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Impacts of Excreta Exposure and Age on Ileal Microbial Communities, Intestinal Permeability, and Corticosterone in Hens Housed in Enriched Colonies and Cage-Free Housing Systems †

Benjamin J. Altendorf [1], Chiron J. Anderson [1,2], Isabella von Seggern [1], Maddison L. Wiersema [1], Stephan Schmitz-Esser [1] and Dawn A. Koltes [1,*]

[1] Department of Animal Science, Iowa State University, Ames, IA 50011, USA; wiersema@iastate.edu (M.L.W.); sse@iastate.edu (S.S.-E.)
[2] Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, IA 50011, USA
* Correspondence: delkins@iastate.edu; Tel.: +1-515-294-5694
† This article is part of the Master's thesis from the author, Maddison L. Wiersema.

Abstract: To tease apart differences between conventional cage (CC) and cage-free (CF) housing systems, this study focuses on the effects of excreta exposure and age by comparing microbial communities, intestinal permeability, and corticosterone in hens in enriched colonies (EC) and CF housing systems during early- and late-lay. Hens were randomly selected from two rooms of CF (n = 20) and EC (n = 20) at 35 and 76 weeks of age. One hour following an oral gavage of fluorescein isothiocyanate dextran (FITC-D), hens were euthanized, and ileal contents and blood were collected. Serum FITC-D using a fluorescent spectrophotometer and corticosterone using a commercial competitive ELISA kit were analyzed. Following DNA isolation from the ileum contents, the V4 region of the 16S rRNA gene was sequenced. Sequence data were filtered in Mothur v1.43.0, followed by *de novo* operational taxonomic unit (OTU) clustering and classifying with the SILVA SSU v138 reference database. Serum FITC-D was altered by housing type, age of hens, and the interaction between housing type and age of hens ($p < 0.001$), with 76-week-old hens housed in EC having the highest FITC-D. Corticosterone increased with age ($p = 0.023$). Microbial community diversity measurements favored hens housed in the CF housing system as ileal contents tended to have increased species evenness ($p = 0.008$) and greater alpha diversity ($p = 0.006$). The majority of the over-representation of OTUs were associated with peak lay.

Keywords: laying hen; housing system; intestinal permeability; corticosterone; microbial community

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1. Introduction

As the egg industry continues to transition to cage-free (CF) housing systems to meet consumer demands [1,2], much remains unclear regarding how these systems alter the physiology of the hens living in them. As hens have increased mobility, hen interactions, and exposure to excreta, their nutrient requirements, skeletal health, basal stress, immune function, and intestinal microbe resilience will likely be altered [3]. Understanding these differences will be critical for maximizing efficiencies and animal welfare. However, when comparing CF to conventional cage (CC) housing systems, it is difficult to tease apart the effects of mobility, hen interactions, and exposure to excreta.

Enriched colony (EC) housing systems are limitedly used in the United States but represent an intermediate housing system between CF and CC housing systems. From a hen housing system design, the EC housing system allows for increased floor space and an increased number of hens per colony compared to caged but less compared to CF. Additionally, similar to CC in an EC housing system, excreta are collected on a manure belt. In commercial settings, EC housing systems are considered to have overall performance that is intermediate to CC and CF. Hens in EC have similar body weight and feed

intake compared to CC, increased hen day egg production compared to both systems, and decreased mortality compared to CF [3,4]. Therefore, the EC housing system provides an ideal environment to tease apart physiological changes associated with social interactions and physical activity compared to interactions with excreta, which can provide insight into the physiological underpinnings of these two factors.

A major difference between EC and CF is the ability of hens to interact with the environment through contact and ingestion of excreta [5,6]. This ingestion can alter microbial presence and diversity [7–11], and given that many disease-causing pathogens, e.g., Eimeria [12], are transmitted through oral–fecal routes, this increased exposure may lead to chronic inflammation, dysbiosis, or enteric disease. Liu et al. [13] observed that during direct exposure of avian hepatitis E virus to hens in CF and CC systems, hens from CF systems had significantly higher serology and excreta shedding. With the advancement of novel sequencing methods, this has increased our understanding of intestinal bacterial communities and has been used to examine pathogenic bacteria, human food-borne pathogenic bacteria, and beneficial bacteria; however, limited studies have examined the relationship between microbial communities in laying hens across housing systems [8,9,14], and even fewer have examined the impacts of time within housing systems [10,14].

This study was a follow-up study to the Wiersema et al. [8] and Anderson et al. 2023 [9] manuscripts which examined the impact of housing system type on intestinal health and microbial communities in commercial laying hens. In this commercial setting, known and novel roles of microorganisms in poultry health were identified. Due to the commercial setting, many questions regarding the age of hen, breed differences, and management differences were not addressed. Additionally, it was not possible to determine if factors were a response to differences in social behaviors and movement or exposure to excreta. Therefore, to dissect the impact of increased exposure to excreta, this study examines the differences in bacterial communities, whole intestine permeability, and circulating corticosterone concentrations between EC and CF hen housing systems during peak and late-lay in a research setting to control for differences in management practices and diet. Additionally, this study examines associations between resident microbes and these health traits

2. Materials and Methods

2.1. Animals

All procedures involving animals were approved by Iowa State University's Institute of Animal Care and Usage Committee (IACUC number 20-033).

At 18 weeks of age (woa), Hy-Line W-80 pullets were housed in group cages at the Robert T. Hamilton Poultry Teaching and Research Farm in March of 2020. At placement, 558 hens were placed in each of the two AVECHII enriched colony rooms (EC), and 2162 hens were placed in each of two NutraSTEP cage-free system rooms (CF), where each room contained one commercial section of each housing unit. Hens underwent typical production practices throughout an 85-week production cycle. At 35 (peak lay) and 76 (late-lay) weeks of age, 20 hens were randomly chosen and weighed from two different rooms of either a CF (n = 40) or EC (n = 40) housing system at each time point.

2.2. Intestinal Permeability

Intestinal permeability was measured in hens selected from CF and EC systems using optimized parameters by Baxter et al. [15]. Briefly, 18 hens were orally gavaged with fluorescein isothiocyanate–dextran (FITC-D; Sigma Aldrich, FD4, Burlington, MA, USA) at a rate of 8.32 mg/kg per room per time point. Two hens per room per time point were not gavaged and were collected for control serum. One hour after hens were gavaged with FITC-D, hens were euthanized via carbon dioxide asphyxiation. Blood was collected from the femoral artery into serum blood collection tubes (BD367815; Fisher Scientific, Hampton, NH, USA). Serum was separated by centrifugation at 1000$\times$ *g* for 15 min, then aliquoted and stored at -80 °C in amber tubes. All samples from hens were diluted at a ratio of 1:5 in saline. Serum from control hens was pooled and used to generate a standard curve.

All diluted samples from hens given FITC-D were plated in triplicate. Fluorescence was measured using a BioTek Cytation fluorescence spectrophotometer (BioTek US, Winooski, VT, USA) with excitation and emission wavelengths of 485 and 528 nm, respectively. For data analysis, triplicates were averaged for each hen.

