MANSOURA JOURNAL OF BIOLOGY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-492X

Bioinformatics Analyses of the 16S rRNA Gene Evaluated the Fluctuation of Bacterial Diversity in the Gut Microbiome of Local Egyptian Chicken

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Received:26/5/2024
Accepted: 25/6/2024

Abstract: The ever-increasing demands for chicken protein by humans highlighted the implications of studying the effects of gut microbiome composition and diversity on this respect. The present study explored the bacterial community profile and its correlation with the different age stages of chicken. Egyptian local chicken were fed on a routine commercial diet and fecal samples were collected at different age points for microbiome analyses. Bacterial profiles of the chicken gut microbiome were investigated using 16S rRNA gene amplicon sequencing and a metagenomics DADA2 pipeline was used for data processing and analysis. The results showed significant diversity among all samples. Alpha diversity and the relative abundance of bacterial taxa first decreased then recovered with time. This was clear with Firmicutes genera that showed a depletion in their abundance followed by a subsequent recovery to the initial levels. However, Actinobacteria increased significantly over time. At the genus level, *Lactobacillus* and *Corynebacterium* declined in abundance, while *Pediococcus* displayed an increase in abundance with time. Similar dynamic patterns were exhibited at different taxonomic bacterial affiliates. While the initial decrease in bacterial diversity may be attributed to the effect of antibiotics, the subsequent attempt of the microbiome to restore its balance, composition and diversity may be driven by the influence of growth-promoting supplements in the diet. In addition, good management of the bacterial changes with chicken age might affect the poultry industry, consumers' health and even environmental health in a positive way.

keywords: Microbiome; Chicken; Metagenomics; 16S rRNA; Diversity

1.Introduction

The high demand for a relatively cheap source of animal protein had directed the attentions to the poultry industry. Since the amounts of chicken meat consumed that exceeded 133 million tons in 2021 [1]. From the microbiological point of view, many scientists had linked a direct correlation between the fodder diets and the chicken microbiomes community. These microbial communities greatly modulated chicken health and its meat production [2]. According to some published records, Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria are the most abundant phyla of bacteria found in the chicken gastrointestinal tract. Proteobacteria was reported to be among the most dominant groups, especially *Desulfohalobium*, *Neisseria*, *Escherichia*, and *Shigella* genera [3]. Moreover, almost 40% of microbiome members belong to the Bacteroidetes. While 31 Firmicutes genera were recorded, mostly Ruminococcus, Eubacterium, Riemeralla, Clostridium, Tanneralla, Paraprevotella, and Prevotella [3].

Some pathogenic bacteria were also reported to be members of the chicken microbiomes such as Campylobacter spp., Salmonella spp., Listeria monocytogenes and Escherichia coli O157:H7 [4-6]. Centers for Disease Control and Prevention [CDC] recorded a link between outbreaks and food from chicken sources which proved to be contained with microbial pathogens like Salmonella typhimurium and Salmonella infantis in 2018 and Salmonella enteritidis and Listeria monocytogenes in 2021

[7].

High-throughput sequencing methods are very powerful approaches to explore microbial profiles in living organisms [8] specifically through amplicon-sequencing of the 16S rRNA gene which contains hyper variable regions that are species-specific. This technology allows accurate exploration of all bacterial members of any microbiome; not only the culturable ones but even the unculturable and nondominant ones [9, 10].

Most of the published Egyptian studies focused on culturing techniques to explore bacterial microbiomes in chicken guts. Despite the validity of these studies, they did account for only a subsection of the real bacterial population of such microbiomes. Since most microbes inside chicken guts are unculturable [9]. For instance, Desouky and her colleagues (2021) isolated and identified the most common and culturable bacteria from chicken guts in Egypt [8]. A recent study investigated the bacterial diversity in Egyptian chicken, *Gallus domesticus,* using metagenomic by Illumina MiSeq sequencing technology [10]. Again, it was restricted to a single point during the life cycle of the chicken. However, the present study was designed to investigate the bacterial community composition and diversity in the Egyptian local chicken *G. domesticus* guts at different lifetime stages using amplicon sequencing after the chicken was fed a routine commercial diet.)

