Postprint: Effects of Different Stocking Densities on Duodenal Gut Microbiota in Caged Laying Hens

Authors: Yu Jiangming, Wang Qiuju, Liu Bolin, Ning Bolin, Wang Long, Zhao Qianyu, Liu Shengjun, Quan Jiahui

Date: 2017-10-10T00:00:00+00:00

Abstract

The present study employed polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) to investigate the effects of different stocking densities on the duodenal intestinal microbiota of caged Hy-Line Gray laying hens. A total of 1,250 11-week-old Hy-Line Gray laying hens were randomly selected and allocated to five groups based on stocking density: Group A, 900 cm^2/bird; Group B, 675 cm^2/bird; Group C, 540 cm^2/bird; Group D, 450 cm^2/bird; and Group E, 380 cm^2/bird, with 50 replicates per group. All five groups were maintained under identical environmental and feeding management conditions. At 16, 26, and 50 weeks of age, five chickens were randomly selected from each group, duodenal contents were collected, and intestinal microbiota analysis was performed using PCR-DGGE. The results demonstrated that within each stocking density, the similarity coefficient of duodenal intestinal microbiota structure in caged laying hens progressively decreased with advancing age. At 16 weeks of age, the structural similarity of duodenal microbiota between high- and low-density groups was high, indicating minimal differences in microbiota composition. Conversely, at 26 and 50 weeks of age, the structural similarity between high- and low-density groups was low, indicating substantial differences in microbiota composition. Consequently, the divergence in duodenal intestinal microbiota structure of caged laying hens increased with rearing age. Two beneficial bacterial species (Lactobacillus gastricus and Lactobacillus alvi) were absent in the high stocking density groups (Groups D and E), and stocking densities higher than 450 cm^2/bird had adverse effects on the duodenal intestinal microbiota structure of caged laying hens.

Full Text

Effects of Different Stocking Density on Duodenal Microbial Flora in Caged Laying Hens

YU Jiangming, WANG Qiuju*, LIU Bolin, NING Bolin, WANG Long, ZHAO Qianyu, LIU Shengjun, **QUAN Jiahui
(College of Animal Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 163319)

Abstract

This study employed polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) to investigate the effects of different stocking densities on the duodenal microbial flora of caged Hy-Line Gray laying hens. A total of 1,250 eleven-week-old Hy-Line Gray laying hens were randomly divided into five groups with different stocking densities: Group A at 900 cm^2/hen, Group B at 675 cm^2/hen, Group C at 540 cm^2/hen, Group D at 450 cm^2/hen, and Group E at 380 cm^2/hen, with 50 replicates per group. All groups were maintained under identical environmental and management conditions. At 16, 26, and 50 weeks of age, five hens were randomly selected from each group, and duodenal contents were collected for microbial analysis using PCR-DGGE. The results demonstrated that within the same stocking density, the similarity coefficient of duodenal microbial community structure decreased progressively with increasing age. At 16 weeks, the duodenal microbial flora showed high similarity between high- and low-density groups, indicating minimal structural differences. However, at 26 and 50 weeks, similarity between high- and low-density groups was markedly low, revealing substantial differences in microbial community structure. These findings indicate that differences in duodenal microbial community structure become more pronounced with advancing age. Moreover, two beneficial bacterial species (*Lactobacillus gastricus* and *Lactobacillus alvi*) disappeared in the high-density groups (Groups D and E), suggesting that stocking densities exceeding 450 cm^2/hen adversely affect the duodenal microbial community structure in caged laying hens.

Keywords: laying hens; stocking density; duodenal bacterial flora; PCR-DGGE

Introduction

As poultry production becomes increasingly intensive, stocking density has emerged as a ubiquitous stressor in the industry. Previous research by Wang Long demonstrated that elevated stocking density intensifies stress responses in stacked-cage systems, manifesting as poorer feather coverage, increased blood corticosterone levels, reduced egg production, and higher mortality rates, thereby severely compromising production performance. Beyond these

external indicators, stress conditions significantly impact internal gut health. The intestinal microbiota serves as a critical indicator of gut health, playing vital roles in nutrient digestion, absorption, and immune function. The small intestine is the primary site for nutrient absorption, with approximately 90% of ingested food absorbed there. The duodenum, being the segment with the largest diameter, deepest position, and most fixed location in the small intestine—and the site where both the pancreatic duct and common bile duct open—directly influences overall animal health. Investigating how stocking density affects duodenal bacterial community structure is therefore essential for elucidating the internal mechanisms governing poultry health.