2.3. Corticosterone ELISA Assay

A subset of serum samples collected for intestinal permeability measurements was used to determine circulating corticosterone in hens following euthanasia. A total of 89 samples (19 early-lay EC, 19 early-lay CF, 18 late-lay EC, 33 late-lay CF) were used for this assay. Selection criteria were based on the amount of serum remaining after FITC-D. Corticosterone was determined using the commercial competitive ELISA following the manufacturer's protocol (K014, Arbor Assays, Ann Arbor, MI, USA). Serum samples were run in duplicate and averaged for statistical analysis.

2.4. Characterization of Bacterial Communities and Sequence Analysis

Bacterial communities were determined from luminal ileal contents in hens selected from CF and EC systems using procedures outlined in Wiersema et al. [8]. Briefly, ileal luminal contents were aseptically removed from a 5 cm section from the ileal cecal junction. Samples were transported to campus and stored at −80 °C until DNA isolation. DNA was extracted using the Qiagen Powerlyzer soil kit following the manufacturer's recommendations. Due to a lack of digesta or low DNA concentrations following extraction, 27 samples were excluded from sequencing. A total of 133 samples were used to amplify bacterial and archaeal 16S rRNA genes. Samples were sequenced using 250 bp paired-end reads for each sample of the V4 region of the 16S rRNA gene (515F, 806R; [16,17]) at the Iowa State University DNA Facility using Illumina MiSeq sequencing technology.

Sequence analysis of 16S rRNA amplicon sequences was completed with mothur V1.43.0 following the mothur [18] MiSeq Standard Operating Procedure [19]. Data were analyzed similar to previous studies [8,9]. Briefly, paired-end reads were merged and quality filters applied using the "make.contigs" and "screen.seqs" commands in mothur. Chimeric sequences were removed with the "Chimera.vsearch" command. For alignment and taxonomic classification of operational taxonomic units (OTUs), the SILVA SSU NR reference database (V138) provided by the mothur website was used. Sequences were clustered into *de novo* OTUs with a cutoff of 99% 16S rRNA gene similarity (=0.01 distance). NCBI BLAST was used on representative sequences to assign additional OTUs taxonomic classification (e.g., genus and species information).

To compare alpha diversity between experimental groups, reads were randomly subsampled to accommodate the sample with the lowest number of reads across data sets (10,114 sequences). Measurements of Chao species richness, Shannon diversity, and Simpson evenness were taken to compare community structures between experimental groups. A principal coordinates analysis (PCoA) plot based on Bray–Curtis distances was generated to visualize differences in community composition between housing system, hen age, and the interaction between housing system and hen age. Numerical comparisons of community structure between housing system, hen age, and the interaction between housing system and hen age were performed using a permutational multivariate analysis of variance and permutational multivariate analysis of dispersion using the Adonis and betadisper commands of the R package vegan v2.5.7 [20].

All plotting was completed using ggplot2, v2_3.1.1 graphing package [21,22] in R 3.6.0. The overall variation in bacterial communities was visualized using PCoA. This information was generated with the Phyloseq (v1.28.0, [23]) and Vegan (v2.5-5, [20]) R packages. The 16S rRNA gene sequences were submitted to the NCBI Sequence Read Archive SRA and are available under the BioProject ID PRJNA1055184. All other data are available from the authors upon request.

2.5. Statistical Analysis

Differences for intestinal permeability and corticosterone concentrations were determined using PROC Glimmix in SAS [24] with housing type and age and their interaction fit as fixed effects and room fit as a random effect. Significance was set at a $p < 0.05$. To determine if specific bacterial OTU abundances were significantly different across housing type, age, and their interaction, data were normalized using the trimmed mean of the M-value (TMM; [25]) for the top 100 OTUs with at least 2 reads in 50% of the samples. Data were then analyzed using PROC Glimmix in SAS for each OTU following a negative binomial distribution and using housing type, age, and their interaction as a fixed effect [24] and room fit as a random effect. q-values were used to control for false discovery rate using the q-value package in R [26]. For OTUs, significance was set at a $p < 0.05$ and $q < 0.05$. To determine potential beneficial or detrimental bacterial communities, correlations were determined between bacterial communities and intestinal permeability or corticosterone concentration using PROC CORR within age and housing type [24]. Significance was set at an $r^2 > |0.35|$. All correlations are included in Supplemental File S1.

3. Results and Discussion

3.1. Animal Parameters

No interaction between housing type and week were observed for body weight ($p = 0.87$; Table 1). The overall body weights between CF and EC housing types were similar over the course of this study ($p = 0.33$). During the peak-lay period, hen body weights were similar between the two housing types ($p = 0.53$). Additionally, hen body weights were similar between CF and EC housing types at late-lay ($p = 0.66$). However, the effect of week was significant ($p < 0.050$) as hens had heavier body weights during the late-lay sampling point. As anticipated, hens in both housing systems increased in body weight over time but were not different across housing systems [27].

Table 1. Least squared means for body weight, intestinal permeability, and serum corticosterone from hens housed in enriched cage and cage-free housing systems during peak and late-lay.

Parameter	Unit	Peak EC	Peak CF	Late EC	Late CF	*SEM*	Time	Housing	Interaction
Body weight	Kg	1.53	1.52	1.62	1.60	*0.03*	<0.05	0.33	0.87
Permeability	ng/mL	160.98 [b]	158.61 [b]	224.03 [a]	173.21 [b]	*5.97*	<0.001	<0.001	0.003
Corticosterone	pg/mL [1]	8241	10,521	11,599	12,732	*1325*	0.023	0.126	0.495
Corticosterone [2]	r^2	0.28	0.01	0.28	−0.28	- [3]	-	-	-

Abbreviations: EC, enriched cage housing system; CF, cage-free housing system; Peak, hen age at week 35; Late, hen age at 76 weeks; SEM, Standard error of the means. [1] Data were back-transformed following analysis. [2] Pearson correlation between corticosterone and intestinal permeability. [3] Not applicable. [a,b] Superscripts represent significant differences within the row ($p < 0.05$).

3.2. Intestinal Parameters

The macromolecular flux of FITC-D, an indigestible sugar, from the lumen of the intestine into circulation was altered by housing type, age of hens, and the interaction between housing type and age of hens ($p < 0.001$, Table 1). The amount of FITC-D observed in circulation was higher in the EC system compared to the CF system ($p < 0.001$). As hens aged, the amount of FITC-D also increased in circulation ($p < 0.001$). Additionally, the amount of FITC-D was the highest from hens during late-lay housed in the EC system ($p < 0.004$).

Differences between EC and CF housing systems were unexpected based on a previous study in our group between CC and CF housing systems [8]. It was anticipated during the design of this study that the EC and CC housing systems would be similar as hens would have limited space movement and limited exposure to excreta and hen interactions compared to CF. However, there are major differences between EC and CC housing systems, such as an increased number of hens which individual hens can interact with, enrichment areas, perches, and nest boxes in EC housing systems. While these differences

may contribute to this discrepancy, a major difference is that this study was conducted in a research setting (e.g., smaller bird numbers, increased space requirements) compared to the Wiersema et al. study that was conducted at a commercial egg farm [8]. At this time, a scale has yet to be established for measuring FITC-D, making it very difficult to interpret data across studies, and only comparisons within studies can be made.

