Original article

# Metagenomics analysis of the fecal microbiota in Ring-necked pheasants (*Phasianus colchicus*) and Green pheasants (*Phasianus versicolor*) using next generation sequencing

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## ARTICLE INFO

## ABSTRACT

Pheasant reintroduction and conservation efforts have been in place in Pakistan since the 1980 s, yet there is still a scarcity of data on pheasant microbiome and zoonosis. Instead of growing vast numbers of bacteria in the laboratory, to investigate the fecal microbiome, pheasants (green and ring neck pheasant) were analyzed using 16S rRNA metagenomics and using IonS5TMXL sequencing from two flocks more than 10 birds. Operational taxonomic unit (OTU) cluster analysis and phylogenetic tree analysis was performed using Mothur software against the SSUrRNA database of SILVA and the MUSCLE (Version 3.8.31) software. Results of the analysis showed that firmicutes were the most abundant phylum among the top ten phyla, in both pheasant species, followed by other phyla such as actinobacteria and proteobacteria in ring necked pheasant and bacteroidetes in green necked pheasant. *Bacillus* was the most relatively abundant genus in both pheasants followed by *Oceanobacillus* and *Teribacillus* for ring necked pheasant and *Lactobacillus* for green necked pheasant. Because of their well-known beneficial characteristics, these genus warrants special attention. Bird droppings comprise germs from the urinary system, gut, and reproductive sites, making it difficult to research each anatomical site at the same time. We conclude that metagenomic analysis and classification provides baseline information of the pheasant fecal microbiome that plays a role in disease and health.

## 1. Introduction

Pheasants are the member of order Galliformes and are known as important environmental indicators. There are total of 49 species of pheasants in the world (McGowan and Garson, 1995) and 5 of them are endemic to Pakistan. Pheasants are threatened and vulnerable owing to expansion in human population, habitat disturbance and poaching. Reintroduction and conservation programs of pheasants are working in Pakistan since 1980s (Zaman, 2008). Although

captive breeding is an authentic tool to conserve declining species, but conservation of pheasants or any other avian species bring the avian pathogens and human in close association. Risk of pathogen transfer within individual of same species and breakout of zoonoses elevates in such scenario. Exploration of type of pathogens, risk factors and cause and effect relation become a dire need in this situation to control the zoonoses outbreak and for adopting the proper protective measure. Understanding of microbes and their relationship with the hosts is necessary to improve the health of host organisms (Gilbert et al. 2016, Roto et al. 2015).

Respiratory microbiota (Shabbir and Muhammad, 2013; Shabbir et al. 2015; Glendinning et al. 2017) and intestinal microbiota (Pedroso et al. 2006; Dumonceaux et al. 2006; Zhou et al. 2007; Gong et al. 2008) of chicken has been reported many times due to their commercial importance, but there is paucity of information on pheasant's microbiota. Examination of Galliformes' cecal microbiome is useful in understanding the sources of

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pathogenic bacteria and could be helpful in management practices of these taxa in captivity (Best, 2017).

Many factors such as nature of organism, rearing conditions, host immune response and exposure to animals and human have an influence on the microbiome and associated diseases (Pan et al. 2012; Meng et al. 2014; Ludvigsen et al. 2016). By using cultural dependent techniques, prevalence and identification of many pathogens have been reported from captive as well as wild avian species throughout the world (Barnes, 1979; Hughes et al., 2009; Smith et al. 202; Benskin et al. 2009; Keller et al. 2011). Even cultural dependent techniques have been used to explore the normal intestinal microbiota of pheasants (Shulin and Xiuli, 1998; Kandričáková A, Lauková, 2014; Lauková and Kandričáková, 2015). But one can never culture all the residential bacteria of intestinal fluid on media in microbiology laboratories.