2. Materials and methods

2.1. Experimental design of growth and feeding

10 recently hatched Egyptian local hens (*G. domesticus*) were obtained from a local hatchery (Mansoura, Dakahlyia, Egypt), transported to a previously prepared place for breeding and allowed to grow freely indoors. They were subjected to three sequential types of feeding routines: a starter diet (first 14 days), a growing diet (from day 15-28), and a finishing diet (from day 29-44). All feed products were purchased from Alqaed, a local feed producer. The contents of each diet type are shown in Table 1. Moreover, growth supplements of vitamins, minerals, antibiotics, prebiotics and probiotics were added according to the commercial practice followed by the local chicken industry (Table 2). The indoor temperature was kept at 32℃, then gradually decreased with ageing to a final room temperature around 25℃, till samples' collection [11].

2.2. Collection of fecal samples

Three fresh fecal samples were collected from each chicken at days 30, 37, and 44 using clean wooden sticks in clean plastic containers. The three samples from each individual chicken were mixed thoroughly to represent the feces microbiome. The collected samples were stored at -20℃ till DNA extractions [12].

2.3. DNA Extraction, 16S rRNA Library Preparation, Sequencing, and Bioinformatic Analysis

2.3.1. DNA Isolation

About 1-2 g of frozen fecal samples were used for the extraction of DNA using a manual protocol. Quality (Purity) and concentration of the extracted DNA were evaluated using a Tecan Spark microplate reader (Tecan Trading AG, Zurich, Switzerland). Extracted DNA of different samples was stored at -20℃ for subsequent steps.

2.3.2. Bacterial 16S rRNA Library Preparation

The V4 region of the 16S rRNA gene (the species-specific segment) was amplified using the dual-index primers 515F and 806R in a 15 µL PCR reaction mixture [13]. The PCR amplification lasted for 25 cycles preceded with an initial denaturation step at 98°C for 3min. Each of the 25 cycles consisted of: denaturation for 30 s at 55°C, annealing for 45 s at 68°C; then each cycle was terminated after a final 4-min hold at 68°C. After amplification, all PCR products were subjected to quality assessment to verify the initial size and ensure amplification using gel electrophoresis with 1.8 % agarose gel (QD LE Agarose, Green Bio Research, Baton Rouge, LA) at 120 V for 55 minutes. Then, primer dimers were removed using a 6X Solid Phase Reversible Immobilization (SPRI) paramagnetic beads according to manufacturer protocol (Agencourt® AMPure®). Concentrations of DNA samples were normalized using Invitrogen Sequal Prep™ Normalization Plate kit (Frederick, Maryland) to 1 – 2 ng and

pooled. After pooling of the library in Eppendorf epMotion (M5073, Germany), it was subjected to purity and concentration examination by qPCR (Quant studio 1, Applied Biosystems) using the KAPA Library Quantification Kit (KK4824, Kapa Biosystems, Wilmington, NC, USA).

Table 1. Constituents of starting, growing and finishing diets.

Starting Diet (from day 1 to day 14)	**Growing Diet (from day 15 to day 28)**	**Finishing Diet (from day 29 to day 44)**
Protein (23%) Fat (5.76%) Fiber (4.2%) Representative energy 3000 kcal/kg of feed Yellow corn Soybean meal Whole-fat extruded soybean meal Korma jowar meal Dicalcium phosphate A mixture of vitamins and mineral salts Hydroxy methionine calcium analogue Sodium bicarbonate Calcium carbonate Table salt	Protein (20%) Fat (4.70%) Fiber (3.88%) Representative energy 3000 kcal/kg of feed Yellow corn Soybean meal Whole-fat extruded soybean meal Korma jowar meal Dicalcium phosphate A mixture of vitamins and mineral salts Hydroxy methionine calcium analogue Sodium bicarbonate Calcium carbonate Table salt	Protein (18%) Fat (3.0%) Fiber (3.0%) Representative energy 3000 kcal/kg of feed Yellow corn Soybean meal Whole-fat extruded soybean meal Korma jowar meal Dicalcium phosphate A mixture of vitamins and mineral salts Hydroxy methionine calcium analogue Sodium bicarbonate Calcium carbonate Table salt

Table 2. Composition of antibiotics and growth supplements.