Traditional microbial cultivation methods can only culture 10–60% of bacteria *in vitro*, providing an incomplete and inaccurate representation of gut microbiota composition. Consequently, these methods have gradually fallen out of favor. The development of molecular techniques based on 16S ribosomal RNA (rRNA) genes, such as DGGE and PCR, has accelerated the detection and characterization of intestinal microbial communities. Currently, the PCR-DGGE method is widely adopted for its speed and detailed resolution in analyzing gut microbiota structure. While DGGE has been extensively applied to detect gastrointestinal bacterial communities in various species, its use in poultry intestinal microbiota analysis remains in its early stages, with few studies examining laying hen gut microbiota. Furthermore, no previous research has investigated the impact of stocking density stress on duodenal microbial community structure in laying hens. This study addresses this knowledge gap by employing PCR-DGGE to analyze how different stocking densities affect duodenal microbial community structure in caged laying hens, providing insights into stocking density-induced stress from a gut health perspective and establishing a foundation for comprehensive analysis of laying hen intestinal microbiota.

Experimental Animals and Design

A total of 1,250 eleven-week-old Hy-Line Gray laying hens (obtained from Heilongjiang Xinghe Biotechnology Co., Ltd.) were randomly allocated into five groups based on stocking density. The cages measured 600 mm × 450 mm × 430 mm (length × width × height). Group A had 900 cm^2/hen (3 hens/cage), Group B had 675 cm^2/hen (4 hens/cage), Group C had 540 cm^2/hen (5 hens/cage), Group D had 450 cm^2/hen (6 hens/cage), and Group E had 380 cm^2/hen (7 hens/cage). Each group comprised 50 replicates, with all hens housed on the same tier (the second level from the ground). Throughout the experimental period, all five groups were maintained under identical environmental conditions and management protocols.

Sample Collection and Processing

Sampling was conducted at 16, 26, and 50 weeks of age. Within each group, 1–2 replicates were randomly selected from every 15 replicates, with five hens chosen from each selected replicate. For lower-density groups (3–4 hens/cage),

six replicates were selected, with five hens chosen from every two replicates. Selected hens were euthanized by severing the jugular vein, and duodenal segments were aseptically isolated and separated. The duodenal contents from five hens within each replicate were thoroughly mixed and placed into a single 15 mL centrifuge tube, labeled, and stored at -80°C until analysis. provides the sample numbering scheme for duodenal contents.

PCR Amplification of Bacterial 16S rDNA Fragments

DNA was extracted from each content sample using the CTAB manual extraction method. Sample genomic DNA served as the template for amplification of 16S rDNA hypervariable regions using bacterial universal primers GC-338F and 518R. Primer details are presented in .

The PCR amplification system (50 L) consisted of: 10× PCR buffer 5 L, dNTPs (2.5 mmol/L) 3.2 L, rTaq polymerase (5 U/ L) 0.4 L, GC-338F primer (20 mol/L) 1 L, 518R primer (20 mol/L) 1 L, template DNA 50 ng, and ddH_2O to a final volume of 50 L.

The PCR amplification program was: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 1 min; final extension at 72°C for 10 min.

DGGE Analysis of PCR Products

Ten microliters of PCR product were analyzed by DGGE using 7% polyacrylamide gels with a denaturing gradient of 35-55%. Electrophoresis was performed at 150 V and 60°C for 5 h in 1× TAE buffer. Following electrophoresis, gels were silver-stained for visualization.

Recovery and Sequencing of Dominant Bands from DGGE Profiles

Target DGGE bands were excised with a sterile scalpel, and DNA was extracted using the OMEGA Poly-Gel DNA Extraction Kit. Two microliters of recovered product served as template for PCR re-amplification using primers 338F/518R. The re-amplified DNA fragments were gel-purified, ligated into pMD18-T vectors, and transformed into DH5α competent cells. Positive clones were sequenced by BGI for the inserted bacterial 16S rDNA fragments.