Metagenomics is capable for analyzing bacterial communities straight forwardly at the nucleic acid level in any environmental sample (Pereira, 2010). Variation in 16S gene sequences has been utilized broadly to characterize different microbial communities. For classification, it is adequate to grouping each hypervariable region rather than the whole 16S gene. Also, the 16S gene contains extremely preserved alignments between hypervariable regions, empowering the design of universal primers. These hypervariable region of the 16S rRNA gene is powerful tool for identification of bacteria. For analyzing all possible residential microbiota either culturable or non-culturable from any kind of environmental sample a powerful tool is under use since 1977 (Kulski, 2016) named Next Generation Sequencing (Schuster, 2008; Wylie et al. 2012). With the advancement of next-generation sequencing (NGS), its practical applications have been extended and improved and have led to the reclassification of bacteria (Pereira et al. 2010). One of the advanced applications of next generation sequencing is to explore the inside of microflora of farm animals and human gut or any other environmental sample, this leads to early detection of pathogens and helps in improving precautionary measure and disease treatment (Ji et al. 2015).

Although, microbial flora of farm animals, poultry and some pet's avian species has been extensively studied, but there is lack of baseline information as which microbial community reside in fecal sample of healthy captive pheasant species. This investigation is important for observing prevalent infections of a specific geographical range, inspecting new or known pathogens, correlating certain commensals or pathogens with disease or health, refining medical and molecular diagnostics, and consequent disease and health control approaches (Berkhoff, 1985; Xenoulis et al. 2010; Brilhante et al. 2010; Shabbir et al. 2015). Since birds can be a reservoir for several zoonotic pathogens (Xenoulis et al. 2010) understanding the fecal microbiota of pheasants is also important for human health (Clemente et al. 2012; Lee et al., 2014). Captive birds are found to be a source of possible diseases, such as transferred through *Campylobacter* sp. and *Clostridium sp.*, all over the world. (Xenoulis et al. 2010; Brilhante et al. 2010). Aim of the study is to investigate the fecal microbiota of pheasants (ring necked and green) using NGS.

## 2. Materials & methods

### 2.1. Fecal sample collection and preparation

Green pheasants (*Phasianus versicolor*) and ring-necked pheasants (*Phasianus colchicus*) were reared privately at Avian Conservation and Research Center, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, Pakistan, and a pooled fecal sample was taken. All the birds used in this study were healthy. Each pooled fecal sample was representative of five birds from the same species. The sample (2–3 g) was collected, following the methodology of Garcia-Mazcorro et al. (2017), in a sterilized falcon tube (10 ml) and stored at −20 °C until processing. All sampling was done in accordance with institutional, national, and international bird care and use norms (Gaunt, et al. 1997). Fecal sample from the flock of both pheasants were collected at the same time (Garcia-Mazcorro et al. 2017). Detail of each flock has shown in Table 1.

### 2.2. Genome DNA extraction

Total fecal DNA was extracted using the CTAB (Cetyl trimethylammonium bromide) method, and the concentration and purity of the collected DNA was determined using gel electrophoresis (1 percent agarose gel) and Nanodrop one (Thermo Fisher Scientific, USA) for NGS. Amplicons were made with a total DNA concentration of at least 2ug.

### 2.3. Amplicon production

16SV4/16SV3/16SV3-V4/16SV4-V5, 18SV4/18S V9, ITS1/ITS2, Arc V4) were amplified using particular primers (e.g. 16SV4: 515F-806R, 18S V4: 528F-706R, 18S V9: 1380F-1510R) with the barcode. Phusion® High-Fidelity PCR Master Mix (New England Biolabs) was used for all PCR experiments.

### 2.4. PCR products qualification and quantification

The PCR products were combined with the same volume of 1X loading buffer containing SYB green, and the amplicons were detected using a two percent agarose gel electrophoresis. For further experimentation, samples with a bright main strip of base pair ranging from 400 to 450 were chosen.

