

Original Article

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Taxonomic composition of bacteria in pigeon droppings revealed by metagenomic analysis targeting V3-V4 region of 16S rRNA

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ABSTRACT

Objective: To investigate the bacterial microbiota in pigeon droppings collected from large food storage facilities in Kannur district of Kerala, India, focusing on the potential public health risks posed by these birds in urban environments using metagenomic analysis targeting V3-V4 region of 16S rRNA .

Methods: The study identifies and catalogs bacteria in both dry (PY1) and fresh pigeon droppings (PY2) using 1 g of pooled samples collected from multiple sites utilizing metagenomic analysis through 16S rRNA gene sequencing.

Results: The results revealed a diverse microbial composition in both fresh and dry pigeon droppings, with notable genera including *Kocuria* (12% in PY1 and 2% in PY2), *Fusobacterium* (48% in PY2), and *Streptococcus* (22% in PY2), among others. The presence of pathogenic bacteria like *Acinetobacter* (1% in PY1) and *Fusobacterium* (48% in PY2) underscored the importance of proper hygiene practices in urban settings to mitigate zoonotic disease transmission.

Conclusions: This study underscored the significance of metagenomic analysis targeting the V3-V4 region of 16s rRNA in elucidating microbial diversity. The observed differences between fresh and dry pigeon droppings highlight the influence of environmental factors on bacterial community composition. These findings emphasized the necessity of continous surveillance to enchance hygeine practices and mitigate contamination risks in food storage environments.

KEYWORDS: Pigeon droppings; Zoonotic pathogens; Metagenome; V3-V4 region; 16s rRNA

1. Introduction

Pigeons are ubiquitous in urban environments, often residing in close proximity to humans. These highly urban birds frequently rely on human-made structures for nesting, roosting, and resting sites[1]. However, their presence poses potential health risks due to their role as natural carriers of pathogens[2]. Moist bird droppings provide an ideal environment for fungal spores and bacteria to proliferate, particularly when they dry or are regularly exposed to rain[1].

Pigeon droppings which are rich in nutrients, can support the growth of infectious organisms and have been linked to disease transmission in several countries[3]. They are known to harbor pathogens such as *Salmonella*, *Listeria*, *Chlamydia* and other microbes that can contaminate food in storage and processing

Significance

Pigeon droppings harbor diverse bacterial communities, which may pose significant health risks due to zoonotic pathogens. This study, employing metagenomic analysis of the V3-V4 region of 16S rRNA, reveals the taxonomic composition of bacteria in droppings, highlighting a critical gap in research regarding bacterial contamination in urban food storage areas frequented by pigeons. These findings underscore the urgent need for improved sanitation, regular monitoring, and effective control measures to mitigate health risks and enhance food safety in urban environments.

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facilities[1]. The availability of food resources and the abundance of buildings in cities and towns are primary factors that attract feral animals, including pigeons[4]. Rodents and birds, such as pigeons, can contaminate food storage areas with feces, leading to structural damage, unsanitary conditions, food contamination, and the spread of harmful parasites[5,6]. Food processing plants are at high risk of contamination because pigeons are attracted to grain spillage. These areas can become hotspots for pathogens carried by pigeons, which may then spread to the food supply. The danger is amplified because pigeons return to the same places repeatedly, causing ongoing contamination. Pigeons often return to the same spots each year, creating piles of droppings, feathers, and nests. They feed on rooftops or grassy areas, with spilled grain near food processing plants as a main food source[1].

Birds are also recognized as frequent vectors of emerging infectious diseases in humans, with over 60% of emerging diseases estimated to originate from wildlife[7]. The public's awareness of zoonotic diseases has recently increased due to their significant public health and economic impacts.

Traditionally, the study of microorganisms has relied on cultivating them in laboratory settings. However, not all microorganisms can be cultured using these methods, prompting a shift towards molecular techniques in modern microbial research[8]. Contemporary studies of microbiomes often employ 16S/18S/ITS ribosomal RNA amplicon sequencing to analyze the taxonomic composition of microbial communities. Specifically, the V3 and V4 regions of the 16S rRNA gene serve as key identifiers for various bacterial and archaeal strains, which are critical for microbial phylogeny and taxonomic classification[9].

This study aims to create a comprehensive catalog of fecal bacteria present in both fresh and dried pigeon droppings collected from large food storage facilities, including grain silos, warehouses, Food Corporation of India (FCI) godowns, ration shops, supplyco warehouses and outlets, public markets, and food processing units. These sites provide an ideal environment for sampling due to their high concentration of pigeons and the availability of shelter, food and water, factors that heighten the risk of pathogen transmission through pigeon droppings.