Data Analysis

Sequences were subjected to homology comparison using the Blast program in GenBank to obtain the most similar reference 16S rDNA sequences. A phylogenetic tree was constructed using MEGA 5 software with the Neighbor-joining method and bootstrap analysis (1,000 replicates). Bacterial diversity indices, which comprehensively evaluate community species richness, abundance, and evenness, were calculated based on band numbers and intensities (gray values)

in the electrophoresis profiles to determine diversity index (H), evenness (E), and richness (S) for each sample.

Results

PCR Amplification of Bacterial 16S rDNA

Amplification with primers GC-338F and 518R yielded DNA fragments of approximately 250 bp from all samples ([Figure 1: see original paper]), confirming suitability for DGGE analysis.

DGGE Analysis of PCR Products

The DGGE analysis results for 16S rDNA PCR products are shown in [Figure 2: see original paper]. Each lane represents a pooled sample from five hens, reflecting the average duodenal microbial community status for each group. Different band positions represent distinct bacterial species, while band quantity indicates microbial population richness (more bands = greater diversity). Band brightness and thickness reflect population abundance (brighter, thicker bands = higher bacterial numbers).

Variations in band position, number, brightness, and thickness among lanes demonstrate differences in duodenal microbial community composition both between stocking densities at the same age and across ages within the same density.

The percentages below [Figure 3: see original paper] indicate overall similarity to Group 1 samples. The highest similarity (48.2%) was observed for Group 3, while the lowest (19.3%) was for Group 14, indicating low similarity and substantial differences between groups. Groups 1–15 contained 17, 14, 22, 22, 15, 22, 14, 15, 18, 12, 17, 22, 24, 14, and 21 bands, respectively. No bands were common to all 15 groups, confirming structural differences in microbial communities.

Bacterial Community Structure Similarity Among Samples

As shown in , similarity indices for duodenal microbial communities within the same stocking density across different ages ranged from 12.1% to 64.5%. The lowest similarity (12.1%) occurred between 16 and 26 weeks in the 6 hens/cage group, while the highest (64.5%) was between 16 and 26 weeks in the 4 hens/cage group. Overall, within each stocking density, similarity coefficients decreased progressively with increasing age, indicating growing divergence in microbial community structure. Thus, age substantially influences duodenal microbiota composition in caged laying hens.

Similarity indices among different stocking densities within the same age ranged from 6.0% to 70.3%. The lowest similarity (5.4%) occurred between Groups B and E at 26 weeks, while the highest (70.3%) was between Groups B and E at

16 weeks. In general, high- and low-density groups showed high similarity at 16 weeks (minimal differences) but low similarity at 26 and 50 weeks (marked differences), indicating that stocking density effects become more pronounced with age.

Sequencing of Dominant Electrophoresis Bands

After excising DGGE bands, PCR re-amplification with primers 338F/518R yielded target DNA fragments. Purified PCR products were ligated into pMD18-T vectors, transformed into DH5α cells, and sequenced. Blast comparison of sequences against GenBank identified the bacterial types represented by each band. Three clones per band were sequenced (). Among 32 sequencing results, microbial homology ranged from 84% to 100%, with most exceeding 98%. Ten bands showed 100% homology, while only six bands had <98% homology. Band 30 showed only 84% similarity to *Pediococcus siamensis*, suggesting it may represent a novel species within Firmicutes.

The analysis detected 26 Firmicutes species, 5 Proteobacteria species, and 1 Actinobacteria species, confirming Firmicutes as the dominant phylum in the duodenal microbiota of laying hens. *Pseudomonas japonica* (Band 28) was unique to Group 14. *Lactobacillus equi* (Band 16) was common to all groups except Group 9. *Romboutsia ilealis* (Band 7), *Lactobacillus gastricus* (Band 8), *Lactobacillus vaginalis* (Band 9), and *Lactobacillus alvi* (Band 10) gradually disappeared with increasing stocking density. *Lactobacillus equi* (Band 17) and *Enterococcus cecorum* (Band 19) were detected in both the lowest- and highest-density groups.

A phylogenetic tree was constructed using MEGA 5 software with the Neighbor-joining method (bootstrap = 1,000) ([Figure 4: see original paper]).

Discussion

During poultry growth and development, intestinal microbial communities produce various metabolites that can be beneficial or detrimental to the host. Interactions between microbiota and gastrointestinal epithelial cells induce structural and functional changes in the digestive tract. Microbiota influence lipid digestion and modify carbohydrate and protein digestion, increase energy and protein requirements, and negatively impact vitamin absorption. Additionally, beneficial bacteria protect against pathogen invasion and constitute part of the intestinal immune system, underscoring the critical role of intestinal microbiota in gut function.