### 2.5. PCR products purification

Libraries were created using the Ion Plus Fragment Library Kit for Thermofisher and quantified using Qubit and Q-PCR. IonS5TMXL (Thermofisher) was used to sequence the data.

### 2.6. Sequencing data processing

Single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. According to the Qiime quality-controlled method (Bokulich et al. 2013), quality filtering on the raw data was conducted under defined filtering settings to generate high-quality clean reads (Magoč and Salzberg, 2011). To find chimaera sequences, the reads were matched to the reference database (Gold database) using the UCHIME Algorithm (Caporaso et al. 2010). After removing chimaera sequences (Edgar et al. 2011), the effective reads were recovered.

**Table 1**
Data of the sampled pheasant flocks from Avian Conservation and Research Center.

| Sample ID | Bird species | No. of birds | Male: Female | Feeding |
|-----------|--------------|--------------|--------------|---------|
| 3L | Ring-necked pheasants (Phasianus colchicus) | 5 | 1:4 | Seeds and grains |
| 6L | Green pheasants (Phasianus versicolor) | 5 | 1:4 | Seeds and grains |

## 2.7. OTU clustering and species annotation

Sequences analyses were performed by Uparse software (Uparse v7.0.1001) (Haas et al. 2011) using all the effective reads. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database (Edgar, 2013) for species annotation at each taxonomic rank (Threshold:0.8 ∼ 1) (kingdom, phylum, class, order, family, genus, species) (Wang et al. 2007). To get the phylogenetic relationship of all OTUs representative sequences, the MUSCLE (Version 3.8.31) was used (Quast et al., 2012).

## 2.8. Sequence data analysis

The data collected from the IonS5TMXL machine was in fastq format, and the raw data was cleaned using Cutadapt software. For OTU clustering and species annotation, the effective data was employed. For taxonomic assignment and OTU selection, downstream statistical analysis was used. Fig. 1 depicts the data analysis workflow.

## 3. Results

### 3.1. Data processing

Amplicon was sequenced on IonS5TMXL to obtain raw reads, which were subsequently filtered using chimaeras to provide the Effective Data. Table 2 displays the data output.

### 3.2. Operational taxonomic Units (OUT) and species annotation analysis

To analyze the bacterial diversity in fecal samples at genus level, all the effective reads were grouped according to their DNA sequence similarity (97%) identity threshold of the 16S gene sequences. For OTUs construction, basic information such as low frequency reads data, effective reads data, and annotation data of Reads from both samples was collected. The statistical dataset for both samples is shown in Fig. 2.

### 3.3. Interactive view of species annotation

Fig. 3 depicts an interactive heat-map of species composition and abundance across multiple samples. The counts on the heat map are colored based on the percentage contribution of each OTU to the overall OTU count in one sample. The red color denotes species that give a small percentage of OTUs to the sample, whereas the green color denotes species that contribute a large percentage of OTUs.

### 3.4. GraPhlAn dispaly

As illustrated in Figs. 4 and 5, GraPhlAn (Asnicar et al. 2015) was used to create a tree graph of species annotation for each group.

### 3.5. Taxonomic tree of ring neck and green pheasants

Using R&D software, specific species related to the top 10 most abundant genus were chosen to create the taxonomy tree (DeSantis et al. 2006) with the reference data Fig. 6 depicts the taxonomy tree of both 3L and 6L samples.

### 3.6. Species distribution

#### 3.6.1. Species relative abundance in phylum

Relative abundance of the top 10 species in the phylum represents in Fig. 7. Fig. 8.