Limited information on the effects of age on intestinal permeability in laying hens in commercial or research settings is known. In this study, intestinal permeability increased from week 35 to week 76 in hens from both the EC and CF housing systems. Drozdowski and Thomson observed a negative effect of aging on organ function and absorption of nutrients [28]; therefore, the increased intestinal permeability observed in this study in older hens would not be unexpected. A study by Gu et al. [29] measured intestinal permeability and egg quality of hens at various ages and found that late-lay hens had increased intestinal permeability as measured by serum d-lactate compared to peak-lay hens. In addition to identifying an increase in EC and late-lay hens, this study observed a greater increase in intestinal permeability of late-lay hens housed in EC compared to late-lay hens in CF. As this is a novel finding, it is unclear if there were additional factors under the research conditions in this study or if additional factors exist with the EC housing system that exacerbated the aging effects in the EC housing system.

3.3. Serum Corticosterone Concentrations

To better understand factors, such as stress, that could increase intestinal permeability with age, serum corticosterone was chosen to be determined retrospectively. In this study, serum corticosterone concentrations were similar across housing type ($p = 0.126$) but increased as hens aged ($p = 0.023$, Table 1). There was no interaction between age and housing type on serum corticosterone concentrations ($p = 0.495$). While serum corticosterone was measured post-mortem, which likely explains the elevated concentrations [30,31], the effect of euthanasia would be consistent for all hens. The lack of difference between housing systems is in contrast to previous reports where corticosterone was increased in hens from CC housing systems compared to cage-free and free range [32,33]. It should be noted that in both studies, they used brown laying hen lines, whereas this study used white laying hens. Despite measuring blood corticosterone over time, Franciosini et al. [33] did not examine the impact of hen age on corticosterone measurements. Additionally, Campbell et al. used feather instead of blood corticosterone measurements which provide an understanding of longer-term stress [32]. Therefore, while we did not observe similar results as others, the lack of differences may be a result of genetic line, age of hens, differences in housing systems, research setting, or changes in management strategies.

Given that serum corticosterone was measured to identify a potential underlying factor that contributes to intestinal permeably, correlations between corticosterone and intestinal permeability within housing system and age were determined (Table 1). No significant correlations were identified, which suggests that there are likely additional mechanisms that increase intestinal permeability between housing systems.

3.4. Ileal Microbial Communities

3.4.1. Taxonomic Assignment

After MiSeq sequencing, 132 samples resulted in 2,758,322 raw sequences. After removing low-quality sequences, 2,108,524 sequences remained, which were clustered into 19,127 OTUs. After the removal of sequences with less than 10 reads, 1284 OTUs were identified. Both the SILVA SSU NR reference database (V138) provided by the mothur website and NCBI Blast on representative sequences were used to assign OTUs taxonomic classification.

3.4.2. Alpha Diversity Measurements

There was a significant effect of housing type for species evenness (Simpson index; $p = 0.008$) and the overall alpha diversity (Shannon index; $p = 0.006$), whereas all other measures of species evenness (Simpson index), estimated richness (Chao1) and overall

alpha diversity which accounts for both evenness and richness (Shannon index) were similar between housing types, hen age, and the interaction between housing type and hen age ($p > 0.05$). For significant indices, the Simpson index of species evenness was higher for hens housed in CF systems than those housed in EC systems (Figure 1). The results from this study suggest that species evenness may be the component driving the changes in the Shannon index. The differences in alpha diversity with higher values in CF for both Simpson and Shannon indices suggest that increased exposure to excreta may allow for an even colonization of microbial communities due to the continuous exposure of organisms that either do not or limitedly colonize. However, this was not observed in our previous study where the Simpson index was found to be similar in hens housed in commercial CF and CC systems [8]. Additionally, the previous study found a trend for species richness to be greater in CF compared to the CC system. This difference in alpha diversity across this study and the Wiersema et al. [8] study are difficult to fully interpret as the biological implications of these indices are not fully understood. Additionally, the inconsistency in alpha diversity across data sets has also been observed with cecal microbial communities between CC and CF systems [7,10].

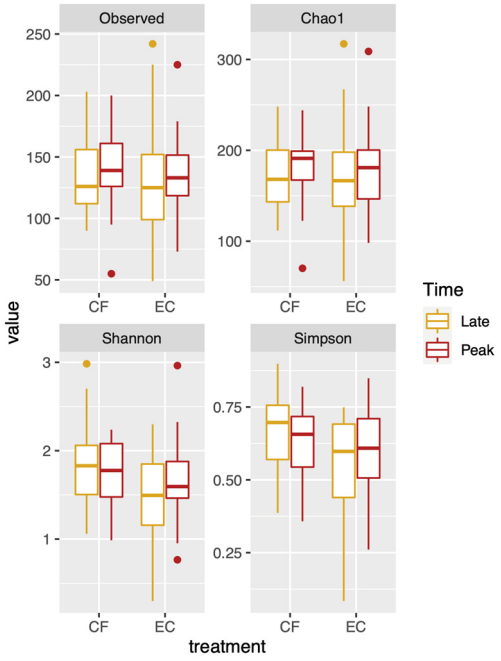


Figure 1. *Boxplots of the alpha diversity measurements of ileal microbiota from hens in late-lay and peak lay housed in cage-free (CF) and enriched colony (EC) systems.* Gold denotes the diversities from hens in late-lay, and red denotes the diversities from hens in peak lay. The parameters displayed are Observed which represents the number of different taxa observed in the sample, Chao1 which represents species richness within a sample, Shannon which represents the Shannon index or an estimate of both species richness and species evenness within a sample, and Simpson which represents species evenness within a sample.

3.4.3. Beta Diversity Measurements

Whole community Beta diversity comparisons of EC and CF, early and late stage of lay, and the interaction between housing systems and age for prokaryotic microbial community samples were made using PERMANOVA. The PERMANOVA test detected a significant difference ($p < 0.001$) in overall community composition between housing types and age, whereas the interaction between housing type and age was not significant ($p = 0.100$). Beta dispersion was similar for housing type and age ($p = 0.207$; $p = 0.581$, respectively). A principal coordinates analysis plot visualizing differences in overall community structure based on Bray–Curtis distances is provided in Figure 2, revealing separation between

centroids clustered by housing type. However, this was not observed in our previous study where hens from commercial CC had higher beta diversity compared to commercial CF systems [8]. This difference was also observed by Adhikari et al. in the cecal beta diversity of EC and CC [11]. These studies suggest that EC and CF are more similar compared to EC and CC and that social hierarchies and movement but not excreta may influence beta diversity. Additionally, Adhikari et al. observed differences in beta diversity across different time points in late-lay (<53 weeks of lay); however, these differences were highly dependent on the strain of the laying hen (Hy-Line Brown compared to Hy-Line W36) [11].