Intestinal microbial composition is complex, with abundance and diversity varying according to poultry species, age, diet, and environment. Even under identical rearing conditions, individual gut microbiota structures differ. To minimize individual variation effects, this study randomly selected 3–6 replicates per group, with five hens per replicate, to detect duodenal contents, thereby

improving data accuracy and PCR-DGGE profile similarity. However, only one bacterial species—*Lactobacillus equi* (Band 16)—was common across all density groups. This substantial variation in gut microbiota among hens reared in the same environment may be attributed to large flock size (30,000 hens in 5-tier cages), where both spatial constraints and group size influence microbial communities. Nevertheless, *Lactobacillus equi*, previously reported as a dominant species in Japanese racehorse feces, appears to be a duodenal dominant bacterium unaffected by age or rearing environment.

Enterococcus cecorum (Band 19) is a known pathogen that causes arthritis and osteomyelitis in poultry. Its detection in both the lowest-density (Group A) and highest-density (Group E) groups suggests that both extremely low and high densities adversely affect duodenal microbial community structure. Numerous studies have identified *Lactobacillus gastricus* and *Lactobacillus alvi* as beneficial bacteria that maintain intestinal balance and prevent pathogen invasion. Their disappearance with increasing stocking density in this study demonstrates that density significantly impacts duodenal microbiota, with high densities exerting detrimental effects.

Research has shown that beneficial bacteria can increase eggshell weight and strength without affecting egg weight and can block pathogen growth in the digestive tract by altering pathogen receptors. Generally, increased stocking density or heat stress exacerbates pathogen damage to beneficial bacteria. Ni Xueqin et al. used PCR-DGGE to analyze effects of age and intestinal segment on microbial community structure and diversity in laying hens, finding that intestinal location determines community structure while age influences bacterial diversity. In subsequent work, they demonstrated that chicken major histocompatibility complex (MHC) genes affect cecal bacterial population structure, with cecal diversity being highest, followed by ileum and jejunum, and lowest in crop and duodenum, with diversity increasing across all segments with age. Li Yongzhu et al. analyzed cecal bacterial community structure in healthy and unhealthy flocks of different breeds and ages, revealing significant compositional differences and substantial impacts on growth performance. Wang Qiuju et al. examined intestinal microbiota in caged Hy-Line Brown pullets, finding that bacterial community structure varied significantly among intestinal segments at the same age.

During normal production, dietary formulations differ between pre-lay and post-lay periods, potentially affecting gut bacterial composition. However, this study found no significant differences in duodenal microbiota structure among density groups at 16 weeks, while marked differences were observed at 26 and 50 weeks when hens were fed the same diet. This indicates that age significantly influences gut microbiota. Furthermore, within each stocking density, similarity coefficients decreased with increasing age, demonstrating that duration of exposure substantially impacts duodenal microbial community structure.

Conclusions

1. Stocking density significantly affects duodenal microbial community structure in caged laying hens. As density increases beyond 450 cm^2/hen, beneficial bacteria (*Lactobacillus gastricus* and *Lactobacillus alvi*) disappear, adversely affecting microbial balance.

2. Within each stocking density, similarity coefficients for duodenal microbial community structure decrease progressively with age, indicating increasing divergence. Thus, age substantially influences structural changes in the duodenal microbiota of laying hens.

References

[1] ONBAŞILAR E E, AKSOY F T. Stress parameters and immune response of layers under different cage floor and density conditions[J]. *Livestock Production Science*, 2005, 95(3): 255-263.

[2] WANG Long. Effects of stocking density on production performance and welfare of laying hens in stacked cages[D]. Master's thesis. Daqing: Heilongjiang Bayi Agricultural University, 2015: 25-29.

[3] SOHAIL M U, HUME M E, BYRD J A, et al. Molecular analysis of the caecal and tracheal microbiome of heat-stressed broilers supplemented with prebiotic and probiotic[J]. *Avian Pathology*, 2015, 44(2): 67-74.

[4] ROBERTS T, WILSON J, GUTHRIE A, et al. New issues and science in broiler chicken intestinal health: intestinal microbial composition, shifts, and impacts[J]. *World' s Poultry Science Journal*, 2015, 71(2): 259-270.