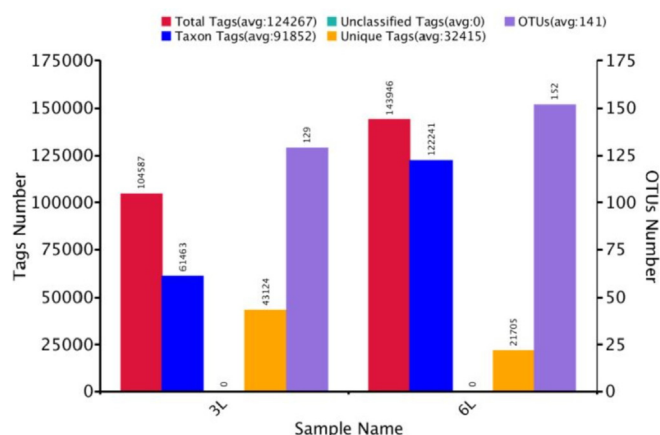**Fig. 1.** Workflow of data analysis.



**Fig. 2.** Analysis of bacterial diversity at genus level in fecal samples of 3L (Ring-neck pheasant) and 6L (Green pheasant) by grouping the effective reads with respect to sequence similarity Notes: "Unique Reads" means the number of reads with a frequency of one and only occurs in one sample.

**Table 2**
Data preprocessing and Initial quality control statistics

| Sample ID | No. of Raw Reads | No. of Clean Reads | No. of Base(nt) in clean reads | Average Length(nt) of clean reads | % base quantity greater than 20 | GC% | Effective % |
|---|---|---|---|---|---|---|---|
| 3L | 152,536 | 104,586 | 45,073,343 | 430 | 82.56 | 54.31 | 68.56 |
| 6L | 146,636 | 143,945 | 62,177,809 | 431 | 83.18 | 54.35 | 98.16 |

**Fig. 3.** Heat map of bacterial species. The top bacterial OTUs for the two samples 3L (ring neck) 6L (Green) Pheasants processed by Quantitative Insights into Microbial Ecology (QIIME).

*3.6.2. Species relative abundance in genus*

Relative abundance of both species for the top 10 species at genus level reveals by different bar colors.

**4. Discussion**

Birds evolve in complex microbial communities and lives near many potentially important pathogenic bacteria. As a result, modern birds have a complicated biochemical mix of microbial and eukaryotic cells, the result of thousands of years of slow and continual adaptation. Significant research on poultry microbes has been published, however it has primarily focused on chickens and other domestic birds. The fecal bacterial makeup of two major pheasant species ring-necked pheasants, and green pheasants is described in this paper. To investigate the microbial flora in feces, a culture-independent approach was used, and sequences were read.

Sample species composition was revealed using the single-end method to construct a small fragment library for single-end sequencing. Through the reads of cut filter, OTUs (Operational Taxonomic Units) clustering species annotation and abundance analysis was done. The current study read a total of 141 OTUs on average. The abundance of species within phyla was found to be *Firmicutes > Actinobacteria > Proteobacteria* in an OTU annotation tree for ring neck pheasants. While, for green neck pheasant the species relative abundance within phyla in fecal sample were as *Firmicutes > Proteobacteria > Bacteroidetes > Actinobacteria*.
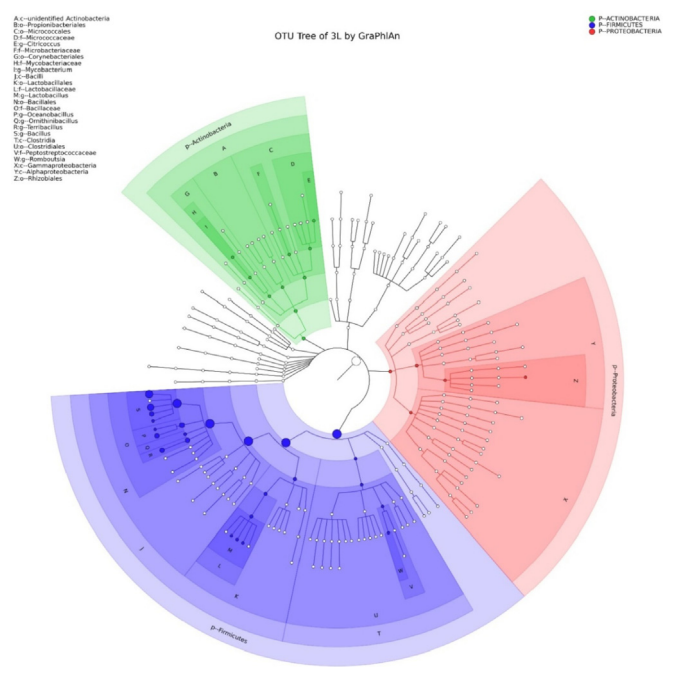
**Fig. 4.** Graphical Phylogenetic Analysis (GraPhlAn) shows microbial community abundance and diversity in ring neck pheasant (3L) Notes: Inside out shows different taxonomic ranks. The size of circles signifies abundance of species. Different colors show different phylum. Solid circles stand for the top 40 species in high abundance.