Ingredient Types	**Ingredients**	**Concentration**
Antibiotics	Anti-coccidia (Diclazuril) Tylosin tartrate Colistin sulphate Doxycycline hydrochloride	10 mg/ml 20 gm/100 gm 120 M.I.U/100 gm 20 gm/100 gm
Probiotics	*Bacillus licheniformis* *Bacillus subtilis* *Saccharomyces cerevisiae*	$4.5x10^{10}$ CFU/ml $5.5x10^{10}$ CFU/ml $2.0x10^{10}$ CFU/ml
Prebiotics	Taurine Methionine Choline chloride Xylanase Alpha-amylase Protease Cellulase	1000 mg 20000 mg 20000 mg 1250 IU 3750 IU 12500 IU 2500 IU
Vitamins	Vitamin A Vitamin D3 Vitamin E	1000000 IU/L 200000 IU/L 200 mg/L
Minerals	Ca Ph Na Mn Zn Fe Cu Se Co K	20000 mg/L 50000 mg/L 50000 mg/L 3000 mg/L 5000 mg/L 4000 mg/L 3000 mg/L 50 mg/L 2000 mg/L 30000 mg/L

2.3.3. 16S rRNA Gene Sequencing

DNA amplicons were subjected to sequencing using the Illumina MiSeq sequencing machine. A dual-index sequencing strategy was implemented using the V2 500 cycle kit for machine operation [13]. Then, adapters of the sequencing machine were removed. Different barcode numbers were used

to create subfolders then the sequences of the barcodes were also removed, after which demultiplexing of the sequences took place. Tracking the status of sequencing was easily followed on the website of Illumina BaseSpace®. The success of the sequencing process was determined.

2.3.4. Bioinformatics Analysis

The Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline used the fastq files after being downloaded from the Illumina BaseSpace® website (forward R1 and reverse R2), which are the final output of the previous step, as an input to generate amplicon sequence variants (ASVs)[14]. Equal sampling depth rarefaction took place. Within the DADA2 workflow, a filtering and trimming step was conducted to exclude and cut primers and low-quality sequences, and then merging and chimera removal took place[14]. Alignment with the Green Genes database was used to get taxonomic affiliation of the generated ASVs. The richness of the gut microbiome was analyzed using the DADA2 pipeline [15] while taxonomic affiliations were obtained through the implementation of the Phyloseq R package [16]. The Shannon and Simpson indices were calculated to investigate alpha diversity [17]. With the R program (v. 4.3.1), output files (ASVs sequences, taxonomy file and count table) were generated ultimately after the DADA2 pipeline.

2.3.5. Statistical Analysis.

Statistical analysis for the alpha diversity indices was performed using the test of Shapiro-Wilk. Principal coordinate analyses were established to visualize the variation in community composition over time between different samples. This was estimated by using the Bray-Curtis unweighted UniFrac distances [18]. Using the relative abundance of the ASVs as input, the heatmap.2 R function [19] was used to create a heatmap to get a significant visualization of differential ASVs.)

3. Results and Discussion

3.1. Bacterial Microbiome Richness and Composition

Diversities for bacterial populations were evaluated according to the indices of Shannon and Simpson (alpha type) and Bray-Curtis UniFrac (beta type). Both indices showed bacterial diversity across different life stages of the local chicken. The significant changes were evident throughout the current study, not only in the alpha diversity for samples taken at 30, 37, and 44 days of age ($P < 0.01$) but also in the beta diversity (Fig. (1 and 2). The blots for both indices, either calculated according to Shannon and Simpson or Bray-Curtis UniFrac showed a decrease of bacterial diversity followed by subsequent recovery to initial levels on Day 44.

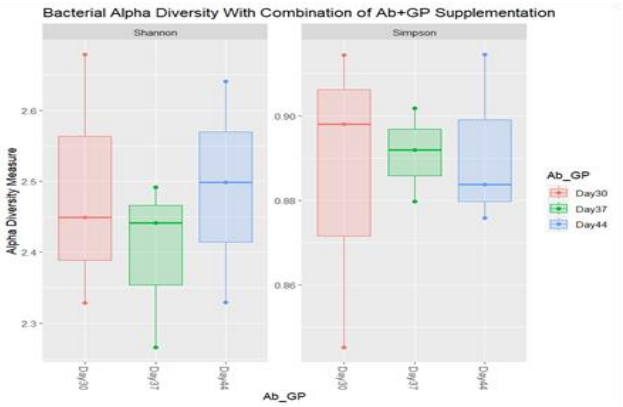

Fig. (1). Bacterial alpha diversity across different time points of chicken age, day 30, 37 and 44 with Shannon and Simpson indices. The diversity decrease is noticeable across all time points before their increase again on Day 44.

The β-diversity of bacteria amongst the chicken gut microbiome resulted in three different clusters reflecting a significant variation in microbiome profiles between the different age stages and bacterial diversity in guts. One cluster of samples collected at day 30 was associated with relatively high diversity. The second cluster of samples collected at the age of 37 days showed decreased diversity levels. The third cluster of samples collected at the age of 44 days showed increased diversity.