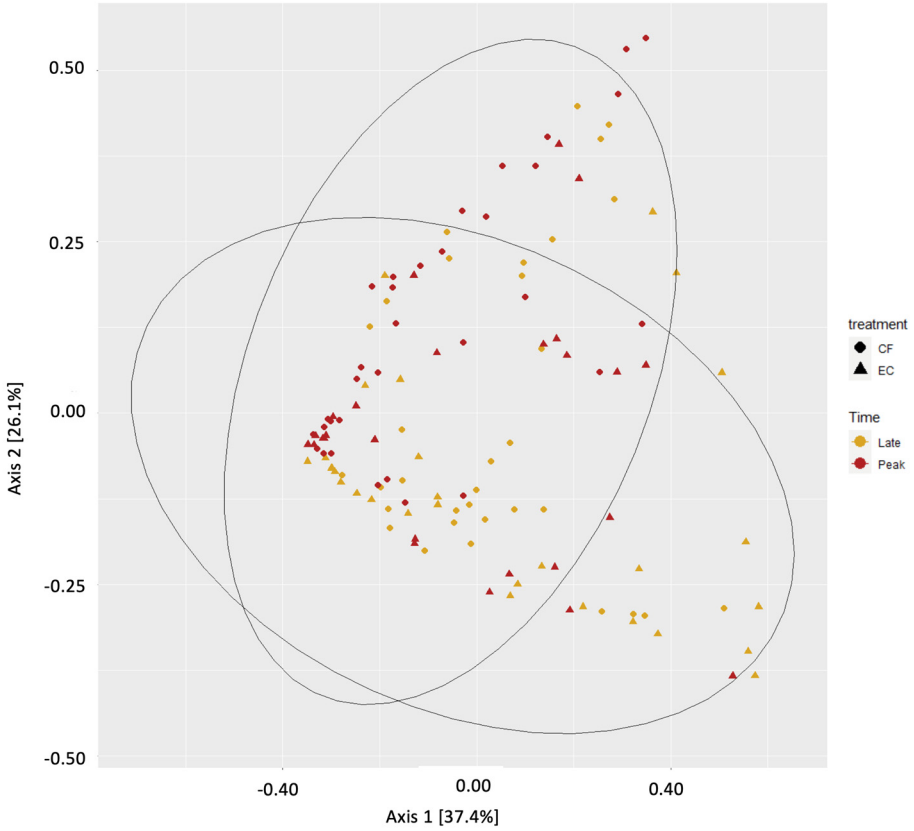


Figure 2. *Principal coordinate analysis comparing the ileal microbiota of hens in cage-free (CF) and enriched colony (EC) systems as well as hens in late-lay and peak lay.* Gold denotes hens in late-lay, and red denotes hens in peak lay. Circles denote hens in CF systems, and triangles denote hens in EC systems.

3.4.4. Ileal Microbial Communities across Housing Systems

At the phylum level, 17 phyla were identified from samples between both housing types. The top 10 phyla can be found in Figure 3A. The most abundant phyla were *Firmicutes* (also known as *Bacillota*; 97.4%), *Proteobacteria* (1.11%), *Actinobacteriota* (0.75%), and *Fusobacteriota* (0.40%). The major genera found in both housing types included mainly *Lactobacillus* (74.6%), *Romboutsia* (13.3%), *Streptococcus* (2.47%), *Turicibacter* (1.65%), *Clostridium_senso_stricto_*1 (1.28%), and *Enterococcus* (1.27%; Figure 4A). The relative abundances of phyla are similar to previously published ileal microbiome communities in laying hens [34,35] and the same as our previous manuscript [8]. While similar genera were observed in this study, the percentages were slightly different when compared to our previous study of commercial hens [8]. In this study, we observed an increase in *Lactobacillus* sp. of 29% and a decrease in *Romboutsia* sp. of 17%, which may be a result of different feed, housing systems, or management strategies.

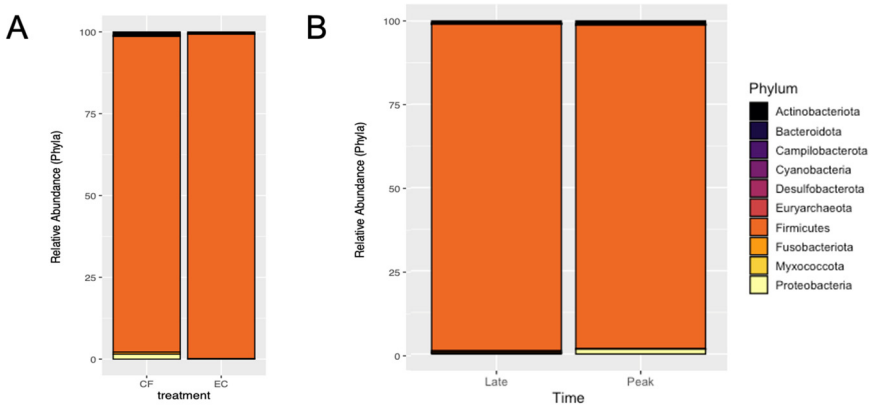


Figure 3. *Relative abundance of phyla of ileal microbiota of hens in cage-free (CF) and enriched colony (EC) systems as well as hens in late-lay and peak lay.* (**A**) Percent of relative abundance of phyla for housing system. (**B**) Percent of relative abundance of phyla for late-lay and peak-lay hens.

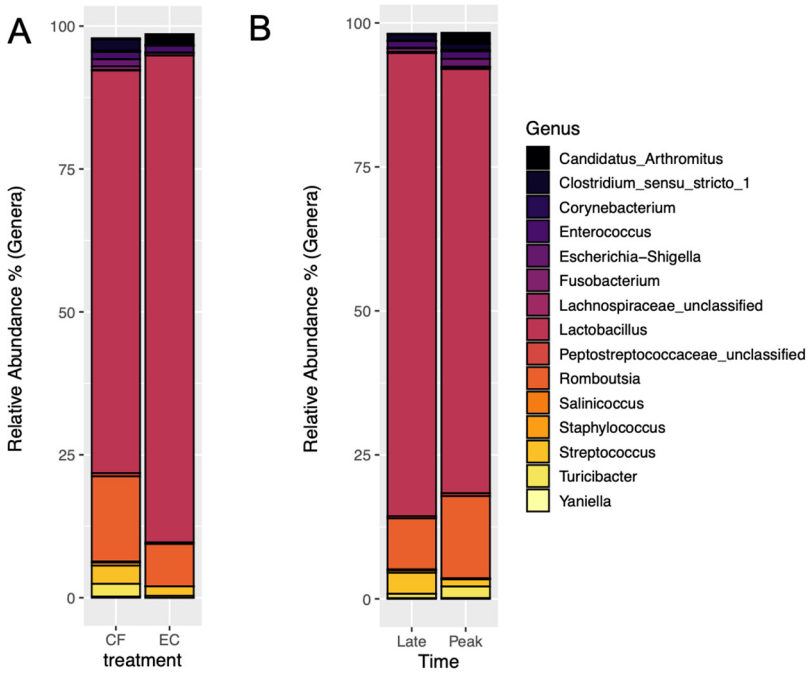


Figure 4. *Relative abundance of top 15 genera of ileal microbiota of hens in cage-free (CF) and enriched colony (EC) systems as well as hens in late-lay and peak lay.* (**A**) Percent of relative abundance of genera for housing system. (**B**) Percent of relative abundance of genera for late-lay and peak-lay hens.

