JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Comparative gut microbiota, growth performances, and cytokine indices in broiler chickens with or without litter
Running Title (within 10 words)	Gut microbiota in broiler chickens with or without litter
Author	Jin Young Jeong [1,*], Seol Hwa Park [1], Minji Kim [1], Hwan Ku Kang [1] and Nam-Geon Park [2]
Affiliation	1Animal Nutrition and Physiology Division, National Institute of Animal Science, Wanju 55365, Korea 2Technology Service Division, National Institute of Animal Science, Wanju 55365, Korea
ORCID (for more information, please visit https://orcid.org)	Jin Young Jeong (https://orcid.org/0000-0002-8670-7036) Seol Hwa Park (https://orcid.org/0000-0002-7218-8212) Minji Kim (https://orcid.org/0000-0003-2106-1921) Hwan Ku Kang (https://orcid.org/0000-0002-4286-3141) Nam-Geon Park (https://orcid.org/0000-0001-6241-2850)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This study was supported by the "Cooperative Research Program for Agriculture, Science, and Technology Development (Project No. PJ015693), Rural Development Administration, Republic of Korea.
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Jeong JY, Park SH. Data curation: Kim MJ. Formal analysis: Jeong JY, Kang HG. Methodology: Park SH, Kim MJ. Software: Jeong JY. Validation: Kang HG, Park NG. Investigation: Park NG. Writing - original draft: Jeong JY Writing - review & editing: Jeong JY, Park SH, Kim MJ, Kang HG, Park NG.
Ethics approval and consent to participate	All animal experiments were approved and reviewed by the National Institute of Animal Science (NIAS) Animal Use and Care Committee (NIAS-2021-0508).

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Jin Young Jeong
Email address – this is where your proofs will be sent	jeong73@korea.kr
Secondary Email address	jeongjinyoung73@gmail.com
Address	Animal Nutrition and Physiology Division, National Institute of Animal Science, Rural Development Administration, Wanju 55365, Korea
Cell phone number	+82-10-9754-5880
Office phone number	+82-63-238-7487
Fax number	+82-63-238-7497

Abstract

Developmental patterns of the gut microbiota are important for improving chicken health and productivity. However, the influence of litter and litter microbes on cecal microbiota is still unclear. This study aimed to identify broiler cecal microbiota at different ages according to litter usage in cage (without litter) and conventional (with litter) conditions. The cecal contents of the broilers from each group were collected from 1–5 weeks. The development and function of the gut microbiota were evaluated using 16S rRNA gene sequencing. The final body weight of the chickens was higher in the cage group than that in the conventional group. In particularly, α-diversity was higher at 3 weeks than that at 1 week. The phyla Firmicutes predominated at 3 weeks. In contrast, the abundance of Bacteroidetes and fibrinolytic bacteria increased significantly at 1 and 2 weeks compared to that at 3 and 5 weeks. *Corynebacterium* was the most abundant genus in the conventional group after 3 weeks. In conclusion, the cecal microbiota are influenced by environmental factors, such as cage, which improves the chicken gut environment.

Keywords: Microbiome, Broiler, Growth performance, Litter

Introduction

In the poultry industry, the immune system and growth performance are governed by changes in the bedding conditions. Particularly, weight gain and gut health of poultry are critical to maintain a healthy population [1–3]. Age and environmental conditions also considerably affect microbial communities [4,5].

The suitability of various materials such as the bedding for chickens has been studied previously[6–8]. Growth performance, health, carcass quality, and welfare are directly affected by litter. Rice hulls can be considered a cost-effective litter source that can be used in place of traditional bedding in rice-producing areas. The use of thick sawdust or rice straw did not significantly affect weight gain and carcass weight [9]. Conversely, broilers reared on rice hull had lower weight gain than other groups [10]. All microorganisms were significantly higher in the rice hull treatment, except total yeast. however, body weight gain and mortality did not show statistically significant differences between treatment groups [11].

The avian gut microbiome varies considerably from that of mammalian. Litter, as bedding material, alters the microbial composition and diversity in the cecum of chickens [12]. Moisture promotes the growth of pathogenic microbes and ammonia production, which adversely affect weight and feed conversion in poultry. Additionally, litter supply and the gut microbiome are related to poultry performance [13]. Bacteroides and Eubacteria are established within 2 weeks, and gut microbes take 6–7 weeks for complete colonization in chickens [14]. The dominant phyla in the cecum of chickens throughout the life cycle are Firmicutes and Bacteroidetes [15–17]. In broiler chickens, gut microbiome colonization and function differ from 1–42 d [18-20].

Changes in gut microbial function and microbial metabolites, such as those of the immune system (cytokines), are simultaneously observed, depending on the litter. However, some studies have found no significant differences in peripheral blood leukocyte counts between cage- and litter laying hens [21]. In general, animals raised in outdoor environments have stronger immune functions [21,22]. Immune functions among animals vary with litter broilers exhibiting higher levels of interleukin-1β (IL-1β) and interferon-γ mRNA than those in caged chickens [12, 23-25]. Free-range and semi-stocked chickens demonstrate higher titers of Newcastle disease virus and infectious bronchitis virus in peripheral blood than those in confined chickens.

A recent study on litter has revealed altered microbial composition and diversity in the cecum [26]. However, it is unclear whether litter and litter microbes can influence the cecal microbiota. This study aimed to determine whether litter affects broiler gut microbiota and growth characteristics.

Materials and Methods

Experimental design and animal care

All animal experiments were approved and reviewed by the National Institute of Animal Science (NIAS) Animal Use and Care Committee (NIAS-2021-508). All broiler chickens were managed

according to the National Research Council specifications. One-day-old broiler chicks (Ross 308) were purchased from a commercial farm and divided into two groups. Each group was assigned to a floor pen (0.93 m × 2.14 m). The size of mesh is 2.54 cm by galvanized steel wire, and bedding materials is used rice hulls. The chickens were fed using a graded feeding program (Table 1) consisting of starters (0–7 days), growers (8–21 days), and finishers (22–35 days); water was provided ad libitum. Feed was supplied as small pellets for the start-up phase and as pellets for the growth and finishing phases. The animals were randomly assigned to one of the six replicate pens per treatment. The experimental groups were divided into cage and cage-free groups, according to litter usage. Room temperature was monitored daily. The light-dark cycle was set from 18 to 6 h during the experimental period. All bedding materials are sterilized and UV irradiated. Additionally, all experimental equipment was brought into the room after a sterilized or sterilized products were used. Body weight and feed intake were recorded weekly. The weight gain and feed conversion ratio (FCR) were then calculated. At 7, 14, 21, 28, and 35 days of age, chickens in the treatment groups were euthanized by anesthesia with carbon dioxide. Blood was collected from the carotid artery or wing vein. Cecal digesta were placed in liquid nitrogen and stored at -80 °C.

