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Impact of Stocking Densities on the Microbiota of the Cloaca, Eggshell, and Egg Content of White Egg Layers in Colony Cages

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Abstract: Food safety is a major concern for commercial poultry producers and consumers. Currently, there is also pressure from retailers and legislators to increase the space per hen in cages. Five different density treatments consisting of six (208 in^2/bird), nine (139 in^2/bird), twelve (104 in^2/bird), fifteen (83 in^2/bird), and eighteen birds (69 in^2/bird) per cage were examined in colony cage environments. Microbiological tests were performed at 39, 55, and 68 weeks of age. The populations of total aerobic bacteria; *E. coli*/coliform; Enterobacteriaceae; and yeasts and molds from an eggshell rinse, egg content, and cloacal swabs were enumerated. The prevalence of *Salmonella* spp. in these samples was also monitored. Overall, no bacteria were detected in any of the egg content, and there were no differences ($p > 0.05$) between treatments for the shell rinse. Stocking density did not influence the eggshell microbiota of the hens. Hens housed at 104 in^2 per hen showed higher levels of total aerobic bacterial counts from the cloaca compared to hens at 208 in^2 and 69 in^2 per hen. Hens housed at 139 in^2 per hen had the highest level of cloacal molds. This research demonstrates that stocking density does not influence eggshell microbiota or *Salmonella* contamination of the eggshell or cloaca, thereby indicating that allowing more space per hen will not positively or negatively affect the prevalence or concentration of foodborne pathogen-associated bacteria in or on the eggs.

Keywords: laying hens; stocking density; food safety; *Salmonella*; microbiota

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1. Introduction

Over the past 100 years, the laying-hen industry has moved from small free-range farms to large cage operations. More recently, a pushback against intensification has been observed. Many animal rights special interest groups believe that current stocking density practices may be detrimental to the health and well-being of the laying hens. These claims, along with other research, have influenced government legislators to restrict some forms of intensive laying-hen housing environments; an example is the banning of cages in California [1]. As these groups claim that the currently utilized practices are unhealthy for production hens, they have worked with consumers, retailers, and legislators to improve these environments [2,3]. The general consumer agrees with the idea that low space per hen can be detrimental to hen health and therefore agrees with the shift towards more extensive systems [4]. While consumers may desire the shift, their willingness to pay for these changes in production systems when given the option is very low [2,5,6]. Therefore, there is a need to evaluate these claims and determine if adding extra space per hen will positively affect the commercial laying hens.

Egg safety is also an issue of particular importance to the egg industry. Higher stress levels have been shown to result in bacterial shedding by hens into the environment or, potentially, into the eggs produced [7]. Due to the potential harm to human health, producers must ensure that all eggs and egg products from their flocks are safe for human

consumption. Microbial contamination can cost producers millions of dollars in recall costs and revenue loss [8]. Microbial contamination, such as *Salmonella* and pathogenic *E. coli*, can also lead to consumer illness and even death, as seen in many cases [9,10]. As production systems evolve, it is important to evaluate the effect of newer production practices and systems on the microbiological profile of the products to understand and mitigate the risks associated with foodborne pathogens.

Research evaluating cloacal and eggshell microbial populations in hens utilizing different densities in the same production system is lacking [11–15]. It is well-understood that eggs contain several physical and antimicrobial barriers, both on the eggshell and from egg white proteins. It has been shown that cloacal swabs and shell rinses from various cage systems produce differences in anaerobic but not aerobic bacterial counts in the cloaca, while no change in both anaerobic and aerobic bacterial counts on the shell [16]. Another study evaluating conventional cages (at high densities) and enriched colony cages (at lower densities) found no change in *E. coli* and Enterococcus populations on the eggshell [17]. Reports on the microbiological profile by age appear to be conflicting, where some papers report no differences, and others report differences between age groups [16–20]. Part of these differences could be due to a seasonality effect varying by region or time of year. Research specifically comparing microbiological profiles across different stocking densities in the same cage environment appears to be lacking.