Although the top ten most prevalent bacterial species in both feces' samples belonged to the bacillus genus. Ring neck pheasants have more *Oceanobacillus*, *Terriobacillus*, and *Lactobacillus* than green neck pheasants.

This is the first study to compare the fecal microbiota of two different pheasant species using metagenomic analysis. We found that there was significant difference in the bacterial communities identified in fecal samples. The most common bacteria identified in fecal samples were members of phylum Firmicutes making 98% of all the bacteria identified in fecal samples of both species. These findings are consistent with those of Cao et al. (2020), who found *firmicutes* to be the most prevalent taxon in the feces of migrating birds *Cygnus cygnus* and *Anser cygnoides*. Firmicute abundance was 58.0 percent and 85.7 percent, respectively, which is lower than the current findings. Garcia-Mazcorro et al. (2017), on the other hand, discovered that the Lactobacillaceae family was the most prevalent among all firmicutes in budgerigar and canary feces. Lactobacillaceae was the second most abundant group among all Firmicutes in our investigation.

Surprisingly, two major taxa, *Oceanobacillus* and *Terribacillus*, were discovered in ring-necked pheasants but not in green-necked pheasants in our investigation. These two genera have never been found in a fecal sample from an avian species before. *Oceanobacillus* is an alkaliphilic Bacillus that is halotolerant. Members of this species are primarily found in the deep sea in nature (Lu et al. 2001), and its extremophilic feature has made it popular in biotechnology. It is possible to isolate this bacterium and use it in biotechnological operations. *Terribacillus* is another