Hematological and cytokine analysis

Blood samples were collected from the carotid artery or wing vein using ethylenediaminetetraacetic acid tubes (BD Vacutainers). An automated hematology analyzer (Mindray BC-5300; Mindray Co., Ltd., Shenzhen, China) was used to assess hematological parameters, such as red blood cell (RBC) count, white blood cell (WBC) count, packed cell volume, hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, erythrocyte sedimentation rate, total protein, and absolute counts of heterophils, lymphocytes, monocytes, eosinophils, and basophils, according to the manufacturer's instructions. Concentration of pro-inflammatory cytokines, including IL-1β, interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α), were measured using commercial chicken enzyme-linked immunosorbent assay kits (AFG Scientific, EK780087, EK780053, EK780062) according to the manufacturer's instructions.

DNA extraction and Microbial Community Analysis

Metagenomic DNA was extracted from broiler cecal samples using the bead-beating (repeated bead-beating plus column) method [27] via a QIAamp DNA kit (Qiagen, Hilden, Germany).

Artificial sequences and low-quality bases in the generated reads were removed using Trimmomatic and TruSeq3-PE. fa:2:30:10:2:True, LEADING:5, TRAILING:20, MINLEN:250 parameters [28]. After raw data QC, the filtered reads were analyzed using QIIME2 [29]. The remaining adapter sequences in the filtered reads were removed using the cutadapt module in the QIIME2 with --p-front-f CCTACGGGNGGCWGCAG and-p-front-r GAC-TACHVGGGTATCTAATCC parameters [30]. The denoising step was conducted using dada2, a denoise-paired module in QIIME2, with parameters–p-trunc-len-f 230 and–p-trunc-len-r 220 [31].

Taxonomic assignment was conducted using the classify-sklearn module with a pretrained silva-138-99-nb-classifer. qza as provided by QIIME2 [32]. After taxonomic assignment, taxa assigned to the mitochondria and chloroplasts and those whose assigned level did not represent the minimum phylum were filtered out.

Statistical analyses

The align-to-tree-mafft-fasttree module [33,34] was used for tree construction of the representative amplicon sequence variant (ASV), and alpha- and beta-diversity were calculated using the diversity module in QIIME2 [35]. For functional pathway prediction of the microbial community, PICRUST2 was employed with a frequency table exported from QIIME2 [36]. Principal Component Analysis (PCA) plots and statistical tests for the predicted pathways were conducted using STAMP with the Kruskal–Wallis test [37]. Differential abundance taxon analyses were conducted using Linear discriminant analysis effect size (LEfSe) [38]. Significant differences in blood results and growth performance were determined at $P < 0.05$, using Prism ver. 9 software.

Results

Growth performances

The effects of environmental bedding conditions on the growth performance of broiler chickens are shown in Table 2. Final body weight, weight gain, and feed conversion ratio were higher in chickens housed in cages (without litter) than those in conventional conditions (with litter) for 5 weeks ($P < 0.01$). However, the average daily feed intake did not differ significantly between the conventional and cage groups.

Blood analysis

Blood hematological and cytokine analyses were performed for the different bedding environmental conditions (Figure 1). Under different conditions, the WBC counts were higher in the cage group than those in the conventional group ($P < 0.001$). The observed increase in white blood cells is proposed to represent a defensive mechanism against external disease or inflammation. However, the RBC count, HGB level, and MCV were not significantly different between the two groups. In addition, TNF-α, IL-1, and IL-6 levels were not differentially regulated between the conventional and cage groups.

Alpha and beta diversity

In the broiler cecum, changes in alpha diversity were confirmed over 5 weeks (Figure 2). The alpha diversity was significantly different from 1 to 2 wks. However, the diversities at weeks 3, 4, and 5 were similar. In the bedding environment, the alpha diversity was not significantly different. Beta diversity clustered from 1 to 5 weeks, similar to the alpha diversity pattern. Beta diversity determined using the PCoA plot was independent of the presence or absence of litter.

Bacteria at the phylum level between the cage and conventional groups by aging

The gut microbiota was dominated by Firmicutes, Proteobacteria, and Bacteroidetes at the phylum level at 1 and 2 weeks, especially, the Firmicutes account for greater than 98% (Figure 3). The gut microflora composition marginally varied between the two groups after one week. *Ruminococcus* was the predominant genus in majority of the samples. In addition, *Lactobacillus* and *Bacillus* corresponding to lactic acid bacteria, *Escherichia-Shigella* including *Escherichia coli*, and *Erysipelatoclostridium* and *Clostridium* were identified as the major genera at 1 week. The gut microflora at 2 weeks was not significantly different from that at the first week. *Faecalibacterium* was dominant at 3 weeks of age. At 4 and 5 weeks, the predominant genera were *Faecalibacterium*, *Lactobacillus*, and *Clostridia*, accounting for more than half of the total population (Figure 4).

Microbial pathway analysis for different bedding conditions

This study attempted to identify significant pathways in individual pathway units. P4-PWY (superpathway of L-lysine, L-threonine, and L-methionine biosynthesis I) and PWY0-781 (aspartate super-pathway) were upregulated in the control group with litter after 1 week (Figure 5). A total of 48 pathways were affected by litter use, among which 32 pathways were upregulated and 16 were downregulated at 2 weeks ($P < 0.05$). At 3 weeks, 21 pathways showed significant differences with respect to litter use, among which 16 pathways were downregulated and five were upregulated in the conventional group ($P < 0.05$). The relative distribution of functional pathways within the intestinal microbial flora was determined to identify clustering patterns between groups using PCA at 3 and 4 weeks. On supplying litter at 3, 4, and 5 weeks, the mycolyl-arabinogalactan-peptidoglycan complex biosynthesis pathway was upregulated in the conventional group (Table 3). LEfSe was used to identify differences depending on litter use. Four differentially abundance taxa at the genus level were discovered at week 1 using LEfSe (Figure 6). The abundance of *Romboutsia* and *Turicibacter* increased in the conventional-litter group whereas that of *Lachonoclostridium* increased in the cage group without litters. In the second week, no significant differences were observed between the cage and conventional cage groups. Relative abundance was not detected at the genus level at week 2 (data not shown). Eight differentially abundance taxa were detected at the genus level at 3 weeks, including five differentially abundance taxa with clear genera. Among these, the abundances of *Corynebacterium* and *Hydrogenoanaerobacterium* were increased in the litter use group, whereas those of *Odoribacter*, *Anaerofustis*, and *Faecalibacterium* were increased in the cage group. Eight differentially abundance taxa were detected at the genus level at 4 weeks. The increased abundances of *Turicibacter* at 1 week and *Corynebacterium* at 3 weeks further increased at 4 weeks. The abundances of *Roseburia*, *Staphylococcus*, *Brachybacterium*, and *Brevibacterium* also increased in the litter-treated groups, whereas that of *Tyzzerella* increased in the cage group. In the fifth week, the four differentially abundance taxa showed differences at the genus level, depending on the litter. The abundances of taxa *Corynebacterium* and *Papillybacter* increased with litter use. In particular, the

abundance of Corynebacter increased at both 3 and 4 weeks. In the absence of litter, an increase in the abundances of two differentially abundance taxa (*Colidextribacter* and *Flavonifractor*) were observed. Relative abundance at the genus level differed based on the type of bedding. The cage and conventional groups are indicated in red and green, respectively. The bacterial taxa were statistically significant ($P < 0.05$) in terms of relative abundance.