Salmonella spp. is one of the most important foodborne pathogens to research regarding human and animal health [21–23]. *Salmonella* contamination is considered dangerous. The ID50 (number of infectious cells required to start an infection with 50% probability) of *Salmonella*, although dependent on stereotypes, can be very low for infection and illness, between seven colony-forming units (CFUs) and thirty-six CFUs [24]. It is understood that as hens age, *Salmonella* spp. contamination becomes more likely [25]. However, other factors can play into the susceptibility of colonization [20]. *Salmonella* can colonize the interior of the egg in two paths: vertically and horizontally. Colonization of the egg interior can be accomplished by colonizing the reproductive system and entering before the eggshell is created (vertical contamination) or by colonizing the shell exterior and penetrating the cuticle and eggshell (horizontal contamination) [15,22,26,27]. Hens infected with *Salmonella* in lower stocking densities shed less fecal *Salmonella* than those in high densities. However, studies evaluating natural infection and the subsequent contamination of the cloaca and eggs in different densities remain to be investigated [7]. The objective of this study was to evaluate the microbiological load and profile, including *Salmonella* and *E. coli*, of the cloaca, eggshell, and egg content of hens kept at various densities. We hypothesized that hens in higher densities would yield higher *Salmonella* spp. loads due to the higher prevalence of feces and higher number of opportunities for inter-cloacal and feces–egg contact.

2. Materials and Methods

2.1. Hens and Housing

Hens raised for this study were placed into colony cages at 17 weeks of age at the North Carolina Department of Agriculture Piedmont Research Station in Salisbury, NC, USA. The study was approved by the Institutional Animal Care and Use Committee of NC State University (IACUC number 19581). The tri-decked colony cages measured 26 in × 48 in with 48 in of feeder space per cage with 4 nipple drinkers. Three hundred and sixty Shaver White laying hens were randomly placed into five density assignments in these cages when the laying cycle began. The treatments were as follows: 208 in^2 per hen (6 hens per cage), 139 in^2/hen (9 hens per cage), 104 in^2 per hen (12 hens per cage), 83 in^2 per hen (15 hens per cage), and 69 in^2 per hen (18 hens per cage). Each replicate consisted of 2 of these cages. Feed was provided to the hens ad libitum and the diet followed a standard mash diet as indicated by Table 1. The hens were on a 14L:10D lighting schedule.

Table 1. Ingredient composition and calculated nutrient analysis for the diet fed to all hens.

Ingredient	Diet (%)
Corn	51.842
Soybean Meal	32.241
Calcium Carbonate	9.42
Dicalcium Phosphate	1.808
Salt	0.38
DL-Methionine	0.181
Soybean Oil	3.729
Santoquin	0.05
Choline Chloride	0.05
NCSU Trace Mineral Premix [1]	0.20
NCSU Vitamin Premix [2]	0.05
NCSU Selenium Premix [3]	0.05
Calculated Values	
Crude Protein (%)	19.50
Metabolizable Energy (kcal/kg)	1328.0
Calcium (%)	4.14
Available Phosphorus (%)	0.45
Total Lysine (%)	1.10
Total Sulfur Amino Acids (%)	0.8245

[1] Mineral premix supplied the following per kilogram of feed: 120 mg of Zn as $ZnSO_4H_2O$, 120 mg of Mn as $MnSO_4H_2O$, 80 mg of Fe as $FeSO_4H_2O$, 10 mg of Cu as $CuSO_4$, 2.5 mg of I as $Ca(IO_3)_2$, and 1.0 mg of Co as $CoSO_4$. [2] Vitamin premix supplied the following per kilogram of feed: vitamin A, 26,400 IU; cholecalciferol, 8000 IU; niacin, 220 mg; pantothenic acid, 44 mg; riboflavin, 26.4 mg; pyridoxine, 15.8 mg; menadione, 8 mg; folic acid, 4.4 mg; thiamin, 8 mg; biotin, 0.506 mg; vitamin B12, 0.08 mg; and ethoxyquin, 200 mg. The vitamin E premix provided the necessary amount of vitamin E as DL-α-tocopheryl acetate. [3] Selenium premix provided 0.3 ppm Se from sodium selenite.