[5] GONG J H, SI W D, FORSTER R J, et al. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca[J]. *FEMS Microbiology Ecology*, 2007, 59(1): 147-157.

[6] JANCZYK P, HALLE B, SOUFFRANT W. Microbial community composition of the crop and ceca contents of laying hens fed diets supplemented with *Chlorella vulgaris*[J]. *Poultry Science*, 2009, 88(11): 2324-2332.

[7] KIM G B, SEO Y M, KIM C H, et al. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers[J]. *Poultry Science*, 2011, 90(1): 75-82.

[8] GABRIEL I, LESSIRE M, MALLET S, et al. Microflora of the digestive tract: critical factors and consequences for poultry[J]. *World' s Poultry Science Journal*, 2006, 62(3): 499-511.

[9] KNARREBORG A, SIMON M A, ENGBERG R M, et al. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages[J]. *Applied and Environmental Microbiology*, 2002, 68(12): 5918-5924.

[10] SUZUKI K, KODAMA Y, MITSUOKA T. Stress and intestinal flora[J]. *Bifidobacteria and Microflora*, 1989, 8(1): 23–38.

[11] ENDO A, OKADA S, MORITA H. Molecular profiling of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species in feces of active racehorses[J]. *Journal of General and Applied Microbiology*, 2007, 53(3): 191–200.

[12] ENDO A, ROOS S, SATOH E, et al. *Lactobacillus equigenerosi* sp. nov., a coccoid species isolated from faeces of thoroughbred racehorses[J]. *International Journal of Systematic and Evolutionary Microbiology*, 2008, 58(4): 914–918.

[13] BORST L B, SUYEMOTO M M, SHIVARAMU K, et al. A chicken embryo lethality assay for pathogenic *Enterococcus cecorum*[J]. *Avian Diseases*, 2014, 58(2): 244–248.

[14] KENSE M J, LANDMAN W J M. *Enterococcus cecorum* infections in broiler breeders and their offspring: molecular epidemiology[J]. *Avian Pathology*, 2011, 40(6): 603–612.

[15] BOERLIN P, NICHOLSON V, BRASH M, et al. Diversity of *Enterococcus cecorum* from chickens[J]. *Veterinary Microbiology*, 2012, 157(3/4): 405–411.

[16] CÁRDENAS N, MARTÍN V, DELGADO S, et al. Characterisation of *Lactobacillus gastricus* strains isolated from human milk[J]. *International Dairy Journal*, 2014, 39(1): 167–177.

[17] MARTÍN V, CÁRDENAS N, JIMÉNEZ E, et al. Genome sequence of *Lactobacillus gastricus* PS3, a strain isolated from human milk[J]. *Genome Announcements*, 2013, 1(4): e00489–13, doi: 10.1128/genomeA.00489-13.

[18] KIM H J, EOM S J, PARK S J, et al. *Lactobacillus alvi* sp. nov., isolated from the intestinal tract of chicken[J]. *FEMS Microbiology Letters*, 2011, 323(1): 83–87.

[19] PANDA A K, REDDY M R, RAMARAO S V, et al. Effect of dietary supplementation of probiotic on performance and immune response of layers in the decline phase of production[J]. *Indian Journal of Poultry Science*, 2000, 35(1): 102–104.

[20] NI Xueqin, GONG J, YU H, et al. Analysis of bacterial community structure and diversity in the intestine of laying hens using PCR-DGGE[J]. *Acta Veterinaria et Zootechnica Sinica*, 2008, 39(7): 955–961.

[21] NI Xueqin, GONG J, YU H, et al. Effect of MHC gene on intestinal bacterial population structure in laying hens analyzed by PCR-DGGE[J]. *Scientia Agricultura Sinica*, 2009, 42(7): 2564–2571.

[22] LI Yongzhu, CUI Y Q. Analysis of cecal microbial community structure in different chicken flocks using PCR-DGGE[J]. *Acta Ecologica Sinica*, 2011, 31(21): 6513–6521.

[23] WANG Qiuju, CUI Yizhe, LIU Shengjun, et al. Analysis of intestinal microbiota in caged Hy-Line Brown pullets using polymerase chain reaction-denaturing gradient gel electrophoresis[J]. *Chinese Journal of Animal Nutrition*, 2014, 26(2): 504-512.

Note: Figure translations are in progress. See original paper for figures.