Discussion

Body weight gain in broiler chickens is influenced by various environmental conditions, including aging, nutrients, microbiome, immunity, and bedding materials [39,40]. In this study, growth performance generally showed a significant difference with or without litter (i.e., cage vs. conventional cage). In particular, although the FCR decreased in broilers in the cage at an early phase, it was ameliorated during the growing phase. Broiler weight gain from days 0–28 was not significantly different between the cage and conventional groups, similar to the findings of a previous study [41].

The productivity and intestinal microbiota were influenced in caged chickens, thus promoting the growth of beneficial microbes and preventing harmful bacteria. Therefore, we investigated the effects of litter use on the gut microbiota of chicken in cages (without litter) and conventional conditions (with litter). The most abundant phyla in the broiler cecum was Firmicutes, which is consistent with previous findings [42,43]. Firmicutes, associated with chicken weight gain, produce compounds in the intestinal wall as an energy source. In this study, the abundance of Firmicutes increased marginally under litter conditions. The abundance of gut bacteria was relatively low in the litter-treated group, as reported in previous studies [44,45].

Ruminococcus was significantly more abundant at all ages. The abundance of Bacteroides and *Ruminococcus* is associated with gut health [46]. The increased abundance of Lactobacilli may inhibit pathogens by producing vitamins and organic acids [47] Increasing the proportion of *Faecalibacterium* in the intestinal microflora positively affects growth [48]. *Faecalibacterium* produces short-chain fatty acids such as acetate, propionate, and butyrate, which are major products of intestinal microorganisms and commensal bacteria [49]. It also produces shikimic and salicylic acids, which are involved in its anti-inflammatory activities. *Faecalibacterium* spp. isolated from chickens with strong immunity may also serve as potential probiotics. Lysine, threonine, and methionine amino acids (AAs) are essential during the early chick phase [50]. The intestine-related inflammatory response can be attributed to β-galactomannan contained in soybeans of broiler fed. The increasing mannan degradation functions in the conventional group improved the abundance of gut microbiota in chickens, which changed with a decrease in intestine-related inflammatory reactions. Mannans are a type of hemicellulose found in a variety of cereals and industrial byproducts utilized in animal feed. While mannans can potentially be detrimental to animals, smaller portions of them offer benefits. The fermentation of mannan polysaccharides and oligosaccharides has been observed to alter the intestinal

microbiota. Therefore, the varying sizes and monosaccharides present in mannan polysaccharides may influence the intestinal microenvironment [51]. Mitigation can improve productivity and alleviate mortality. The abundance of *Faecalibacterium* increased in the cage group compared to that in the conventional group. Therefore, it is expected to play an important role in the health of individual species at 3 weeks of age owing to increased immunity. Increasing AAs in chickens housed without litter can enhance chicken health through intestinal microbial flora.

Five microbes were detected at the genus level. The abundances of *Corynebacterium* and *Hydrogenoanaerobacterium* increased in the conventional group while those of *Odoribacter*, *Anaerofustis*, and *Faecalibacterium* were enhanced in the cage group at 3 weeks. *Corynebacterium* can cause diseases in various livestocks [52]. After the third week, the use of litter for 3 weeks induced *Corynebacterium* growth. *Brachybacterium* and *Brevibacterium* species at 4 weeks associated with growth performance are frequently found in the microbial flora of dust and feces [53]. Forty-eight pathways showed significant differences after two weeks. Among these, 32 pathways were upregulated in the conventional group with litter and 16 pathways were downregulated in the cage group without litter. The upregulation of biosynthesis-related pathways and downregulation of decomposition-related pathways were observed.

In this study, the pathways identified based on the graphical analysis at weeks 3, 4, and 5 did not significantly affect the intestinal microbial flora during litter use. However, the three common pathways influencing the mycolyl-arabinogalactan-peptidoglycan complex biosynthesis increased at weeks 3, 4, and 5 compared to 1 and 2 wks. However, this pathway is unlikely to be directly related to the effect of litter, since it is specific to cell wall synthesis. *Romboutsia* was an uncharacterized bacterial genus. However, the fungal species in the gut microbiota of young hens showed differences when Astragalus was used as a feed additive [54]. *Romboutsia* is the major genus involved in functioning of the intestinal microbial flora of chicken [55]. In addition, *Turicibacter* is present at residual levels in the feed intake of chickens [56]. Feed intake and average weight gain of groups depended on litter use. The *Lachnoclostridium* strain can be used to regulate body weight and drip loss associated with meat quality and body weight in broilers [57]. This suggests that meat quality can be improved by regulating the intestinal microbiota. The genus Corynebacterium can cause diseases in various animals and its growth is positively reduced by lactic acid bacteria or feed additives [58]. Therefore, if the abundance of related species increases in the intestinal microbial flora, litter use may not be considered positive after the third week. In this study, the abundance of *Odoribacter*, a key bacterial species in feed additives consisting of phages, increased in the conventional groups without litter. *Anaerofustis* is related to energy metabolism and is positively correlated with the accumulation of abdominal fat in chickens [59]. Although this genus needs further evaluation, it is unlikely to positively affect growth rate. *Faecalibacterium* positively affects the growth of intestinal microflora [48]. In this study, *Faecalibacterium* was established as the dominant species in the cage group without litter from 3–5 weeks. During this period, the unuse of litter is preferable based on the existing known intestinal microorganisms.

Brachybacterium is mainly found in dust and fecal samples from poultry farms with poor breeding performance [60]. However, increase in the abundance of this species in litter has not been evaluated. In addition, *Brevibacterium* is also abundant on farms with poor performance [60]. Herein, considering these bacterial species markers to evaluate the use of litter in intestinal microorganism research may not yield good results. *Papillibacter* is a pathogenic bacterium with considerably reduced abundance in chickens when *Lactobacillus casei* is used as a feed additive. Increase in litter use did not positively affect intestinal microorganisms, even in the fifth week. Therefore, various evaluations may be necessary for related bedding, depending on the use of litter from the third week onwards.

In summary, all the bacterial species that increased in abundance in the cage (without litter) group are known to be associated with generally beneficial functions, such as improving growth performance or regulating immune responses. However, in this study, the intestinal microbial flora composition was more remarkably affected by the growth period than that by bedding use. In particular, chicken intestinal microbial flora was established, and the major dominant species did not change after the third week. In particular, the abundance of *Cornynebacterium* increased in the litter group from 3–5 weeks. Increased bacterial abundance in the litter had a negative effect in this study. Hence, it is necessary to consider the benefits of using litter by analyzing the intestinal microbiota. In contrast, improvement in the FCR and relative abundance of beneficial gut microbiota was observed in cages (without litter) compared to those in conventional-supplied litter. Hence, it is recommended that the use of litter should be avoided after three weeks when intestinal microorganisms are established.