2.2. Microbialogical Analysis

Microbiological analyses were collected at 39 (May), 55 (August), and 68 (November) weeks of age and performed by collecting biological samples including cloacal swabs, exterior eggshell rinses, and internal egg contents. The same three hens in each replicate were swabbed at each time point (two hens from one cage and one hen from the other). The microbes analyzed included the total aerobic count (TAC), *E. coli*, total coliforms, Enterobacteriaceae (EB), yeasts, molds, and presence of *Salmonella* spp. During each sample period, the cloaca of three hens per replicate (nine hens per treatment) were swabbed, and six eggs per replicate (eighteen eggs per treatment) were taken for sampling. Once the eggs were collected, they were placed in an egg cooler for 24 h prior to testing. Cloacal swabs were performed using cotton swabs that were placed in 1 ml of phosphate-buffered saline (PBS) for transport. The microbial sampling method was modified from Jones et al. [28]. In the lab, each egg was placed in a bag with 10 mL PBS solution and shaken for 30 s. Eggs were then removed from the bags, dipped in 95% ethanol, and set aside to dry. Every six shell rinse bags were pooled and serially diluted for plating. Once eggs were dried, they were cracked open, and the contents were pooled in bags and serially diluted. Finally, cloacal swabs were serially diluted for plating. All samples were plated utilizing 3M Petrifilms, specifically, total aerobic count, Enterobacteriaceae, coliforms/*E. coli*, and yeast/molds (3M, Saint Paul, MN, USA). Total aerobic count, Enterobacteriaceae, and coliforms/*E. coli* were incubated at 35 °C for 24 h, and yeast/mold Petrifilms were incubated at 25 °C for approximately 72 h. Plates were counted and data are expressed as $\log_{10}$CFU/mL. For *Salmonella* analysis, the rest of the samples, after plating on Petrifilms, were enriched individually in buffered peptone water at a 1:5 ratio at 35 °C for 24 h. After the primary enrichment, 0.1 mL of the samples were transferred to 10 mL of Rappaport–Vassiliadis (RV) at 42 °C for 24 ± 2 h. One loop of the enrichment was streaked on xylose lysine deoxycholate, Hektoen enteric, and bismuth sulfate agars; incubated for 24 h at 36 °C; and checked for typical *Salmonella* colonies. The colonies were randomly picked to confirm *Salmonella* by BAX® System standard PCR assay. The detection limit for the

shell microbial loads, *Salmonella* detection presence, and egg contents microbial loads was 1 CFU/mL, and the detection limit for cloacal swab microbial loads was 10 CFU/mL.

2.3. Statistical Analysis

Statistical analysis was performed using R 3.3.0 with packages tidyverse and reshape2 for data preparation [29–31]. For analysis of variance, packages agricolae and AICcmodavg were utilized [32,33]. The GLM function was used for analysis of variance for microbial counts, and means were separated using Tukey's HSD. Density, age, and interaction were analyzed for this study. For *Salmonella* analysis, a Chi-squared test was run to determine significant differences in percent positives between treatments and ages. Multiple comparisons for Pearson's Chi-squared tests were performed using the Bonferroni correction with the package rstatix [34]. p values less than or equal to 0.05 were considered statistically significant and p values between 0.05 and 0.1 were considered statistically trending. No outliers were excluded in analysis, although yeasts and molds at 55 weeks of age were, unfortunately, excluded due to improper handling of samples. The replicate of 2 cages was considered the statistical unit of this study. Correlative analysis was performed utilizing Spearman's correlation utilizing package, Hmisc [35]. R values over 0.6 were considered a strongly positive correlation and below −0.6 were considered a strongly negative correlation.

