the experiment and next generation sequence with guidance from W.C. and H.M. C.H., L.Z. and Y.Z. conducted bioinformatics analyses. H.M., P.S. and Y.Z. prepared the manuscript of this publication.

Additional information