Acknowledgments

This study was supported by the "Cooperative Research Program for Agriculture, Science, and Technology Development (Project No. PJ015693), Rural Development Administration, Republic of Korea.

References

1. Dahiya DK, Renuka MP, Puniya M, Shandilya UK, Dhewa T, Kumar N, et al. Gut microbiota modulation and its relationship with obesity using prebiotic fibers and probiotics: a review. Front Microbiol. 2017;8:563. https://doi.org/10.3389/fmicb.2017.00563

2. Yin D, Yin X, Wang X, Lei Z, Wang M, Guo Y, et al. Supplementation of amylase combined with glucoamylase or protease changes intestinal microbiota diversity and benefits for broilers fed a diet of newly harvested corn. J Anim Sci Biotechnol. 2018;9:24. https://doi.org/10.1186/s40104-018-0238-0

3. Kumar D, Pornsukarom S, Thakur S. Food safety in poultry meat production. antibiotic usage in poultry production and antimicrobial-resistant salmonella in poultry. Cham. 2019;pp. 47–66.

4. Bodogai M, O'Connell J, Kim K, Kim Y, Moritoh K, Chen C, et al. Commensal bacteria contribute to insulin resistance in aging by activating innate B1a cells. Sci Transl Med. 2018;10:eaat4271. https://doi.org/10.1126/scitranslmed.aat4271

5. You I, Kim MJ. Comparison of gut microbiota of 96 healthy dogs by individual traits: breed, age, and body condition score. Animals (Basel). 2021;11:2432. https://doi.org/DOI:10.3390/ani11082432

6. Bilgili SF, Montenegro GI, Hess JB, Eckman MK. Sand as litter for rearing broiler chickens 1. J Appl Poult Res. 1999;8:345–351. https://doi.org/DOI:10.1093/japr/8.3.345

7. Atapattu NSBM, Wickramasinghe KP. The use of refused tea as litter material for broiler chickens. Poult Sci. 2007;86:968–972. https://doi.org/DOI:10.1093/ps/86.5.968

8. Garcia RG, Almeida Paz ICL, Caldara FR, Nääs IA, Pereira DF, Ferreira VMOS. Selecting the most adequate bedding material for broiler production in Brazil. Braz J Poult Sci. 2012;14:121–127.

9. Costa HDA, Vaz RGMV, Silva MCD, Rodrigues KF, Sousa LF, Bezerra LDS, et al. Performance and Meat Quality of Broiler Chickens Reared on two Different Litter Materials and at two Stocking Densities. Br Poult Sci. 2021;62: 396–403. https://doi.org/10.1080/00071668.2020.1864810

10. Toghyani M, Gheisari A, Modaresi M, Tabeidian SA, Toghyani M. Effect of different litter material on performance and behavior of broiler chickens. Appl Anim Behav Sci. 2010;122:48-52. https://doi.org/10.1016/j.applanim.2009.11.008.

11. Durmuş M, Kurşun K, Polat Açık I, Tufan M, Kutay H, Benli H, et al. Effect of different litter materials on growth performance, the gait score and footpad dermatitis, carcass parameters, meat quality, and microbial load of litter in broiler chickens. Poult Sci. 2023;102:102763. https://doi.org/10.1016/j.psj.2023.102763.

12. Song Bochen, Li P, Xu H, Wang Z, Yuan J, Zhang B, et al. Effects of rearing system and

antibiotic treatment on immune function, gut microbiota and metabolites of broiler chickens. J Anim Sci Biotechnol. 2022;13:144. https://doi.org/DOI:10.1186/s40104-022-00788-y

13. De Toledo TDS, Roll AAP, Rutz F, Dallmann HM, Prá MAD, Fábio Pereira Leivas Leite FPL, et al. An assessment of the impacts of litter treatments on the litter quality and broiler performance: A systematic review and meta-analysis. PLoS One. 2020;15:e0232853. https://doi.org/DOI: 10.1371/journal.pone.0232853

14. Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr. 1999;69:1035S–1045S. https://doi.org/DOI:10.1093/ajcn/69.5.1035s

15. Rychlik I. Composition and function of chicken gut microbiota. Animals (Basel) 2020;10:103. https://doi.org/DOI:10.3390/ani10010103

16. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, et al. The chicken gastrointestinal microbiome. FEMS Microbiol Lett. 2014;360:100–112. https://doi.org/DOI:10.1111/1574-6968.12608

17. Nordentoft S, Mølbak L, Bjerrum L, De Vylder J, Van Immerseel F, Pedersen K. The influence of the cage system and colonisation of Salmonella enteritidis on the microbial gut flora of laying hens studied by T-RFLP and 454 Pyrosequencing. BMC Microbiol. 2011;11:187. https://doi.org/DOI:10.1186/1471-2180-11-187

18. Huang P, Zhang Y, Xiao Kangpeng, Jiang F, Wang Hengchao, Tang D, et al. The chicken gut metagenome and the modulatory effects of plant-derived benzylisoquinoline alkaloids. Microbiome. 2018;6:211. https://doi.org/DOI:10.1186/s40168-018-0590-5

19. Hu Y, Wang L, Shao D, Wang Q, Wu Y, Han Y, et al. Selectived and reshaped early dominant microbial community in the cecum with similar proportions and better homogenization and species diversity due to organic acids as AGP alternatives mediate their effects on broilers growth. Front Microbiol. 2019;10:2948. https://doi.org/DOI:10.3389/fmicb.2019.02948

20. Li MH, Meng JX, Wang W, He M, Zhao ZY, Ma N, et al. Dynamic description of temporal changes of gut microbiota in broilers. Poult Sci. 2022;101:102037. https://doi.org/DOI:10.1016/j.psj.2022.102037

21. Abo Ghanima MM, El-Edel MA, Ashour EA, Abd El-Hack ME, Othman SI, Alwaili MA, et al. The influences of various housing systems on growth, carcass traits, meat quality, immunity and oxidative stress of meat-type ducks. Animals (Basel). 2020;10:410. https://doi.org/DOI:10.3390/ani10030410

22. Braghieri A, Pacelli C, Verdone M, Girolami A, Napolitano F. Effect of grazing and homeopathy on milk production and immunity of Merino derived ewes. Small Rumin Res. 2007;69:95–102. https://doi.org/DOI:10.1016/j.smallrumres.2005.12.014

23. Yan L, Lv ZZ, An S, Xing K, Wang ZG, Lv MB, et al. Effects of rearing system and narasin on

growth performance, gastrointestinal development, and gut microbiota of broilers. Poult Sci. 2021;100:100840. https://doi.org/DOI:10.1016/j.psj.2020.10.073

24. Song Bochen, Yan S, Li P, Li G, Gao Mingkun, Yan L, et al. Comparison and correlation analysis of immune function and gut microbiota of broiler chickens raised in double-layer cages and litter floor pens. Microbiol Spectr. 2022;10:e0004522. https://doi.org/DOI:10.1128/spectrum.00045-22