3. Results

3.1. Cloacal Swabs

Table 2 presents the microbiological profiles from the cloacal swabs. *E. coli*, total coliforms, Enterobacteriaceae, and yeasts were not found to be statistically different between density treatments. Furthermore, there was no difference found in the amounts of *E. coli*, total coliforms, and Enterobacteriaceae when comparing ages in this study. However, hens in the 104 in^2 density had higher total aerobic bacteria numbers than hens in the 208 in^2 density, and the other densities were not statistically different. Furthermore, it was found that hens in the 139 in^2 density had higher numbers of molds than the 208 in^2, 104 in^2, and 83 in^2 densities. Hens were also found to have the most aerobic bacteria at 55 weeks of age and the least number of aerobic bacteria at 39 weeks of age. Furthermore, hens aged 39 weeks were found to have higher cloacal yeast numbers than at 68 weeks of age. Finally, at 55 weeks of age, molds were only found in the cloaca with no detection at any other tested time point.

Table 2. The effect of stocking density and hen age on cloacal microbial profile of eggs from commercial white egg layers (log_{10}CFU/mL).

	TAC	***E. coli***	**Coliforms**	**EB**	**Yeasts**	**Molds**
Density/bird						
208 in^2	4.19 ^{b}	3.04	3.12	3.13	0.584	0 ^{b}
139 in^2	4.32 ^{a,b}	3.04	3.23	3.33	0.336	0.251 ^{a}
104 in^2	4.69 ^{a}	3.18	3.43	3.38	0.236	0 ^{b}
83 in^2	4.47 ^{a,b}	2.70	3.19	3.04	0.407	0 ^{b}
69 in^2	4.5 ^{a,b}	2.75	3.03	3.29	0.226	0.033 ^{a,b}
SEM	0.263	0.288	0.23	0.235	0.166	0.04
***p*-Value**	0.015	0.698	0.875	0.875	0.489	0.018
Age						
39 weeks	3.48 ^{c}	3.04	3.15	3.20	0.565 ^{a}	0 ^{b}
55 weeks	5.06 ^{a}	2.79	3.25	3.20	0.445 ^{a,b}	0.17 ^{a}
68 weeks	4.79 ^{b}	3.10	3.17	3.38	0.064 ^{b}	0 ^{b}
SEM	0.084	0.28	0.195	0.176	0.114	0.034
***p*-Value**	0.0001	0.482	0.920	0.752	0.0173	0.016
Density × age *p*-Value	0.241	0.401	0.276	0.707	0.542	0.005

^{a,b,c} Signifies statistical significance of $p < 0.05$.

3.2. Shell Rinse and Egg Contents

Table 3 details the shell rinse data for density treatments as well as ages. There were no differences in the microbial load between density treatments for any of the microbes measured. However, differences were detected in several microbes measured across ages. Eggs from hens aged 39 weeks were shown to house more *E. coli* than hens at any other age. This trend repeated for total coliforms, Enterobacteriaceae, and molds, demonstrating that eggs from the younger hens contained more total coliforms, Enterobacteriaceae, and molds on the shells than eggs from older hens. Finally, no bacteria were detected in the egg contents.

Table 3. The effect of stocking density and hen age on eggshell microbial profile of eggs from commercial white egg layers (log_{10}CFU/mL).

	TAC	***E. coli***	**Coliforms**	**EB**	**Yeasts**	**Molds**
Density/bird						
208 in^2	3.87	0.347	0.602	0.218	1.69	1.61
139 in^2	3.81	0.499	0.591	0.229	1.59	1.63
104 in^2	3.86	0.173	0.386	0.443	1.90	1.85
83 in^2	3.87	0.404	0.514	0.509	1.81	1.72
69 in^2	3.83	0.251	0.542	0.711	1.66	1.46
SEM	0.236	0.177	0.202	0.216	0.16	0.245
***p*-Value**	0.557	0.404	0.876	0.557	0.634	0.284
Age						
39 weeks	3.97	0.861 [a]	1.060 [a]	1.260 [a]	1.88	2.144 [a]
55 weeks	3.80	0.519 [b]	0.296 [b]	0.371 [b]	--	--
68 weeks	3.77	0.092 [b]	0.224 [b]	0.182 [b]	1.58	1.16 [b]
SEM	0.049	0.083	0.113	0.104	0.0942	0.0757
***p*-Value**	0.055	0.001	0.001	0.001	0.052	0.001
Density × age *p*-value	0.979	0.474	0.543	0.450	0.442	0.986

[a,b] Signifies statistical significance of $p < 0.05$.