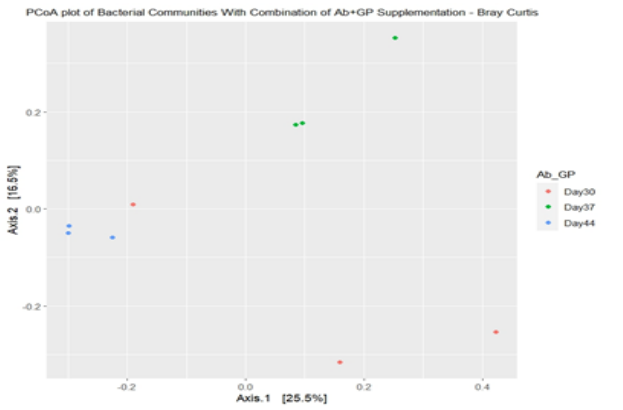

Fig. (2). Bacterial beta diversity profiles across different time points of chicken age, d-30, 37

and 44. Using the principal coordinate analysis plot, a distinct clustering pattern of samples of the same time point was revealed.

Moreover, the "abundance profile" of bacteria reflected the abundance and dominance of bacterial phylum, class, order, families, genera, and species in different collected gut microbiome samples. Bacteria belonging to the Firmicutes phylum were the most dominant followed by the Actinobacteria (Fig. (4). Regarding the class levels, class Bacilli was the most common in all samples while Actinobacteria and Clostridia showed lower levels in all samples (Fig. (5). At the order level, Lactobacillales was highly abundant in all samples followed by Actinomycetales and Clostridiales (Fig. (6). At the family level, Lactobacillaceae showed the highest abundance among all other families followed by Corynebacteriacea and Ruminococcaceae (Fig. (7). Family Lachnospiraceae was only abundant at day 30 of age. The genus Pediococcus was highly abundant among genera. At the same time, the genera Corynebacterium and Lactobacillus were also abundant but less than Pedicoccus's relative abundance percentage (Fig. (8). At the species level, Corynebacterium stationis and Corynebacterium pilosum were the most abundant species followed by Lactobacillus ruminis and Streptococcus equi (Fig. (9).

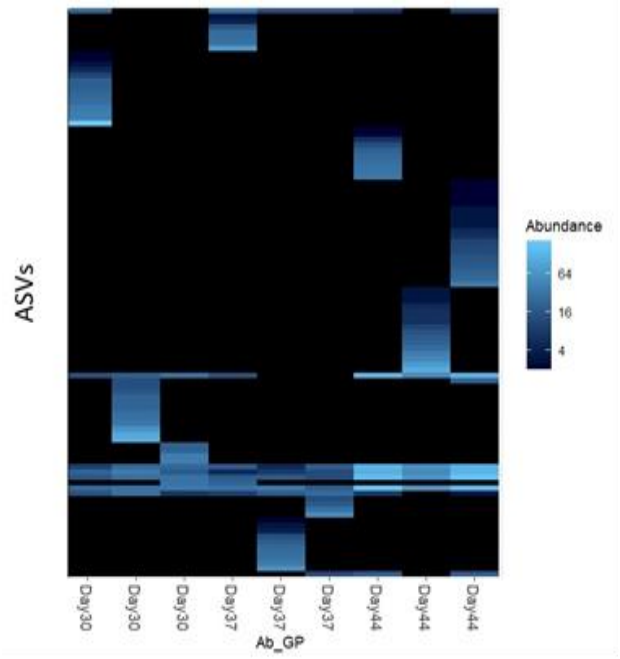

Fig. (3). Bacterial ASVs abundance profiles across different time-points of chicken age, d-30, 37 and 44 using heatmap.

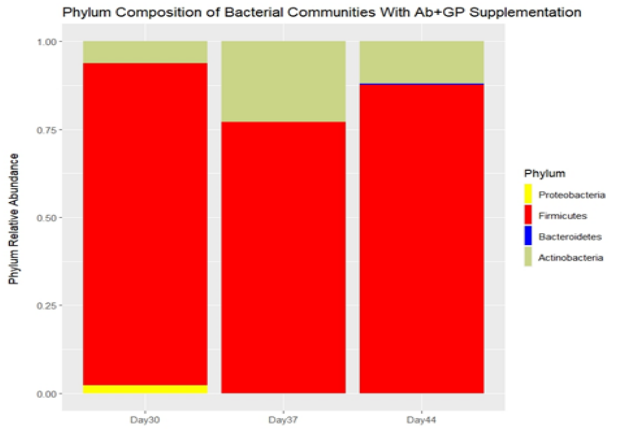

Fig. (4). Bar plots demonstrating the relative abundance profiles of bacterial phyla. Firmicutes and Actinobacteria showed the highest abundance ratio among all other present phyla.