25. Al-Otaibi MIM, Abdellatif HAE, Al-Huwail AKA, Abbas AO, Mehaisen GMK, Moustafa ES. Hypocholesterolemic, antioxidative, and anti-inflammatory effects of dietary spirulina platensisis supplementation on laying hens exposed to cyclic heat stress. Animals (Basel). 2022;12:2759. https://doi.org/DOI:10.3390/ani12202759

26. Bindari YR, Moore RJ, Van TTH, Hilliar M, Wu SB, Walkden-Brown SW, et al. Microbial communities of poultry house dust, excreta and litter are partially representative of microbiota of chicken caecum and ileum. PLoS One. 2021;16:e0255633. https://doi.org/10.1371/journal.pone.0255633

27. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 2004;36:808. https://doi.org/10.2144/04365ST04

28. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170

29. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–857. https://doi.org/10.1038/s41587-019-0209-9

30. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17:10–12. https://doi.org/10.14806/ej.17.1.200

31. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–583. https://doi.org/10.1038/nmeth.3869

32. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–D596. https://doi.org/10.1093/nar/gks1219

33. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30:772–780. https://doi.org/10.1093/molbev/mst010

34. Price MN, Dehal PS, Arkin AP. FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One. 2010;5:e9490. https://doi.org/10.1371/journal.pone.0009490

35. Vázquez-Baeza Y, Pirrung M, Gonzalez A, Knight R. EMPeror: a tool for visualizing high-

throughput microbial community data. GigaScience. 2013;2:16. https://doi.org/10.1186/2047-217X-2-16

36. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, et al. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol. 2020;38:685–688. https://doi.org/10.1038/s41587-020-0548-6

37. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics. 2014;30:3123–3124. https://doi.org/10.1093/bioinformatics/btu494

38. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60. https://doi.org/10.1186/gb-2011-12-6-r60

39. Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME. Microbiota, gut health and chicken productivity: what is the connection? Microorganisms. 2019;7:374. https://doi.org/10.3390/microorganisms7100374

40. Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes. 2014;5:108–119. https://doi.org/10.4161/gmic.26945

41. Bilal K, Mehmood S, Akram M, Imran S, Sahota AW, Javed K, et al. Growth performance of broilers under two rearing systems in three different housing zones in an environmentally controlled house during winter. J Anim Plant Sci. 2014;24:1039–1044.

42. Adewole D. Effect of dietary supplementation with coarse or extruded oat hulls on growth performance, blood biochemical parameters, ceca microbiota and short chain fatty acids in broiler chickens. Animals (Basel). 2020;10:1160. https://doi.org/10.3390/ani10081429

43. Adewole D, Akinyemi F. Gut microbiota dynamics, growth performance, and gut morphology in broiler chickens fed diets varying in energy density with or without bacitracin methylene disalicylate (BMD). Microorganisms. 2021;9:787. https://doi.org/10.3390/microorganisms9040787

44. Line JE. Aluminum sulfate treatment of poultry litter to reduce Salmonella and Campylobacter populations. Poult Sci. [Abstr.] 1998;77:S364.

45. Line JE. Campylobacter and Salmonella populations associated with chickens raised on acidified litter. Poult Sci. 2002;81:1473–1477. https://doi.org/10.1093/ps/81.10.1473

46. Hollister EB, Gao C, Versalovic J. Compositional and functional features of the gastrointestinal microbiome and their effects on human health. Gastroenterology. 2014;146:1449. https://doi.org/10.1053/j.gastro.2014.01.052

47. Radka CD, Frank MW, Rock CO, Yao Jiangwei. Fatty acid activation and utilization by Alistipes

finegoldii, a representative Bacteroidetes resident of the human gut microbiome. Mol Microbiol. 2020;113:807–825. https://doi.org/10.1111/mmi.14445

48. Martín R, Rios-Covian D, Huillet E, Auger S, Khazaal S, Bermúdez-Humarán LG, et al. Faecalibacterium: a bacterial genus with promising human health applications. FEMS Microbiol Rev. 2023;47:fuad039. https://doi.org/10.1093/femsre/fuad039

49. Parada Venegas DP, De la Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, et al. Short chain fatty acids (scfas)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. Front Immunol. 2019;10:277. https://doi.org/10.3389/fimmu.2019.00277

50. Lee CY, Song AAL, Loh TC, Abdul Rahim RahaA. Effects of lysine and methionine in a low crude protein diet on the growth performance and gene expression of immunity genes in broilers. Poult Sci. 2020;99:2916–2925. https://doi.org/10.1016/j.psj.2020.03.013

51. Wang J, Ke S, Strappe P, Ning M, Zhou Z. Structurally Orientated Rheological and Gut Microbiota Fermentation Property of Mannans Polysaccharides and Oligosaccharides. Foods. 2023;12:4002. https://doi.org/10.3390/foods12214002.

52. Yeruham I, Braverman Y, Shpigel NY, Chizov-Ginzburg A, Saran A, Winkler M. Mastitis in dairy cattle caused by Corynebacterium pseudotuberculosis and the feasibility of transmission by houseflies. I Vet Q. 1996;18:87–89. https://doi.org/10.1080/01652176.1996.9694623

53. Bindari YR, Moore RJ, Van TTH, Walkden-Brown SW, Gerber PF. Microbial taxa in dust and excreta associated with the productive performance of commercial meat chicken flocks. Anim Microbiome. 2021;3:66. https://doi.org/10.1186/s42523-021-00127-y

54. Qiao H, Zhang L, Shi H, Song Y, Bian Chuanzhou. Astragalus affects fecal microbial composition of young hens as determined by 16S rRNA sequencing. AMB Express. 2018;8:70. https://doi.org/10.1186/s13568-018-0600-9

55. Yang Q, Liu J, Wang X, Robinson K, Whitmore MA, Stewart SN, et al. Identification of an intestinal microbiota signature associated with the severity of necrotic enteritis. Front Microbiol. 2021;12:703693. https://doi.org/10.3389/fmicb.2021.703693

56. Siegerstetter SC, Petri RM, Magowan E, Lawlor PG, Zebeli Q, O'Connell NE, Metzler-Zebeli BU. Feed restriction modulates the fecal microbiota composition, nutrient retention, and feed efficiency in chickens divergent in residual feed intake. Front Microbiol. 2018;9:2698. https://doi.org/10.3389/fmicb.2018.02698

57. Lei J, Dong Yuanyang, Hou Q, He Y, Lai Y, Liao C, et al. Intestinal microbiota regulate certain meat quality parameters in chicken. Front Nutr. 2022;9:747705. https://doi.org/10.3389/fnut.2022.747705

58. Sgobba E, Blöbaum L, Wendisch VF. Production of food and feed additives from non-food-competing feedstocks: valorizing N-acetylmuramic acid for amino acid and carotenoid

fermentation with Corynebacterium glutamicum. Front Microbiol. 2018;9:2046. https://doi.org/10.3389/fmicb.2018.02046

59. Chen Y, Akhtar M, Ma Z, Hu T, Liu Q, Pan H, et al. Chicken cecal microbiota reduces abdominal fat deposition by regulating fat metabolism. NPJ Biofilms Microbiomes. 2023;9:28. https://doi.org/10.1038/s41522-023-00390-8