extremophilic bacteria genus that has never been found in a bird's feces and this genus' members have the potential to be utilized in biotechnological procedures (Essghaier et al. 2014). Members of the *Lactobacillus* genus were the other top 10 bacteria found in feces. *Lactobacilli* are commonly seen in the gastrointestinal tract and feces (Sohail et al. 2015). *Lactobacillus* sp. can be isolated and employed as a species-specific probiotic formula in domestic birds,
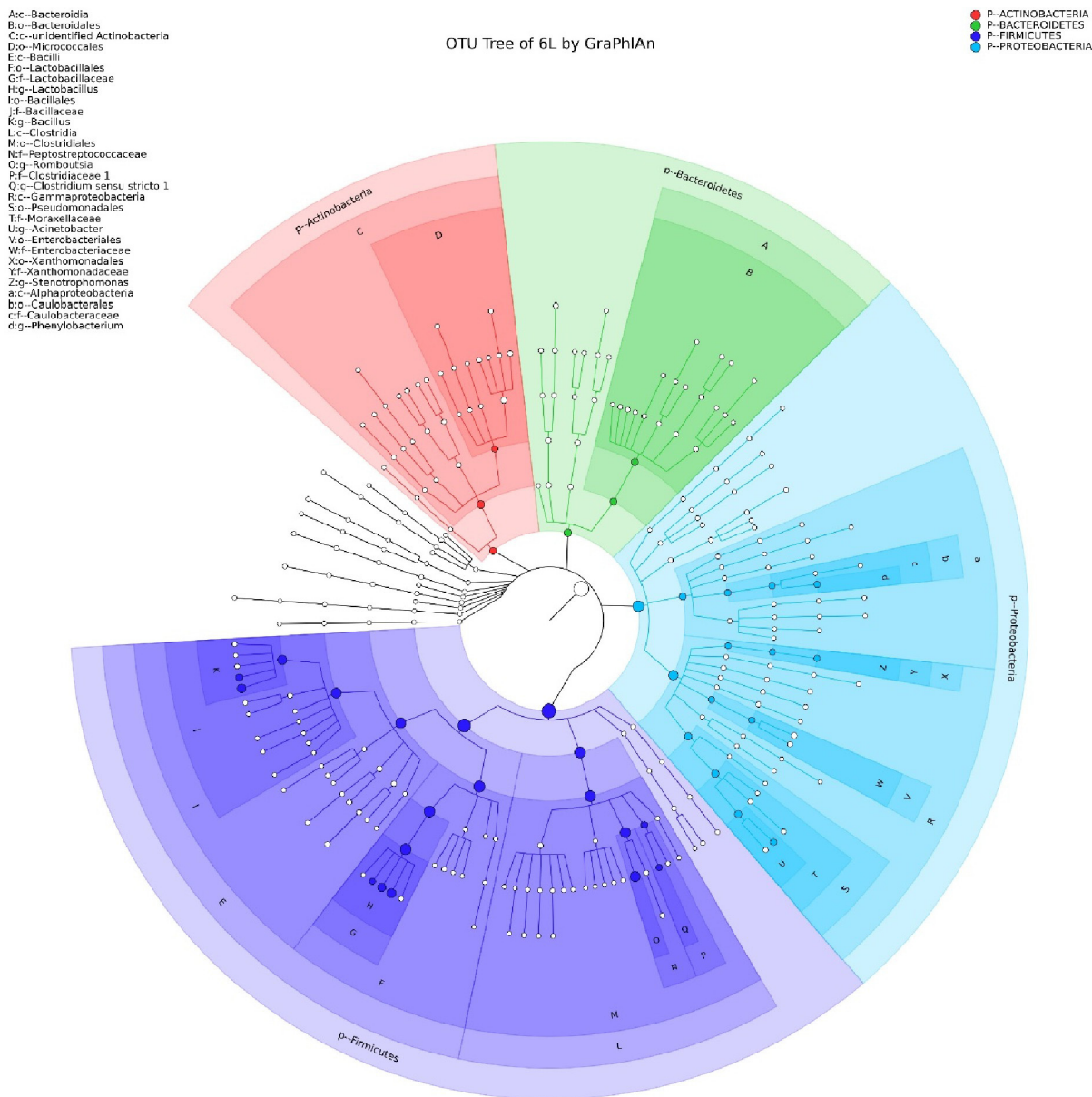
**Fig. 5.** Graphical Phylogenetic Analysis (GraPhlAn) shows microbial community abundance and diversity in Green pheasant (6L)Notes: Inside out shows different taxonomic ranks. The size of circles signifies abundance of species. Different colors show different phylum. Solid circles stand for the top 40 species in high abundance.

which is a topic of great interest (Cisek and Binek, 2014). In fecal microbiota of ring neck pheasant, the second top most abundant phyla was actinobacteria these findings also coincides with the findings of Cao et al. (2020) they found *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* as four dominant phyla in all species, accounting for 75–98% of the total bacterial community in migratory birds. However, in our study *Bacteroidetes* were only abundant phyla in green neck pheasant, a major group for mammalian gut health involved in energy production and obesity in humans (Garcia-Mazcorro et al. 2017). Though, recent studies in poultry microbes have found low levels of Bacteroidetes ranging from 7 to 11 % of all sequences in fecal microbiota of chickens (Xiao et al. 2011; Zhao et al. 2013) and less than 0.1 % of all sequences in fecal microbiota of budgerigars (Garcia-Mazcorro et al. 2017).