3.3. Salmonella

Table 4 houses information on the samples positive for *Salmonella* related to density and age for both shell rinses and cloacal swabs. There was no difference between density treatments for both the shell rinses and the cloacal *Salmonella* presence. Furthermore, no difference between ages was found for the shell rinses. However, the cloacal swabs were found to be significantly different across ages with 0% positives at 39 weeks, 64% positives at 55 weeks, and 58% positives at 68 weeks. Finally, no *Salmonella* was detected in the egg contents.

Table 4. The effect of stocking density and hen age on presence of *Salmonella* in hen cloaca and eggshells of commercial white egg layers.

	Shell Rinse *Salmonella*	**Cloacal Swabs *Salmonella***	**Egg Content *Salmonella***
Density/bird			
208 in^2	11%	44%	0%
139 in^2	22%	56%	0%
104 in^2	11%	41%	0%
83 in^2	11%	37%	0%
69 in^2	22%	26%	0%
***p*-Value**	0.9075	0.2659	1.000
Age			
39 weeks	0%	0% [b]	0%
55 weeks	27%	64% [a]	0%
68 weeks	20%	58% [a]	0%
***p*-Value**	0.111	0.001	1.000

[a,b] Signifies statistical significance.

3.4. Correlative Analysis

A correlative analysis between the microbes measured is presented in Table 5. Cloacal *E. coli* were found to be positively correlated to cloacal coliforms (r = 0.851) and cloacal Enterobacteriaceae (r = 0.666). Cloacal coliforms were also positively correlated with cloacal Enterobacteriaceae (r = 0.68). Cloacal aerobic counts were found to be negatively correlated with eggshell *E. coli* (r = −0.622) and eggshell molds (r = 0.646). Eggshell *E. coli* were found to be positively correlated with eggshell coliforms (r = 0.804), eggshell Enterobacteriaceae (r = 0.73), and eggshell molds (r = 0.632). Eggshell coliforms were found to be positively correlated with eggshell Enterobacteriaceae (r = 0.841). Eggshell Enterobacteriaceae were found to be positively correlated with eggshell molds (r = 0.791). Cloacal aerobic bacteria were also found to be negatively correlated with eggshell *E. coli* (r = −0.622). Finally, the number of eggshell aerobic bacteria was found to be positively correlated with eggshell molds, as well (r = 0.712). All other microbiological correlations were found to have r values of less than 0.6 or greater than −0.6.

Table 5. Correlative analysis between cloacal and eggshell microbiological profiles of commercial white egg layers.

		Cloacal						Eggshell					
		E. coli	**Coliforms**	**Enterobac**	**Aerobic**	**Yeast**	**Mold**	***E. coli***	**Coliforms**	**Enterobac**	**Aerobic**	**Yeasts**	**Molds**
Cloacal	*E. coli*	1											
	Coliforms	0.851 (<0.001)	1										
	Enterobac	0.666 (<0.001)	0.680 (<0.001)	1									
	Aerobic	0.069 (0.704)	0.207 (0.225)	0.194 (0.229)	1								
	Yeasts	0.036 (0.842)	0.173 (0.304)	0.133 (0.408)	−0.273 (0.073)	1							
	Molds	−0.161 (0.362)	−0.176 (0.299)	−0.213 (0.182)	0.325 (0.031)	−0.015 (0.922)	1						
Eggshell	*E. coli*	0.195 (0.267)	0.131 (0.439)	−0.095 (0.554)	−0.622 (<0.001)	0.324 (0.030)	−0.201 (0.186)	1					
	Coliforms	0.305 (0.076)	0.308 (0.064)	0.010 (0.950)	−0.150 (<0.001)	0.336 (0.024)	−0.193 (0.205)	0.804 (<0.001)	1				
	Enterobac	0.169 (0.267)	0.260 (0.120)	−0.005 (0.974)	−0.535 (<0.001)	0.383 (0.009)	0.209 (0.168)	0.730 (<0.001)	0.841 (<0.001)	1			
	Aerobic	0.130 (0.469)	0.068 (0.695)	0.132 (0.417)	−0.209 (0.179)	0.159 (0.168)	0.040 (0.799)	0.316 (0.037)	0.249 (0.103)	0.281 (0.065)	1		
	Yeasts	0.251 (0.273)	0.262 (0.238)	0.284 (0.151)	−0.204 (0.289)	0.283 (0.129)	-	0.209 (0.268)	00.253 (0.177)	0.255 (0.173)	0.389 (0.037)	1	
	Molds	0.257 (0.261)	0.273 (0.218)	0.101 (0.617)	−0.646 (0.002)	0.554 (<0.001)	-	0.632 (0.002)	0.395 (0.002)	0.791 (<0.001)	0.712 (<0.001)	0.599 (0.004)	1