60. Muyyarikkandy MS, Parzygnat J, Thakur S. Uncovering changes in microbiome profiles across commercial and backyard poultry farming systems. Microbiol Spectr. 2023;11:e0168223. https://doi.org/10.1128/spectrum.01682-23

462 Table 1. Nutrient levels in the diets used during different growth periods

Items	Starter	Grower	Finisher
Crude protein (%)	24.15	23.47	23.01
Crude fat (%)	9.41	6.13	4.65
NDF (%)	9.23	12.35	8.51
ADF (%)	4.24	3.94	3.66
Ash (%)	8.35	5.87	6.01

463 NDF, Neutral detergent fiber; ADF, acid detergent fiber.

464

Table 2. Growth performance of broiler chickens according to bedding conditions

Items	Conventional cage with litter (n =150)	Cage without litter (n =150)	P value
IBW, g (1 wk)	38.17±0.23	38.27±0.22	0.7470
FBW, g (5 wk)	2,329±17.34	2,444±38.66	0.0087
ADFI, g	93.49±0.65	92.76±1.32	0.6111
ADG, g	65.47±0.50	68.75±1.11	0.0088
FCR, g/g	1.43±0.01	1.35±0.02	<0.001

Values are mean ± standard error of the mean. IBW, initial body weight; FBW, final body weight. ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

469 **Table 3.** The influence of gut microbiota on signaling pathways, based on aging

Pathway	3 wks	4 wks	5 wks
Mono-trans, poly-cis decaprenyl phosphate biosynthesis (PWY-6383)	Down	Up	Up
Mycolyl-arabinogalactan-peptidoglycan complex biosynthesis (PWY-6397)	Up	Up	Up
Mycothiol biosynthesis (PWY1G-0)	Down	Up	Up

470
471

A B

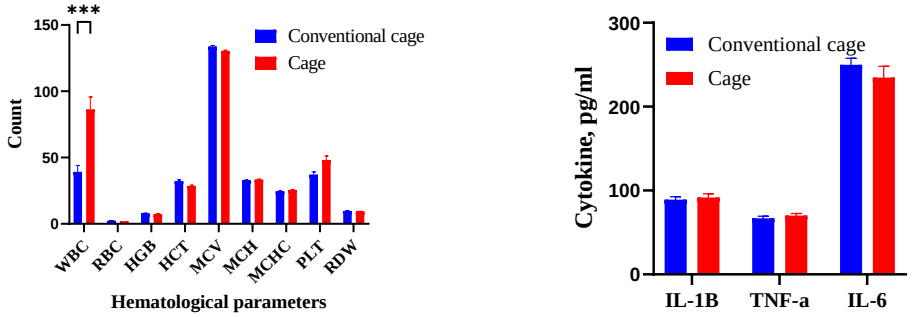
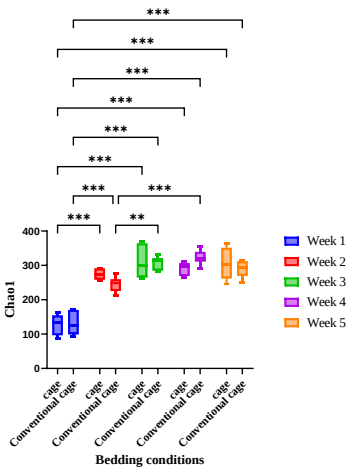
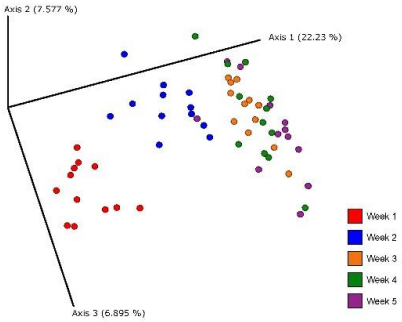


Figure 1. Hematological (A) and cytokine (B) analyses of broiler chickens according to bedding conditions. Data are shown as mean and standard error of the mean. n= 6. For statistical analysis, unpaired Stu-dent's T-test was used to compare the means of two populations. WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, Hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, Platelet; RDW, red cell distribution width. *** $P < 0.001$ (highly significant).

A



B



C

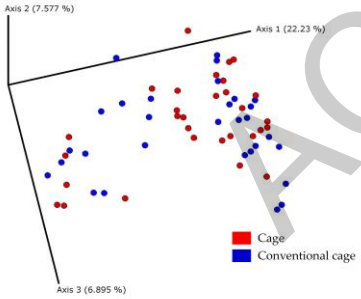


Figure 2. Microbiota diversity indices of the gut microbiota between the five age groups and bedding condition. n = 6. (A) Alpha-diversity using the Chao 1 index. (B) Beta diversity principal coordinate analysis (PCA) plot using Bray Curtis dissimilarity measure in the five age groups. (C) Beta diver-sity PCA plot using Bray Curtis dissimilarity measure between cage and conventional groups. The P value was tested using a nonparametric Kruskal-Wallis test with a Bonferroni post hoc test. ** $P < 0.01$; ***, $P < 0.001$.