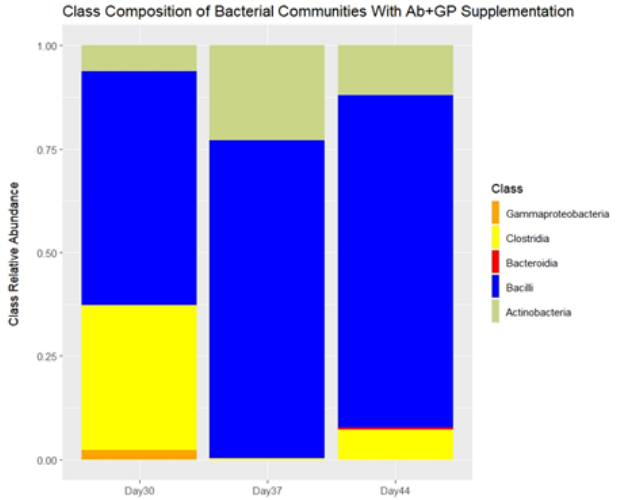

Fig. (5). Bar plots demonstrating the relative abundance profiles of bacterial Classes. Bacilli was the most abundant class followed by Actinobacteria and Clostridia in all samples at different age levels.

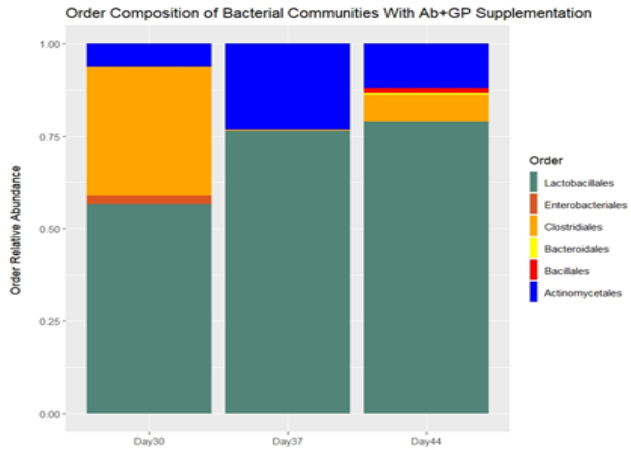

Fig. (6). Bar plots demonstrating the relative abundance profiles of bacterial orders. Lactobacillales was the most abundant order at

all. Actinomycetales and Clostridiales were also among the abundant orders.

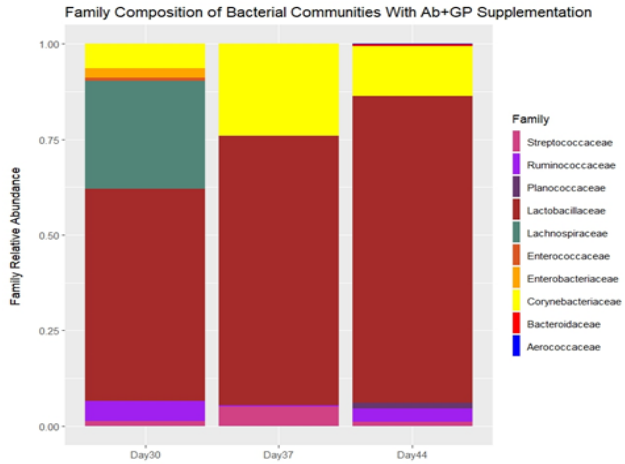

Fig. (7). Bar plots demonstrating the relative abundance profiles of bacterial families. Lactobacillaceae was the most abundant family followed by Corynebacteriacea and Ruminococcaceae.