Numbers in parentheses indicate *p*-value while numbers in standard font indicate R-value.

4. Discussion

4.1. Cloacal Swabs

The differences in aerobic counts found between the 104 in^2 and 208 in^2 densities may be due to the house position, as two replicates of the 104 in^2 density were closer to the entryway door than the 208 in^2 density replicates, although it is unknown if door proximity would affect the cloacal microbial load. Dust particulates and other small fomites carrying these microbes may enter in through the door, as the house is under negative pressure ventilation, and land in the cages close to the door when the door is open. We theorize that this may be due to the hen's proximity to the entry door. Hens closer to the entry may exhibit higher aerobic microbial loads as well as a higher yield of molds. This may explain the data found in this study, as the extremes did not exhibit differences and the house was ventilated with negative pressure. Furthermore, we are unsure as to why the 139 in^2 density contained more molds, and we theorize that this may be due to random chance as the cage positions of these treatments were evenly distributed through

the house. Comparatively, the characterization of cloacal microbiota across several stocking densities is a novel comparison, and therefore, more comparisons must be performed to validate the results found in this study. When looking at studies that compared different cage systems that utilized different densities, Sharma et al. found that conventional hen cages at 120 in^2 per hen and enriched colony cages at 233 in^2 per hen did not exhibit any difference in cloacal aerobic or coliform microbial loads, which agrees with the findings of the present study [16]. In relation to age, Sharma et al. also found that hens at 38 weeks of age demonstrated less CFUs of cloacal aerobic bacteria than 45-week-old hens, which agrees with this study's findings. However, Sharma et al. [16] also indicated that cloacal colonization increased as the hen aged, which is not reflected in the present study, where the aerobic bacteria CFUs decreased at 68 weeks of age.

4.2. Shell Bath

The past literature covering eggshell microbiota is more extensive than that on cloacal microbiota. Research explicitly addressing the effect of the density per hen on eggshell microbiota is still lacking. In the present study, the eggs were placed in an egg cooler almost immediately after laying, thereby reducing the outgrowth of the bacteria on the eggshell. Furthermore, about an hour before oviposition in the shell gland, the hen deposits the cuticle on the eggshell as the first line of defense, protecting against microbial penetration to the egg contents [36]. The cuticle, which has been shown to contain antibacterial properties such as C-lysosome, ovotransferrin, and ovocalyxin-32, works as not only a barrier to entry by preventing bacterial penetration of the eggshell but also impedes microbial proliferation [37–39]. The action of these antimicrobial elements, coupled with the colder environment and the absence of food materials for bacteria on the eggshell, may have prevented proliferation. Therefore, these reasons may have caused no treatments to be statistically different in the presence of microbes on the eggshell. Sharma et al. also found similar results to the present study, finding no difference between aerobic bacteria and coliforms on the eggshell between conventional cages at 120 in^2 per hen and enriched colony cages at 233 in^2 per hen [16]. Englmaierová et al. also found no change in the total eggshell microbial count as well as the eggshell populations of *E. coli* and Enterococcus between hens housed in conventional cages at 85.3 in^2 per hen and hens housed in enriched colony cages at 116.3 in^2 per hen [17]. De Reu et al. found that hens in furnished cages at 116.3 in^2 per hen and hens in conventional cages housed at 99.2 in^2 displayed no difference in eggshell populations of aerobic bacteria or Gram-negative bacteria, indicating an agreement with the findings of this study [40]. Given the past documentation and the results from the present study, we hypothesize that the quality of the cuticle does not differ between density treatments. However, more research will be required to confirm this claim, as measuring the cuticle was not part of this study. As no bacteria were detected in the egg contents from any treatment, it can also be hypothesized that either the cuticle quality did not change as density increased, albumen quality did not change as density increased, or both predictions are accurate. Finally, due to the lack of presence of *Salmonella* in the egg content, we hypothesize that no *Salmonella* colonization of the reproductive tract occurred.