In hens from the EC system, *Lactobacillus* was the most abundant genus (85.16%), followed by *Romboutsia* (7.40%) and *Candidatus Arthromitus* (a member of the segmented filamentous bacteria (SFB), 1.86%). In hens from the CF system, *Lactobacillus* was the most abundant genus (70.41%), followed by *Romboutsia* (14.87%) and *Streptococcus* (3.18%). The 15 most abundant genera and their relative abundances by housing system can be found in Figure 4B.

To determine specific OTU abundance differences across housing types, data were analyzed in SAS following abundance normalization, which accounts for the number of sequencing reads. Of the 100 OTUs analyzed, 7 OTUs were differentially abundant between housing types (Tables 2 and 3). Six OTUs were over-represented in CF compared to EC systems (Table 2). One of the OTUs was associated with a *Lactobacillus* species that aligned to the *Lactobacillus reuteri* sequence at higher than 98% using BLAST. A study on young chickens that were orally administered *Lactobacillus reuteri* showed that the administration of this species significantly enhanced lysozyme mRNA expression to improve intestinal

innate mucosal immunity when compared to the control group [36]. This suggests positive probiotic capability and the beneficial nature *Lactobacillus reuteri* has on the intestinal health of chickens. The other over-represented OTUs have not been studied in poultry.

Table 2. Operational taxonomic units over-represented in ileal digesta of hen housed in cage-free systems compared to enriched colony systems.

Group [a]	Fold Change [b]	*p*-Value	q-Value	Genus Taxonomy [c]	Taxonomy Based on NCBI BLASTn Search [d]
OTU00044	4.2552	1.95×10^{-7}	1.29×10^{-5}	*Staphylococcaceae_* unclassified	*Mammaliicoccus lentus*; *M. sciuri*
OTU00006	1.4212	0.0169	0.0382	*Lactobacillus*	*Lactobacillus vaginalis*; *L. reuteri*
OTU00030	12.7917	0.0063	0.0200	*Salinicoccus*	*Salinicoccus roseus*
OTU00051	16.8406	0.0127	0.0310	*Brachybacterium*	*Brachybacterium paraconglomeratum*
OTU00034	4.0001	0.0041	0.0150	*Corynebacterium*	*Corynebacterium casei*; *Corynebacterium ammoniagenes*
OTU00003	0.3166	0.0002	0.0022	*Romboutsia*	*Uncultured Romboutsia* sp.

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Fold change is expressed relative to an enriched colony system. [c] Taxonomic assignments are based on the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

Table 3. Operational taxonomic units over-represented in ileal digesta of hen housed in enriched colony systems compared to cage-free systems.

Group [a]	Fold Change [b]	*p*-Value	q-Value	Taxonomy [c]	Taxonomy Based on NCBI BLASTn Search [d]
OTU00012	0.9383	0.0023	0.0223	*Lactobacillus*	*Lactobacillus acidophilus*; *L. crispatus*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Fold change is expressed relative to a CF system. [c] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

The remaining OTU was over-represented in the EC system (Table 3). The OTU was aligned to the *Lactobacillus acidophilus* sequence at higher than 98% using BLAST [37]. This species has been isolated from the intestine of chickens and studied for its possible probiotic capabilities. Additionally, previous work from our lab identified that *Lactobacillus* sp. and specifically *Lactobacillus acidophilus* sequences were over-represented in hens housed in the CF housing system compared to hens housed in CC systems [8]. While the exact effect on commercial laying hens is still unclear, the dietary supplementation of *Lactobacillus acidophilus* has been shown to increase FCR and overall egg production [38].

Ileal microbial communities across stage of lay were analyzed. The majority of phyla were *Firmicutes* (97.4%), *Proteobacteria* (1.11%), *Actinobacteriota* (0.75%), and *Fusobacteriota* (0.40%; Figure 3B). In hens that were sampled during peak lay, *Lactobacillus* was the most abundant genus (73.65%), followed by *Romboutsia* (14.24%) and *Turicibacter* (1.99%). In hens that were sampled during late-lay, *Lactobacillus* was the most abundant genus (80.41%), followed by *Romboutsia* (8.85%) and *Streptococcus* (3.65%). The 15 most abundant genera and their relative abundances by stage of lay can be found in Figure 4B.

Of the 100 OTUs analyzed, 25 OTUs were differentially abundant between stage of lay (Tables 4 and 5). Nineteen OTUs were over-represented in peak lay compared to late-lay (Table 4). The majority of these OTUs comprised *Lactobacillus* sp. (11/19; 58%), and upon NCBI BLASTn search, several of the sequences aligned with *Lactobacillus crispatus* at higher than 98% using BLAST [37]. *Lactobacillus crispatus* has been observed to inhibit *Salmonella enteritidis*, and *Escherichia coli* showed promising results of *Lactobacillus crispatus* being a possible probiotic candidate [39].

Table 4. Operational taxonomic units over-represented in ileal digesta from peak-lay hens compared to late-lay hens.

Group [a]	Fold Change [b]	*p*-Value	q-Value	Genus Taxonomy [c]	Taxonomy Based on NCBI BLASTn Search [d]
OTU00044	5.3506	4.39×10^{-8}	1.41×10^{-6}	*Staphylococcaceae*_unclassified	*Mammaliicoccus lentus*; *M. sciuri*
OTU00085	4.6941	1.04×10^{-6}	1.67×10^{-5}	*Rothia*	*Rothia nasarium*; *R. terrae*
OTU00023	1.2685	7.77×10^{-5}	0.0008	*Lactobacillus*	*Staphylococcus equorum*
OTU00074	2.2309	0.0004	0.0028	*Lactobacillus*	*Lactobacillus saerimneri*; *L. ghanensis*
OTU00006	0.7515	0.0004	0.00281	*Lactobacillus*	*Lactobacillus vaginalis*; *L. reuteri*
OTU00051	2.8098	0.0008	0.0040	*Brachybacterium*	*Brachybacterium paraconglomeratum*
OTU00037	1.2035	0.0010	0.0045	Chloroplast_ge	*Campylotropis* sp.
OTU00012	0.8056	0.0016	0.0065	*Lactobacillus*	*Lactobacillus acidophilus*; *L. crispatus*
OTU00087	1.3369	0.0025	0.0088	*Streptococcus*	*Streptococcus pluranimalium*
OTU00039	0.9714	0.0034	0.0094	*Lactobacillus*	*Lactobacillus acidophilus*; *L. crispatus*
OTU00036	0.8861	0.0035	0.0094	*Lactobacillus*	*Uncultured Olsenella* sp.
OTU00083	1.2870	0.0043	0.0096	*Romboutsia*	*Uncultured Bacterium*
OTU00034	0.7768	0.0045	0.0096	*Corynebacterium*	*Corynebacterium casei*; *C. ammoniagenes*
OTU00088	1.4737	0.0056	0.0111	*Lactobacillus*	*Lactobacillus acidophilus*; *L.s crispatus*
OTU00052	2.1456	0.0094	0.0158	*Lactobacillus*	*Lactobacillus ingluviei*
OTU00058	1.1406	0.0123	0.0198	*Lactobacillus*	*Uncultured Prokaryote*
OTU00050	1.1645	0.0183	0.0280	*Veillonellaceae*_unclassified	*Veillonella seminalis*; *V. ratti*
OTU00071	0.7040	0.0226	0.0330	*Lactobacillus*	*Lactobacillus acidophilus*
OTU00040	0.6189	0.0376	0.0483	*Lactobacillus*	*Lactobacillus acidophilus*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Fold change is expressed relative to the late-lay system. [c] Taxonomic assignments are based on the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

Table 5. Operational taxonomic units over-represented in ileal digesta from late-lay hens compared to peak-lay hens.