Metagenomics offers a powerful approach for analyzing bacterial communities directly at the nucleic acid level in environmental samples[10]. The variability in 16S gene sequences has been widely utilized to characterize microbial communities, with the hypervariable regions serving as effective markers for classification. Instead of sequencing the entire 16S gene, targeting specific hypervariable regions is often sufficient for taxonomic identification. Additionally, the highly conserved sequences flanking these hypervariable regions enable the design of universal primers. The hypervariable regions of the 16S rRNA gene are thus valuable tools for bacterial identification.

This study conducts a metagenomic analysis of bacterial communities in pigeon droppings using the V3-V4 regions of the 16S rRNA gene to elucidate the taxonomic composition of bacteria present in these samples.

2. Materials and methods

2.1. Sample collection and DNA extraction

The droppings of pigeon were collected from areas around large food storage of Kannur district, India, and 1 g of the pooled samples from these locations was used for further analysis. Collection of samples were done using non-invasive methods using sterile spatulas and placed into pre-weighed sterile tubes. The samples were then transferred on ice to the laboratory and kept for analysis. Each sample was weighed. Two groups were formed based on the sample condition: Dry pigeon dropping sample named as PY1 and fresh pigeon dropping sample as PY2. All dry samples were pooled together, and all fresh samples were pooled separately to maintain consistency across sample types.

The samples were then desiccated until all moisture was fully drawn from the sample. The metadata for the analyzed samples, along with the bioinformatics pipeline, is publically accessible at Github repository[11]. The DNA was extracted from the homogenised samples using the DNeasy Powersoil Pro Kit (Qiagen) following the manufacturer's protocol.

The research utilized only naturally shed pigeon droppings, which are considered animal-derived waste materials and do not constitute animal subjects requiring ethical review. All sample collections were carried out without disturbing pigeons, altering their behavior, or impacting their natural environment.

2.2. PCR amplification

Bacterial microbiota analysis from the samples was carried out based on the V3-V4 hypervariable regions of 16S rRNA genes. The sequence of primer sets used were V3F (5′CCTACGGGNBGCASCAG-3′) and V4R (5′GACTACNVGGGTATCTAATCC-3′) in a 20 μL reaction with Takara Emerald Amp GT PCR Master Mix, extracted DNA, nuclease-free water, and 10 picomoles of each primer.

The DNA amplification using PCR involved initial denaturation at 98 ℃ for 2 min, followed by cyclic denaturation at 98 ℃ for 10 s, primer annealing at 60 ℃ for 30 s, extension at 72 ℃ for 30 s, and a final extension step at 72 ℃ for 5 min. The PCR products were then run on a 2% agarose gel for detection (Figure 1).

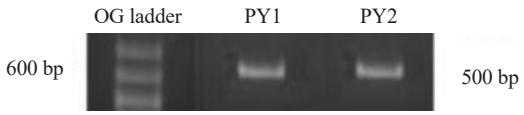


Figure 1. Samples amplified using V3F and V4R primers.

2.3. Sequencing library preparation

After the amplification process, the resulting amplicons were purified using a bead-based method. The amplicon libraries were constructed using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs Inc, USA) following the manufacturer's recommendations, and then added index codes. The validated libraries were used for sequencing on the Illumina MiSeq platform generating 250 bp paired-ends read.

After sequencing, the resulting FASTQ files were processed using QIIME2 (version 2023.5) and DADA 2 plugin. Quality filtering, denoising, and chimera removal were performed. Taxonomic assignment was conducted using the Greengenes 99% database as a reference, ensuring precise sequence classification within the specific gene region.

The step by step pipeline of bioinformatics analysis including preprocessing, quality control and taxonomic classification is available at our GitHub repository[11]. Krona plots of both samples were generated to visualize the hierarchical relationships of identified taxa (Supplementary Figure 1 and Supplementary Figure 2). Summary of bacteria identified up to species level from V3-V4 region based metagenomic study of pigeon dropping samples is represented in Table 1.

Table 1. Bacteria identified up to species level from the metagenomic study in pigeon droppings collected from Kannur district, Kerala, India.