Analyzing *E. coli* and other coliforms is an important indicator of the contamination and safety of food products. Firstly, coliforms are a group of bacteria that are commonly found in the intestines of warm-blooded animals [41]. Due to their habitat, the presence of *E. coli* and other coliforms can be an indicator of fecal contamination. Furthermore, certain strains of *E. coli* can be pathogenic, such as *E. coli* O157:H7, and are known to cause severe human illnesses which can lead to poor public perception, expensive recalls, or even losses of life [42–45]. A final reason for analyzing *E. coli* relates to regulatory requirements. Many regulatory bodies, such as the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), have established guidelines and regulations regarding the acceptable levels of *E. coli* and coliforms in various food products [46,47]. By age, *E. coli*, coliforms, Enterobacteriaceae, and mold counts were higher on eggshells from younger hens than those from older ones. Sharma et al. found no difference in aerobic counts on

eggshells as hens aged. Sharma also found that coliform presence increased with age, which disagrees with the results of the current study [16]. However, it is also known that different genetic hen strains can be more resistant to bacterial colonization and contamination, which could explain why the results of Sharma's study and this investigation differ [48–50]. It is unknown why the bacterial colonization of eggs decreased as the hens aged. It could be partly due to a poorer quality cuticle at an early age, or the eggs could have been collected slightly later than the other collection dates as they could have been exposed to the environment for longer.

Furthermore, seasonal climate changes could have also influenced these results. While the house was environmentally controlled, there was still a temperature region, which was considered within the optimal range for these hens. As the trial ran for an entire year, the changes in seasonal temperature could explain the differences we saw in age. However, we did not record temperatures for this study. Further research is needed to evaluate the external temperature and humidity effects on microbes from indoor poultry production.

4.3. Salmonella

Salmonella is considered one of the most important microbes in food safety and gut health as it is one of the most common enteric disease-causing organisms [51,52]. Gast et al. studied the effect of density on the presence of *Salmonella* in laying hens' internal organs by orally inoculating them with *Salmonella*. They concluded that stocking density affects internal organ colonization, specifically showing more colonization in the ovaries in higher-density hens than in lower-density hens [53]. Gast et al. purposely infected the hens with *Salmonella*. However, this paper indicates that hens living at higher densities might be predisposed to colonization by *Salmonella*. By density treatment, our study did not report any change in the *Salmonella* positivity rates between densities for neither eggshells nor cloacal swabs.

Furthermore, no presence of *Salmonella* was detected within the egg contents. As a note, as part of the farm's egg safety plan, FDA environmental swabs were taken and were negative for *Salmonella* Enteritidis. This indicates that without vectors such as the feces removed in the cage system, it is more difficult for *Salmonella* to colonize key body systems. Eggs also contain several antibacterial defense mechanisms to protect the embryo from contamination during development, which further explains the absence of *Salmonella*. *Salmonella* is commonly present in the chicken's lumen, thus potentially explaining why the *Salmonella* presence was higher on the cloacal swabs than on the eggshell [54]. Feces were removed from the hens as a design of the cage system. However, some feces can be caught on the wire floor and transfer bacteria to the egg if contact is made. For this reason, ensuring a low *Salmonella* presence in the digestive tract is critical in preventing egg contamination by *Salmonella*.