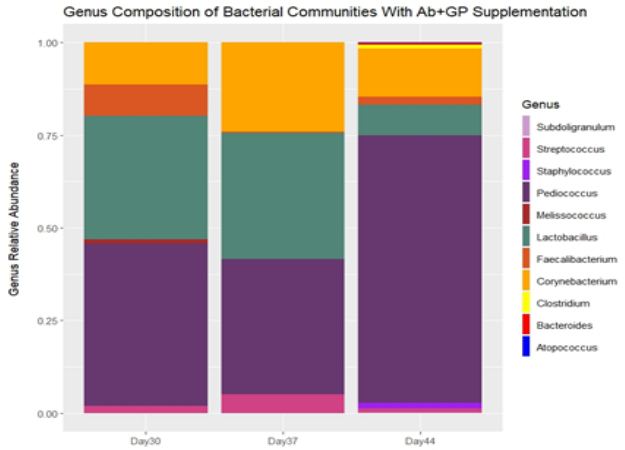

Fig. (8). Bar plots demonstrating the relative abundance profiles of bacterial genera. Pediococcus was the most abundant genus followed by Lactobacillus and Corynebacterium.

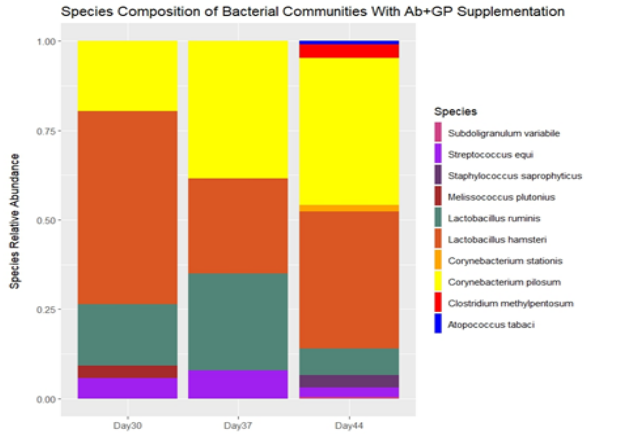

Fig. (9). Bar plots demonstrating the relative abundance profiles of bacterial species. *Lactobacillus hamsteri* and *Corynebacterium pilosum* were the most abundant species followed by *Lactobacillus ruminis*.

Previous studies on microbiome profiles supported the fluctuation trends shown in our study in terms of the microbial communities' profiles throughout the growth stages of the chickens or the dominance-specific taxa. Changes in the community dynamics were attributed to factors like farming conditions, type of dietary supplements, and environmental stresses [18-20]. In addition, pieces of evidence were found to support the strong effect of different regimes of diet on microbiome communities' composition of poultry [21]. This effect may include changes in the rate of occurrence of each taxon, where some taxa show a higher rate and others a lower rate and vice versa, and this change would probably affect the health status of the poultry [22]. In a recent study, an elevation has been recorded in the abundance of Proteobacteria in response to heat in comparison with cooler environmental conditions [23]. Moreover, Firmicutes was reported to show an alteration in its abundance due to different genetics and rearing processes [24, 25].

Overall, it is noticeable that microbial diversity in the chicken gut could be affected by the age stage of the chicken and other internal or external parameters such as the type of diet used to feed the poultry. This outcome ensures the importance of performing studies that focus on gut microbial populations to improve rearing practices and dietary composition. Such improvements would help to optimize health performance and increase the productivity of the poultry industry.

The present study has recorded a significant fluctuation in the microbiome over time. There was a decrease in Firmicutes at day 37 of age then a significant increase took place to recover the original abundance level that occurred at day 30 of age. However, Actinobacteria showed an increase concurrently with the decrease in Firmicutes at day 37 of age, where they also returned to their initial relative abundances at day 44 of age. The current study shows that diversity changes dynamically with no uniform pattern of increase or decrease with age. As revealed by the above results, diversity was the highest at the 30-day and 44-day age of

chicken while in between at day 37, diversity was the lowest. These findings contradict the results obtained in the study of [26] as they stated that diversity measurements for samples of fecal origin increased with age progress in conventional and non-conventional farms. However, other studies support our finding as they detected a dynamic variability in alpha diversity calculations across chicken life and showed a decrease in the diversity between days 28 and 42 of chicken age[27]. In addition, the initial decrease in bacterial diversity may be linked to the effect of antibiotics supplementation in commercial diet, and the dynamic patterns of microbiome to recover its initial balance may be driven by the influence of growth-promoting supplements which could be explored in future studies.)

4. Conclusion

The present findings support the opinion that a dynamic change, with no predictable specific patterns, can occur to alter the microbial diversity of chicken guts over time. These results, along with studying the effect of different production systems on microbiome community profiles, would help address the impacts on poultry performance and subsequently safety of food and public health. Understanding these implications would improve the way of growing poultry to optimize the best practices for high-quality production, increased productivity and elimination of adverse implications on animals, human beings and the environment.

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