Proportions of Bacteroidetes in feces of birds varies widely depending on the specific species (Waite and Taylor, 2014). The high proportion of Bacteroidetes in this current study is interesting because in humans, two of its most important and abundant members (Bacteroides and Prevotella) have been associated with diets rich in protein and animal fat and carbohydrates respectively (Wu et al. 2011). Regardless, it is unclear what the bacterial group Bacteroidetes truly represents (Hoyles and McCartney, 2009) an important issue that to our knowledge has not been discussed in the animal or avian literature.

It is crucial to remember, however, that fecal microorganisms in birds come from a variety of sources, including the gut, the reproductive tract, and the urinary tract (Fricke et al. 2014) the fact that feces do not exclusively come from the intestines of birds is equally significant.

Despite these limitations, the current study is thought to provide a foundation for future research on microbial communities in other birds and mammals, as it sheds light on the complex bacterial communities observed in pheasant droppings. Importantly, the fecal microbiome includes bacteria from not only the stomach but also the reproductive and urinary systems. More study is needed to examine fecal bacteria and metabolic profiles in more
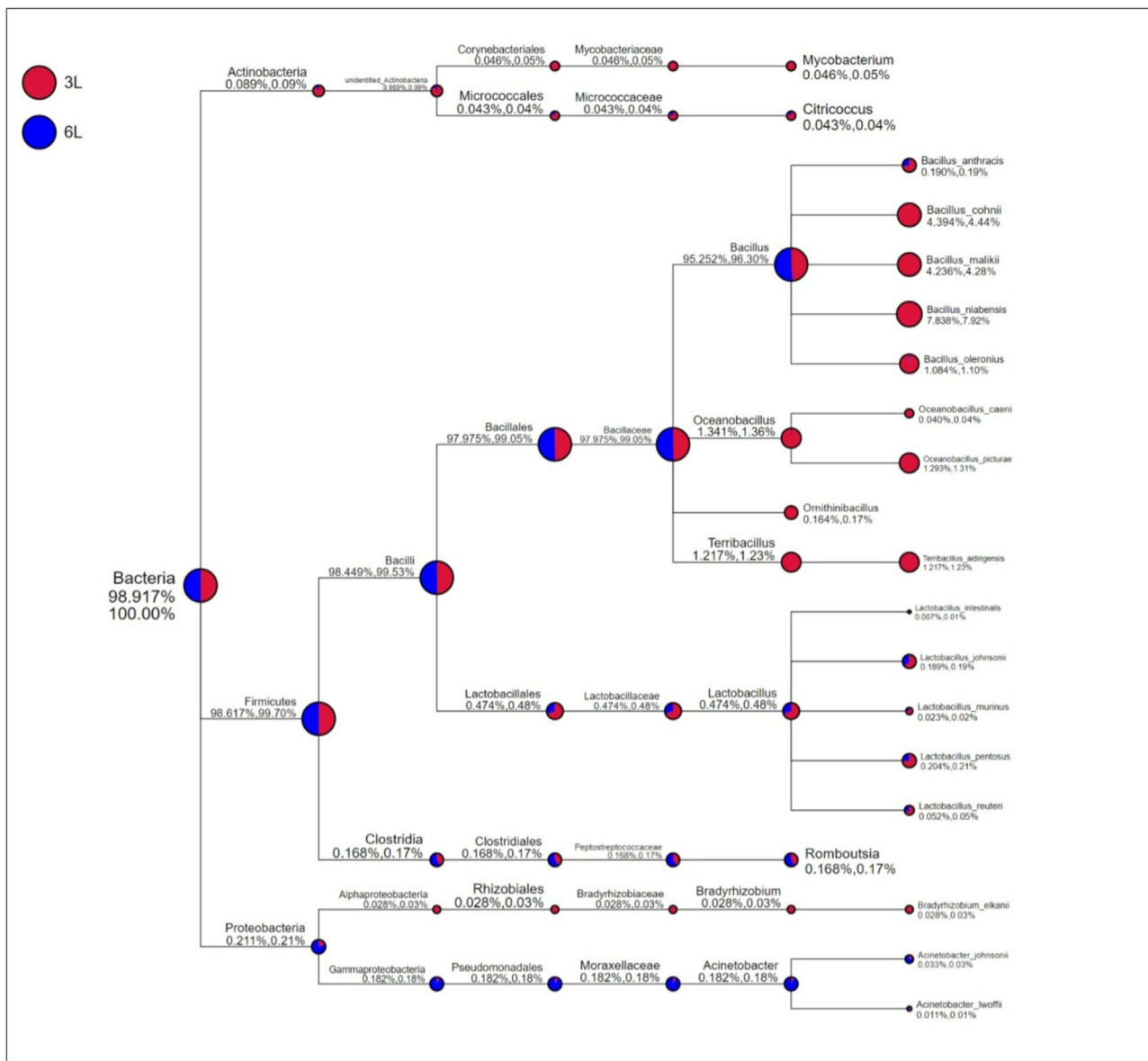
**Fig. 6.** Taxonomy tree depicts the lines of evolutionary descent of different species Notes: Distinct colored sectors reflect different samples. The relative abundance is indicated by the size of the sector. The first number below the taxonomic name denotes the proportion of the entire taxon, whereas the second number denotes the percentage of the selected taxon.
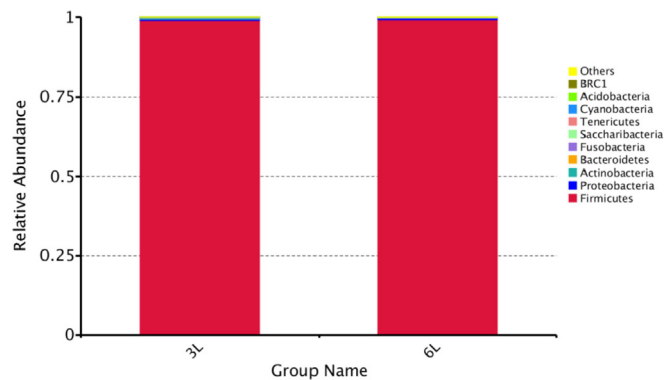