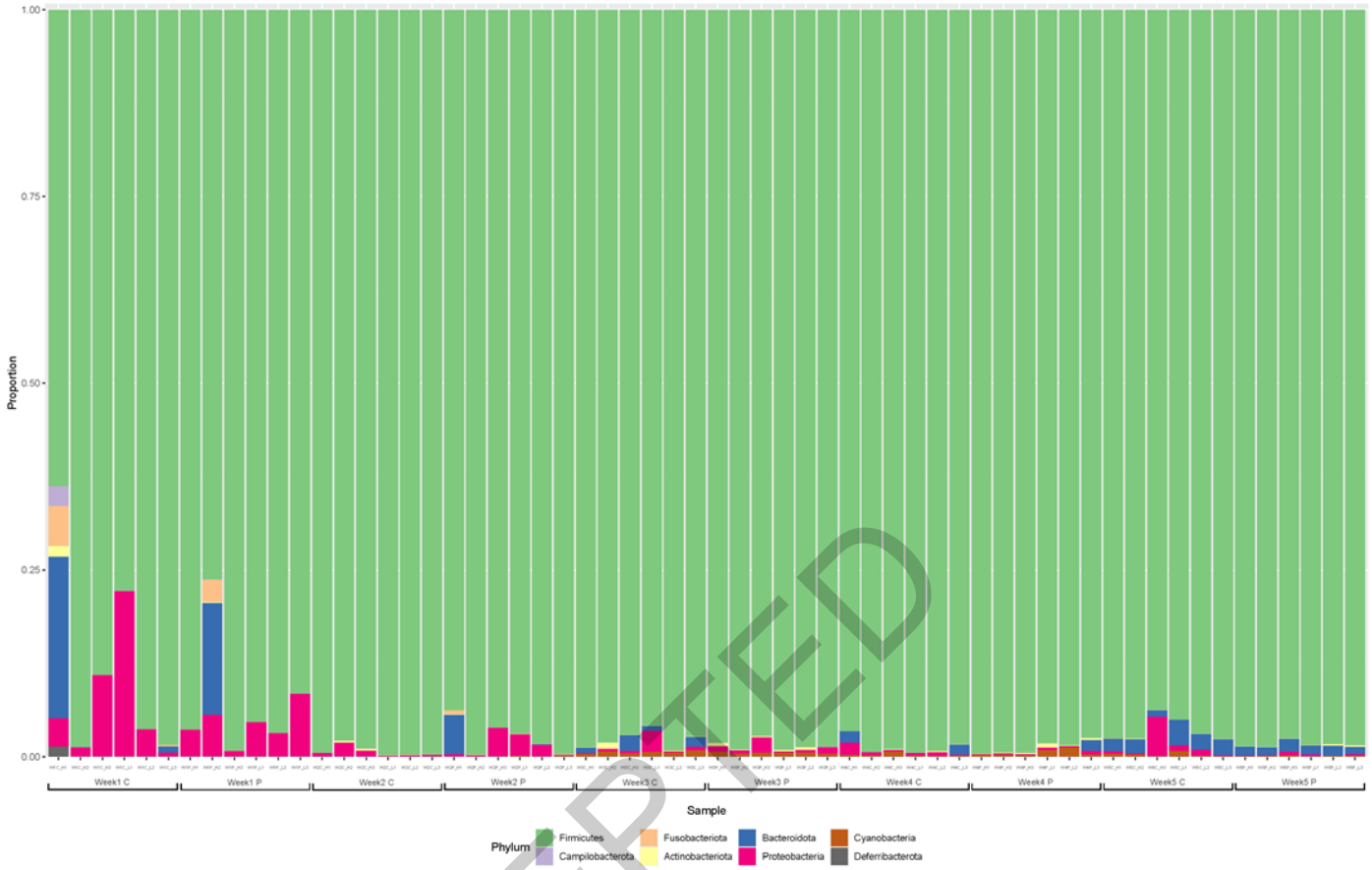


Figure 3. The relative abundances of Firmicutes at the phylum level by aging between the cage (without litter) and conventional (with litter) groups. The percentages of Firmicutes were 87.39±5.72, 99.31±0.31, 97.73±0.50, 98.70±0.45, and 96.44±0.67 % at 1,2,3,4 and 5 weeks, respectively, among chickens housed in cages without litter. The percentages of Firmicutes were 92.63±3.42, 97.47±0.94, 98.53±0.29, 98.83±0.35, and 98.36±0.16 % at 1,2,3,4, and 5 weeks, respectively, among chickens housed in conventional conditions (with litter). n= 6.

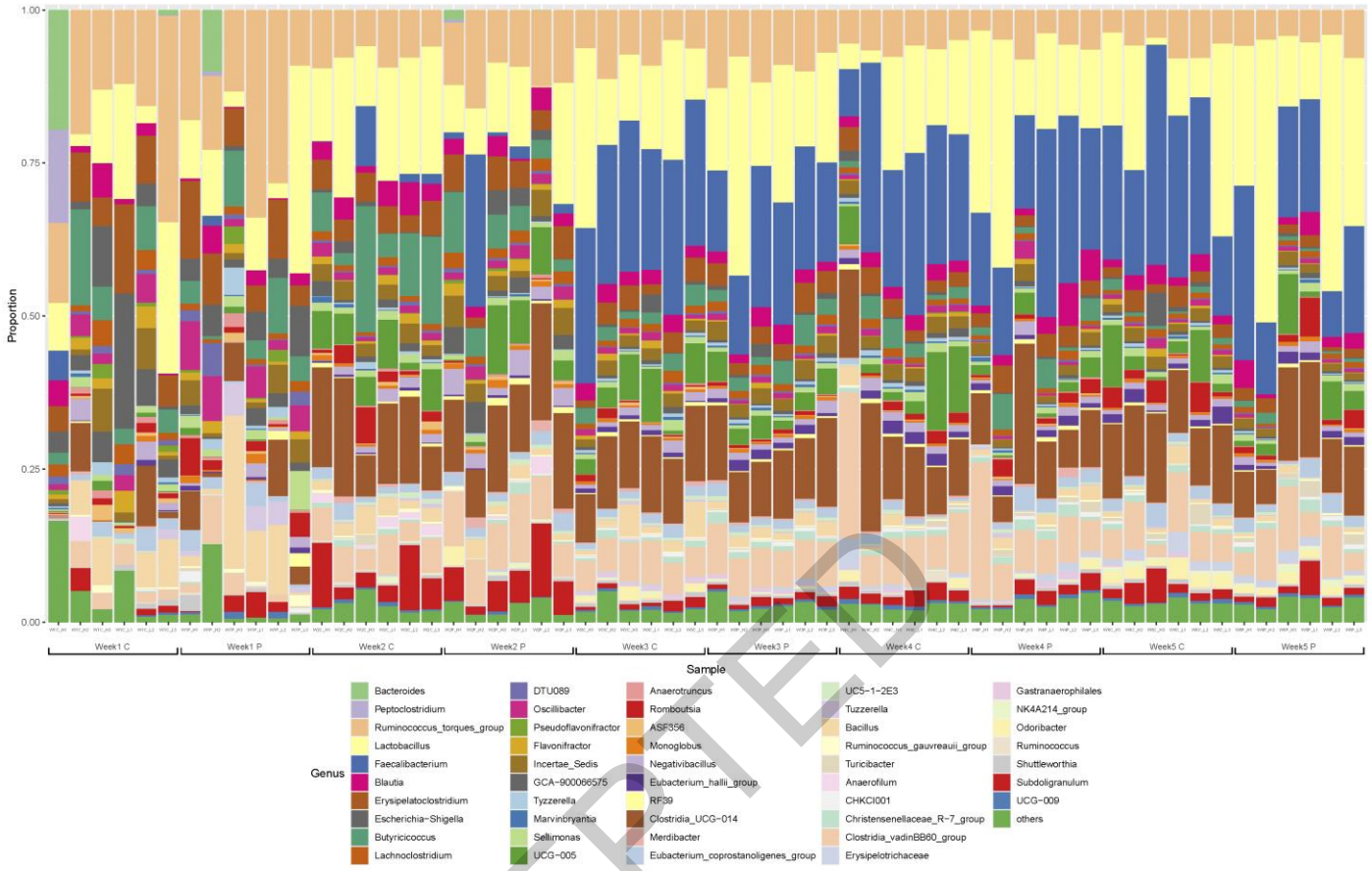
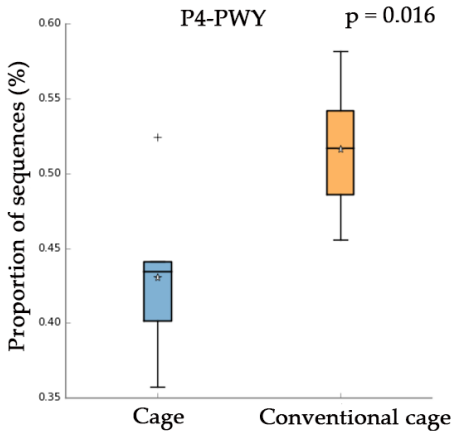


Figure 4. The relative abundances of Lactobacillus at the genus level by aging between the cage (without litter) and conventional (with litter) groups. The percentages of Lactobacillus were 11.34±3.70, 17.11±2.10, 15.38±3.20, 11.67±2.86, and 13.97±4.44 % at 1,2,3,4, and 5 weeks, respectively, among chickens housed in cages without litter. The percentages of Lactobacillus were 11.26±4.76, 9.94±2.67, 19.23±3.65, 19.34±4.65, and 26.50±6.21 %, respectively, at 1,2,3,4, and 5 weeks among chickens housed in conventional conditions (with litter). n = 6.