Group [a]	Fold Change [b]	*p*-Value	q-Value	Genus Taxonomy [c]	Taxonomy Based on NCBI BLASTn Search [d]
OTU00011	0.3077	0.0028	0.0088	*Lactobacillus*	*Lactobacillus acidiophilus*; *L. cripatus*
OTU00007	0.6988	0.0042	0.0096	*Lactobacillus*	*Lactobacillus reuteri*
OTU00093	1.4180	0.0059	0.0111	*Enterococcaceae*	*Enterococcus faecium*
OTU00072	0.7234	0.0077	0.0137	*Lactobacillus*	*Lactobacillus kitasatonis*
OTU00090	2.8311	0.0330	0.0461	*Megamonas*	*Megamonas funifromis*
OTU00097	0.6304	0.0370	0.0483	*Lactobacillus*	*Lactobacillus pontis*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Fold change is expressed relative to the peak lay. [c] Taxonomic assignments are based on the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

The remaining six OTUs were over-represented in late-lay (Table 5). The majority of these OTUs comprised *Lactobacillus* sp. (4/6; 67%). In addition to identifying *Lactobacillus crispatus*, our BLASTn [37] search further identified one of the Lactobacillus OTUs as *Lactobacillus kitasatonis*. This species was also prevalent in a previous study by our lab [8], and it has been studied recently for its potential role as a competitive inhibitor of pathogenic *Salmonella*; however, *L. kitasatonis* has no significant contribution towards inhibiting said pathogens [40,41].

Ileal microbial communities across housing systems and stage of lay were analyzed. To explore ecological changes in each of the housing systems over time, the interaction of housing system and stage of lay was examined. Of the 100 OTUs analyzed, nine were differentially abundant across both housing type and stage of lay (Figure 5). Four patterns of ecological changes were observed. First, two OTUs decreased over time in CF hens but remained relatively stable in EC hens. These were OTU00044, which aligned to *Staphyloccaceae*, and OTU00051, which aligned to *Brachybacterium*. Upon further investigation, it was found that OTU00051 aligned with the *Brachybacterium paraconglomeratum* sequence at higher than 98% using BLASTn [37]. While little is known about this specific species of *Brachybacterium*, the genus as a whole has been found in the dust or excreta associated with low-performing poultry farms [42]. Further investigation is required to

make any judgments about what specific *Brachybacterium* species could be doing. Second, one OTU decreased in EC hens but remained relatively stable in CF hens over time. This was OTU00052, which aligned to *Lactobacillus*. Using BLASTn, OTU00052 aligned with *Lactobacillus ingluviei* at higher than 98% similarity [37]. While current research is lacking in describing the function of *Lactobacillus ingluviei* in poultry, it has been shown to have a strong antagonistic relationship with *C. perfringens*, a well-known poultry pathogen [43]. Third, two OTUs decreased in CF hens but increased in EC hens over time. These were OTU00008, which aligned to *Turicibacter*, and OTU00027, which aligned to *Peptostreptococcaceae*. Maki et al. [44] reported that *Turicibacter* was associated with improved FCR in broilers. More specifically, *Turicibacter sanguinis* is an immunomodulating bacteria that may lead to secondary infections [45]. Additionally, *Turicibacter sanguinis* has been associated with bile salt reabsorption and intestinal serotonin production; thus, it is unclear what role *Turicibacter sanguinis* has in regulating intestinal physiology. *Peptostreptococcaceae* is a family associated with increases in antimicrobial resistance [46,47]. Lastly, four OTUs increased in CF hens but decreased in EC hens. These were OTU00035, which aligned to *Olsenella*, OTU00041, which aligned to *Blautia*, OTU00005, which aligned to *Streptococcus*, and OTU00065, which aligned to *Subdoligranulum*. Unfortunately, these genera have not been associated with functions in poultry; however, *Blautia* have been associated with probiotics in mammals [48].

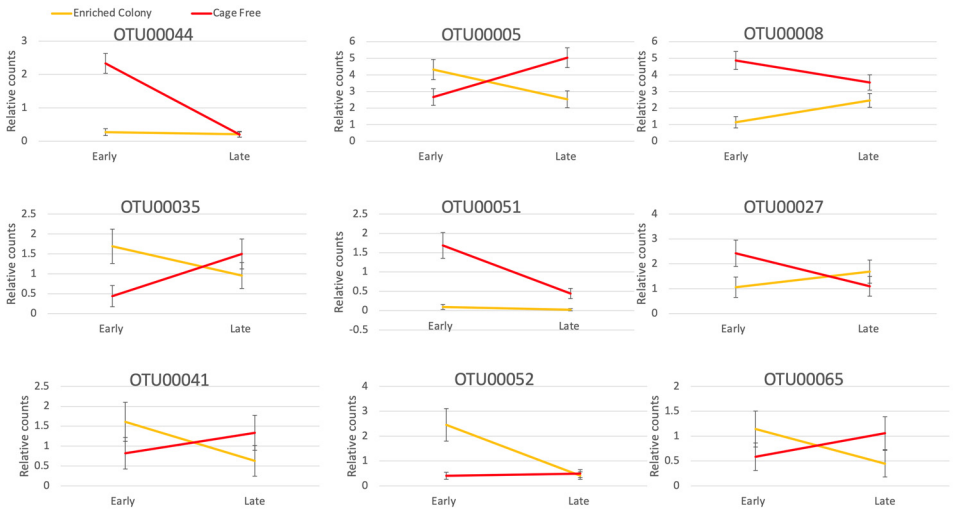


Figure 5. *Changes in ileal microbiota from hens in enriched colonies (EC) and cage-free (CF) systems across peak and late-lay.* Each graph depicts different significant ($p < 0.05$, $q < 0.05$) interaction for individual operational taxonomic unit (OTU). Gold lines represent average relative counts from ileal sample collected from enriched colonies. Red lines represent average relative counts from ileal sample collected from cage-free system.