As the hens aged, the presence of *Salmonella* increased on the cloacal swabs but not on the eggshells. When experimentally inoculated, it is known that as hens age, eggs become more susceptible to invasion by microbes such as *Salmonella* [28]. In the present study, however, natural colonization did not yield any difference in contaminated shells as the hens aged. Finally, no *Salmonella* was detected inside the egg. Following being laid, the eggs were collected aseptically within one hour after the lights were on and stored in a cool, dry environment. Handling the eggs in this manner prevents egg sweating, which can assist in the penetration of the eggshell by microorganisms. Therefore, *Salmonella* had less time to infiltrate the egg than in an egg processing facility [23,55,56].

Furthermore, the absence of *Salmonella* in the egg interior suggests that the reproductive tract of the hens in this study was not naturally colonized by *Salmonella*, which some researchers claim is the primary route of egg content colonization [22,26,57]. This further emphasizes the importance of producers practicing good biosecurity and management practices to control this *Salmonella* contamination [58]. However, due to the lower sample sizes, further research should be performed.

4.4. Correlative Analysis

It was expected that cloacal *E. coli*, coliforms, and Enterobacteriaceae as well as eggshell *E. coli*, coliforms, and Enterobacteriaceae would be positively correlated, as these microbes are in the same family. It was unexpected, however, that the eggshell and cloacal microbes did not have strong correlations between them. However, this is understandable, as the reproductive tract everts through the cloaca and deposits the egg without cloaca–egg contact. Furthermore, as mentioned previously, the eggshell contains antibacterial properties that guard against contamination. These reasons explain why the eggshell and cloacal microbiological profiles were not strongly correlated. Another interesting and unexpected find was that eggshell molds were positively correlated with many other eggshell bacteria, while cloacal molds were not correlated with cloacal bacteria. It is known that molds are sustained well in ventilation systems, with growth promoted in the environment and the air. Given these characteristics of mold growth, the cloaca may be inhospitable for some molds compared with the eggshell in the environment.

Furthermore, the cloaca is not as exposed to the outside air as the eggshell is [59,60]. Molds in the cloaca could also be competitively inhibited by other bacteria. This agrees with the findings of Shi et al., who found that lactic acid bacteria can competitively inhibit mold growth in vitro [61]. The negative correlation between cloacal aerobic bacteria and eggshell *E. coli* indicates a competition between these two types of bacteria, which is further confirmed by several in vitro studies identifying that Lactobacillus can competitively exclude *E. coli* [62]. Unfortunately, the full relationship between these microbes in this environment is not fully understood, and therefore, more research is needed to understand the mechanisms at work. Furthermore, more research is needed to expand on these relationships and understand the mechanisms behind the negative correlations.

5. Conclusions

In summary, this study found that increasing the space per hen from the currently utilized levels did not affect the eggshell microbiological profiles or the *Salmonella* prevalence for cloacae nor eggshells. This study also did not detect any contamination inside the egg contents, indicating that *Salmonella* had yet to naturally colonize the reproductive tract, nor had any microbes been able to penetrate the eggshell. We did find that the hens in the lowest density were found to have the lowest total aerobic bacteria count compared to the hens kept at 139 in^2 which demonstrated the highest cloacal molds, possibly due to the position of the cages. As the hens aged, the eggshell counts of several microbes including *E. coli*, total coliforms, Enterobacteriaceae, and molds decreased while cloacal *Salmonella* positives increased over time. The correlative analysis showed that cloacal and eggshell microbes were not strongly correlated, likely due to the separation from the reproductive tract and cuticle at the time of lay. Finally, the results of this study indicate that increasing density does not affect egg safety at the point of collection. Unfortunately, this study did not include any animal health variables or microbial compositions found. In future studies, swabbing the egg collection belt as well as identifying *Salmonella* strains and enumerating *Salmonella* could provide some valuable insight. Furthermore, analysis of animal health parameters as well as microbial compositions would be a valuable addition to this research to determine the presence of disease-causing organisms as well as microbiome changes.

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