A



B

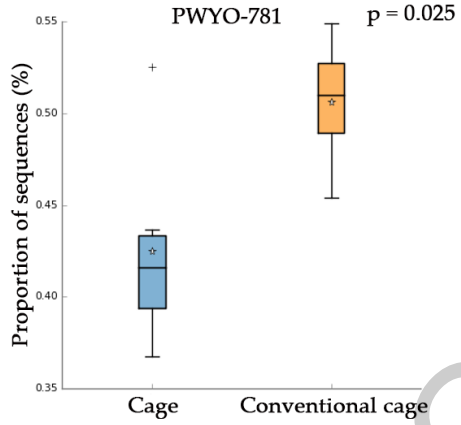
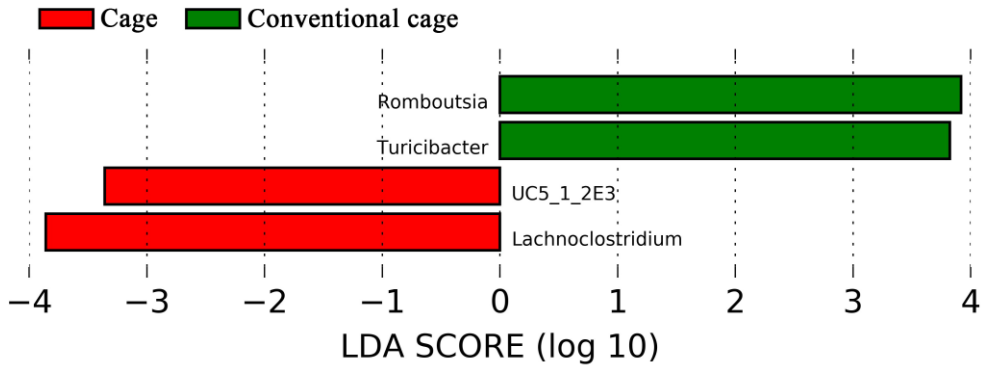
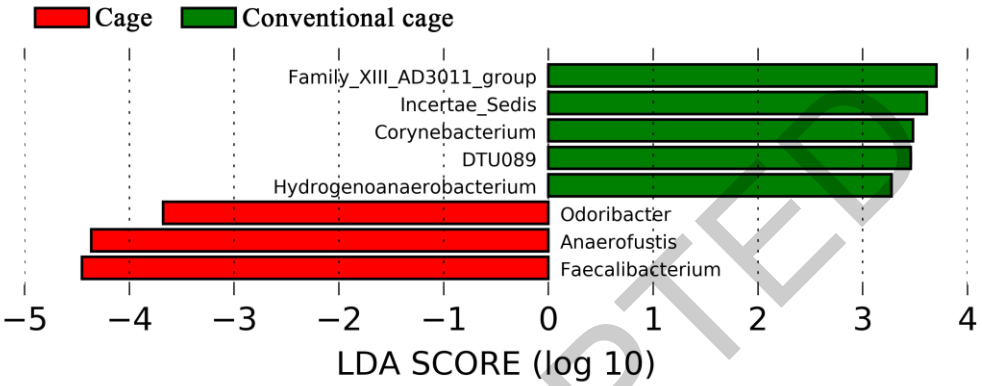


Figure 5. Microbial pathway abundance box plots between the cage and conventional groups. (A) P4-PWY (superpathway of L-lysine, L-threonine, and L-methionine biosynthesis I) at 1 week. (B) PWY0-781 (spartate superpathway) at 1 week.

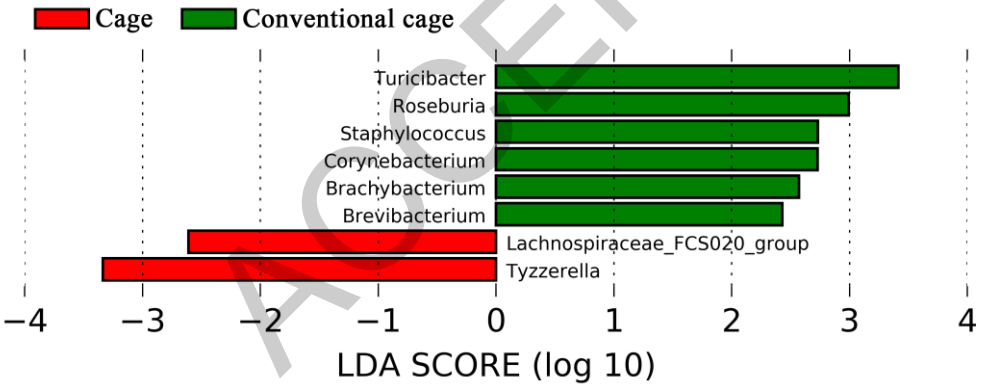
A



B



C



D

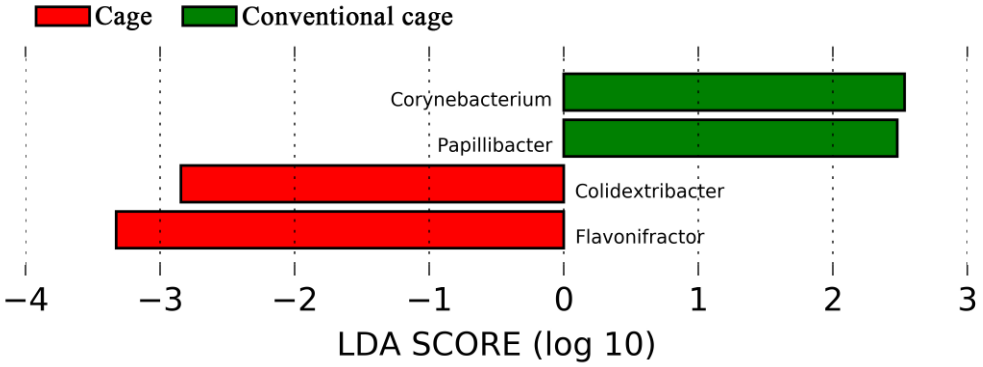


Figure 6. Graphical representation of Linear discriminant analysis (LDA) effect size (LEfSe) of cecal microbiota in broiler chickens among the cage and conventional groups. (A-D) show the LEfSe results at weeks 1, 3, 4, and 5, respectively. The horizontal bar represents the $\log_{10}$ transformed LDA score