3.5. Associations between Health Parameters and Microbial Communities

3.5.1. Correlation of OTUs with Intestinal Permeability

To determine if intestinal permeability, a measure of the rate of FITC-D flux from the intestine into circulation, was associated with specific OTUs, Pearson correlations were determined between OTUs and FITC-D. To prevent confounding with significant differences from the housing system, age, and the interaction of the housing system and age, correlations were run within each of the four groups (peak EC, peak CF, late EC, late CF). A total of 31 OTUs were correlated ($r^2 > |0.35|$) with intestinal permeability (Tables 6 and 7; all correlations are provided in Supplemental File S1). The majority of correlations (28/31) occurred during peak lay and were positive (23/28). In this instance, a positive correlation would be considered unfavorable as the increased presence of the sequence is associated with increased intestinal permeability. With hens housed in EC during peak lay, four of the nine positive correlations were associated with sequences from *Romboutsia* (4/9).

Wiersema et al. also observed *Romboutsia* as a genus positively correlated with FITC-D in CC [8]; however, it is unclear how this genus is associated with intestinal permeability. The other sequences were aligned to *Lactobacillus* (2/9), *Peptostreptococcaceae* (1/9), *Megamonas* (1/9), and *Enterococcus* (1/9). The negative correlations or favorable relationships were exclusively aligned with *Lactobacillus* sp. (5/5). Of these species, *Lactobacillus pontis* and *Lactobacillus acidophilus* have been associated with probiotics [49,50]. With hens housed in CF during peak lay, the genus of OTUs correlated with intestinal permeability are more diverse. The most common genus was *Salinicoccus* (2/12) which belongs to the family Staphlococcaceae. However, no known functions or diseases are associated with this genus. Other genera with positive or negative correlations were represented with a single OTU, and intestinal permeability is listed in Table 6.

Table 6. Correlation of operational taxonomic units from ileal digesta from peak-lay hens housed in EC and CF systems for intestinal permeability.

Housing	Group [a]	Correlation [b]	Taxonomy [c]	BLAST Search [d]
Enriched Cage	OTU00003	0.52	*Romboutsia*	*Uncultured Romboutsia* sp.
	OTU00024	0.46	*Peptostreptococcaceae*	*Uncultured Romboutsia* sp.
	OTU00080	0.46	*Lactobacillus*	*Lactobacillus acidophilus*; *L. crispatus*
	OTU00029	0.44	*Romboutsia*	*Uncultured bacterium*
	OTU00090	0.41	*Megamonas*	*Megamonas funifromis*
	OTU00025	0.41	*Romboutsia*	*Uncultured bacterium*
	OTU00010	0.40	*Enterococcus*	*Enterococcus cecorum*
	OTU00070	0.39	*Lactobacillus*	*Lactobacillus acidophilus*; *L. kitasatonis*
	OTU00057	0.37	*Romboutsia*	*Uncultured bacterium*
	OTU00028	−0.38	*Lactobacillus*	*Lactobacillus pontis*; *L. paracollinoides*
	OTU00053	−0.36	*Lactobacillus*	*Lactobacillus acidophilus*; *L. kitasatonis*
	OTU00096	−0.38	*Lactobacillus*	*Lactobacillus pontis*
	OTU00086	−0.46	*Lactobacillus*	*Lactobacillus pontis*
	OTU00004	−0.57	*Lactobacillus*	*Lactobacillus pontis*
Cage-free	OTU00054	0.63	*Brachybacterium*	*Brachybacterium faecium*; *B. paraconglomeratum*
	OTU00087	0.60	*Streptococcus*	*Streptococcus pluranimalium*
	OTU00085	0.59	*Rothia*	*Rothia nasimurium*; *R. terrae*
	OTU00076	0.53	*Jeotgalicoccus*	*Jeotgalicoccus aerolatus*; *J. halotolerans*
	OTU00079	0.53	*Salinicoccus*	*Uncultured Salinicoccus bacterium*
	OTU00030	0.51	*Salinicoccus*	*Salinicoccus roseus*
	OTU00091	0.48	*Brevibacterium*	*Uncultured Brevibacterium* sp.
	OTU00060	0.42	*Lactobacillus*	*Lactobacillus kitasatonis*; *L. acidophilus*
	OTU00026	0.40	*Yaniella*	*Uncultured Yaniella* sp.
	OTU00034	0.37	*Corynebacterium*	*Corynebacterium casei*; *C. ammoniagenes*
	OTU00061	0.37	*Dietzia*	*Dietzia* sp.
	OTU00044	0.35	*Staphylococcaceae_unclassified*	*Mammaliicoccus lentus*; *M. sciuri*
	OTU00050	−0.35	*Veillonellaceae*	*Veillonella seminalis*; *V. ratti*
	OTU00014	−0.39	*Escherichia*-Shigella	*E. coli*
	OTU00083	−0.39	*Romboutsia*	*Uncultured bacterium*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Correlations were determined as Pearson correlations. [c] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

Unlike peak lay, the number of OTUs correlated with intestinal permeability during late-lay was limited to three. Two OTUs were positively correlated with intestinal permeability in late-lay hens housed in EC systems, whereas one OTU was negatively correlated with intestinal permeability in late-lay hens housed in CF systems. These OTUs did not carry over from peak lay in either system. Unfortunately, limited information is available about *Acidibacter* and *Sediminibacterium*, and given that these were associated with uncultured bacteria, it is likely that any function assigned would not be accurate. However, *Lactobacillus mucosae* has been associated with favorable intestinal health and has been included as a probiotic. This species was also observed to be correlated with CC and CF in our previous study, however, in an unfavorable association (positive correlation) but associated with favorable ileal morphology (positive correlation) [8]. Therefore, these

studies would suggest that *Lactobacillus mucosae* may support ileal health, but additional studies examining the direct interactions are needed.

Table 7. Correlation of operational taxonomic units from ileal digesta from late-lay hens housed in EC and CF systems for intestinal permeability.

Housing	Group [a]	Correlation [b]	Taxonomy [c]	BLAST Search [d]
Enriched Cage	OTU00045	0.38	*Acidibacter*	Uncultured bacterium
	OTU00046	0.38	*Sediminibacterium*	Uncultured bacterium
Cage-free	OTU00016	−0.36	*Lactobacillus*	*Lactobacillus mucosae*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Correlations were determined as Pearson correlations. [c] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

3.5.2. Correlations of OTUs with Corticosterone

Circulating corticosterone in birds was determined post-mortem to identify if stress due to the housing system may influence intestinal microbial ecosystems. Similar to intestinal permeability correlations, correlations were run within each of the four groups (peak EC, peak CF, late EC, late CF). A total of 34 OTUs were correlated ($r^2 > |0.35|$) with serum corticosterone (Tables 8 and 9; all correlations are provided in Supplemental File S1). The majority of significant correlations (26/34) occurred during peak lay and were positive (20/26). In this instance, a positive correlation would be considered unfavorable as the increased presence of the sequence is associated with increased circulating corticosterone. With hens housed in EC during peak lay, four of the eleven positive correlations were associated with sequences from *Romboutsia*. In swine undergoing repeated social stress, *Romboutsia* was observed to be enriched [51]. Together, these data provide evidence to support this relationship; however, additional investigations are needed to fully explore this relationship. Two of the eleven positive correlations were associated with *Lactobacillus*. All other OTUs were associated with a single genus (Table 8). The negative correlations or favorable relationship was primarily with Lactobacillus (2/3). In both of these cases, Lactobacillus was also associated with Lactobacillus acidophilus. This microorganism has been used as a probiotic [49,50], and in laying hens, when fed as a direct-fed microbial, it mitigated circulating corticosterone when hens were challenged with lipopolysaccharide (LPS) [52]. For hens housed in CF during peak lay, the genera of OTUs correlated with corticosterone are similar in correlations with intestinal permeability. The most common genera were *Staphylococcus* (2/9) and *Lactobacillus* (2/9). Other genera with positive or negative correlations were represented with a single OTU and corticosterone and are listed in Table 8.