**Fig. 7.** Species relative abundance in phylum of top ten bacterial species in the fecal sample of ring-neck (3L) and green pheasants (6L).
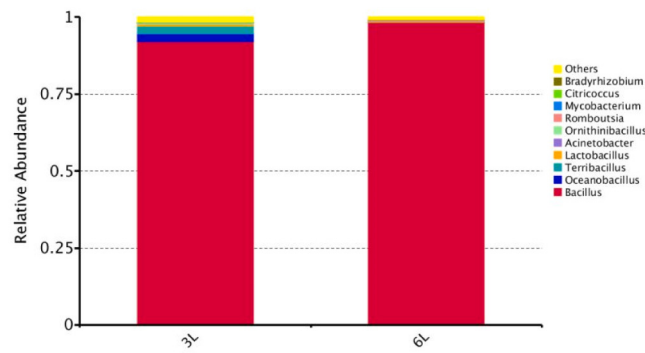


**Fig. 8.** Specie Relative abundance in genera of top ten bacterial species in the fecal sample of ring-neck (3L) and green pheasants (6L).

avian species to better understand how microorganisms coevolve with their hosts.

## 5. Conclusion

We report the microbial community of pheasants by amplifying the hyper variable region of 16S via NGS. The NGS permits a comprehensive clarification of the bacterial communities in birds. The NGS cannot only improve our understanding of diversity and abundance of pathogenic and non-pathogenic bacteria in birds but also organisms ingested as part of the diet in birds and humans.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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