Unlike peak lay, the number of OTUs correlated during late-lay was more limited, with a total of eight OTUs correlated with corticosterone. Three OTUs were negatively correlated with serum corticosterone in late-lay hens housed in EC and CF systems (1 and 2, respectively) aligned to *Lactobacillus.* All other OTUs aligned to a novel genus with one OTU positively correlated and one additional OTU negatively correlated with corticosterone in late-lay hens housed in EC systems. In late-lay hens housed in CF systems, two OTUs were positively correlated, and one additional OTU was negatively correlated with serum corticosterone. As *Lactobacillus* continues to be a genus that was correlated in both peak and late-lay hens with corticosterone, this genus continues to need additional research to explore its relationship with corticosterone.

Table 8. Correlation of operational taxonomic units from ileal digesta from peak-lay hens housed in EC and CF systems for corticosterone.

Housing	Group [a]	Correlation [b]	Taxonomy [c]	BLAST Search [d]
Enriched Cage	OTU00022	0.56	*Staphylococcus*	*Staphylococcus equorum*; *S. arlettae*
	OTU00037	0.49	Cholorplast_ge	*Campylotropis* sp.
	OTU00001	0.46	*Lactobacillus*	*Lactobacillus acidophilus*; *L. kitasatonis*
	OTU00003	0.42	*Romboutsia*	*Uncultured Romboutsia* sp.
	OTU00025	0.42	*Romboutsia*	*Uncultured bacterium*
	OTU00029	0.40	*Romboutsia*	*Uncultured bacterium*
	OTU00046	0.40	*Sediminbacterium*	*Uncultured bacterium*
	OTU00085	0.38	*Rothia*	*Rothia nasimurium*; *R. terrae*
	OTU00083	0.38	*Romboutsia*	*Uncultured Bacterium*
	OTU00070	0.36	*Lactobacillus*	*Lactobacillus acidophilus*; *L. kitasatonis*
	OTU00087	0.35	*Streptococcus*	*Streptococcus pluranimalium*
	OTU00093	−0.41	*Enterococcus*	*Enterococcus faecium*
	OTU00012	−0.47	*Lactobacillus*	*Lactobacillus acidophilus*; *L. crispatus*
	OTU00071	−0.53	*Lactobacillus*	*Lactobacillus acidophilus*; *L. kitasatonis*
Cage-free	OTU00058	0.53	*Lactobacillus*	*Uncultured Prokaryote*
	OTU00082	0.44	*Eggerthellaceae*	*Eggerthellaceae bacterium*
	OTU00009	0.41	*Lactobacillus*	*Lactobacillus gasseri*; *L. johnsonii*
	OTU00056	0.39	*Peptostreptococcaceae*	*Peptococcus niger*
	OTU00051	0.37	*Brachybacterium*	*Brachybacterium paraconglomeratum*; *B. sacelli*
	OTU00022	0.37	*Staphylococcus*	*Staphylococcus equorum*; *S. arlettae*
	OTU00092	0.36	*Terrisporobacter*	*Uncultured Bacterium*
	OTU00044	0.35	*Staphylococcaceae_unclassified*	*Mammaliicoccus lentus*; *M. sciuri*
	OTU00045	0.35	*Acidibacter*	*Uncultured bacterium*
	OTU00004	−0.36	*Lactobacillus*	*Lactobacillus pontis*
	OTU00055	−0.40	*Clostridium*	*Brachybacterium* sp.
	OTU00077	−0.40	*Romboutsia*	*Uncultured bacterium*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Correlations were determined as Pearson correlations. [c] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

Table 9. Correlation of operational taxonomic units from ileal digesta from late-lay hens housed in EC and CF systems for corticosterone.

Housing	Group [a]	Correlation [b]	Taxonomy [c]	BLAST Search [d]
Enriched Cage	OTU00044	0.41	*Staphylococcaceae*_unclassified	*Mammaliicoccus lentus*; *M. sciuri*
	OTU00011	−0.37	*Lactobacillus*	*Lactobacillus kitasatonis*; *L. acidophilus*
	OTU00010	−0.42	*Enterococcus*	*Enterococcus cecorum*
Cage-free	OTU00037	0.50	Cholorplast_ge	*Campylotropis* sp.
	OTU00032	0.38	*Peptostreptococcaceae*	*Uncultured Peptostreptococcaceae bacterium*
	OTU00097	−0.35	*Lactobacillus*	*Lactobacillus pontis*
	OTU00095	−0.35	*Erysipelotrichaceae*	*Uncultured Erysipelotrichaceae bacterium*
	OTU00043	−0.36	*Lactobacillus*	*Lactobacillus ruminis*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 100 taxonomic groups. [b] Correlations were determined as Pearson correlations. [c] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 138). [d] BLASTn search results were reported if the similarity was higher than 97%.

4. Conclusions

Changes in housing type, particularly with increased exposure to excreta during a production cycle of laying hens, may have an impact on overall intestinal health, stress, and microbial communities. The present study observed differences in intestinal permeability as measured by FITC-D across housing systems, age, and the interaction between housing systems and age, suggesting some intestinal distress may be occurring as hens age, and this change may be impacted by housing system. To determine if this increased intestinal stress was a result of overall stress, serum corticosterone was measured and found to increase as hens age but did not significantly differ between housing systems, suggesting other factors outside of corticosterone are increasing intestinal permeability in hens housed in EC. Similar to Wiersema et al. [8], no single housing type had OTUs that were significantly

over-represented that were classified as known primarily pathogenic bacteria, and limited OTUs were over-represented in one housing system over the other. However, between peak and late-lay, it was observed that *Lactobacillus* OTUs shifted over time, which could lead to changes in whole microbial communities. Lastly, we explored the correlation of bacterial communities with health traits. A primary finding of this study was that a higher number of correlations were observed between both intestinal permeability and corticosterone and ileal microbial communities during peak lay. This suggests that as the hens age, their microbial communities may become more resilient to intestinal stressors. Therefore, as we explore the impact of shifts in microbial communities in future studies, the stage of lay and housing systems will be important factors to consider.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/poultry3030020/s1, Table S1: Correlations for EC and CF.xlsx.

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Institutional Review Board Statement: All procedures involving animals were approved by Iowa State University's Institute of Animal Care and Usage Committee (IACUC number 20-033).

Informed Consent Statement: Not applicable.

Data Availability Statement: The 16S rRNA gene sequences have been submitted to the NCBI Sequence Read Archive SRA and are available under the BioProject ID PRJNA1055184. All other data are available from the authors upon request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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