Phylum	Class	Order	Family	Species
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Kordiimonadaceae	*Kordiimonas* sp002401685
	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas faecalis*
	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	*Methylobacterium jeotgali*
	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Ensifer adhaerens*
	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas terrae*
	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Altererythrobacter aerius*
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter pittii*_659518
	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	*Methylobacterium* sp001424705
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter* sp000632455
Chloroflexota	Chloroflexia	Thermomicrobiales	Thermomicrobiaceae	*Sphaerobacter thermophilus*
	Anaerolineae	Anaerolineales	EnvOPS12	OLB14 sp001567105
Patescibacteria	Saccharimonadia	Saccharimonadales	Nanosynbacteraceae	*Nanosynbacter lyticus*
	Saccharimonadia	Saccharimonadales	UBA1020	UBA1020 sp002348615
Firmicutes_A	Clostridia	Peptostreptococcales	Peptostreptococcaceae	*Romboutsia_B maritimum*
	Clostridia	Clostridiales	Clostridiaceae	*Dwaynesavagella* sp 000270205
	Clostridia	Peptostreptococcales	Anaerovoracaceae	*Eubacterium_B sulci*
	Clostridia	Tissierellales	Peptoniphilaceae	*Peptoniphilus_A olsenii*
	Clostridia	Tissierellales	Peptoniphilaceae	*Anaerococcus degeneri*
	Clostridia	Peptostreptococcales	Peptostreptococcaceae	*Peptostreptococcus canis*
	Clostridia	Clostridiales	Clostridiaceae	*Clostridium_T celatum*
Deinococcota	Deinococci	Deinococcales	Deinococcaceae	Deinococcus_Baluminii
Firmicutes_D	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus equinus*
	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus nasalidis*
	Bacilli	Mycoplasmatales	Mycoplasmoidaceae	*Mycoplasma_L_271074 liparidae*
	Bacilli	Lactobacillales	Aerococcaceae	*Aerococcus viridans*_322136
	Bacilli	Lactobacillales	Lactobacillaceae	*Ligilactobacillus agilis*
Cyanobacteria	Cyanobacteriia	Cyanobacteriales	Chroococcidiopsidaceae	*Chroococcidiopsis thermalis*
Fusobacteriota	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	*Fusobacterium gastrosuis*
Actinobacteriota	Actinomycetia	Actinomycetales	Bifidobacteriaceae	*Galliscardovia ingluviei*
	Actinomycetia	Actinomycetia	Micrococcaceae	*Kocuria marina_A_384466*
	Actinomycetia	Propionibacteriales	Propionibacteriaceae	*Cutibacterium modestum*
	Actinomycetia	Actinomycetales	Bifidobacteriaceae	*Aeriscardovia aeriphila*
	Actinomycetia	Actinomycetales	Actinomycetaceae	*Bowdeniella nasicola*_A_386203
	Actinomycetia	Actinomycetales	Micrococcaceae	*Micrococcus antarcticus*
	Actinomycetia	Mycobacteriales	Micromonosporaceae	*Rugosimonospora acidiphila*
	Actinomycetia	Mycobacteriales	Mycobacteriaceae	*Lawsonella clevelandensis*
	Actinomycetia	Mycobacteriales	Jatrophihabitantaceae	*Jatrophihabitans fulvus*
	Actinomycetia	Actinomycetales	Quadrisphaeraceae	*Pseudokineococcus lusitanus*

3. Results

The diversity of bacterial communities in pigeon droppings was analyzed using the 16S rRNA gene amplicon sequencing method; the results were interpreted with reference to existing literature on microbial contamination in food storage facilities. The bacterial community was visualized through a Krona plot which provides a hierarchical representation of bacterial taxa, illustrating the composition and relative abundance of various bacterial groups within the sample. The Krona plot's concentric circles represent different taxonomic levels, progressing from the innermost circle, which represents the entire bacterial community, to the outermost circles that reflect increasing specificity in bacterial classification.

The first concentric ring denotes the domain bacteria, indicating that all identified bacteria belong to this domain. The second ring categorizes these bacteria into major phyla. The third ring provides a breakdown of each phylum into classes. This classification is further refined in subsequent rings, subdividing the classes into orders, families, genera, and potentially species (Supplementary Figure 1 and Supplementary Figure 2).

In the dry pigeon droppings sample (PY1), genera such as *Kocuria*, *Galliscardovia*, and *Kordiimonas* each accounted for 12% of the bacterial population, highlighting their significant role in the ecological dynamics of pigeon droppings. Other notable genera include *Corynebacterium* (8%), *Brevundimonas* (7%), and *Cutibacterium* (6%), along with smaller proportions of *Methylobacterium* (5%), *Nanosynbacter* (4%), and *Bifidobacterium* (2%). In contrast, the fresh pigeon droppings sample (PY2) was dominated by the genus *Fusobacterium* (48%) from the Fusobacteriaceae family and *Streptococcus* (22%) from the Streptococcaceae family. *Galliscardovia* and *Bifidobacterium* were also notable, accounting for 13% and 7% of the population, respectively. Other bacterial groups, including *Lactobacillus* (2%), *Liquorilactobacillus* (1%), and various minor genera, were present in trace amounts (Table 2, Supplementary Figure 1 and Supplementary Figure 2).

The study identified significant communities in both samples, including classes such as *Clostridia*, *Alphaproteobacteria*, *Fusobacteriia*, and *Bacilli*, along with a diverse range of orders and genera, such as *Kocuria*, *Streptococcus*, *Liquorilactobacillus*, *Lactobacillus*, *Corynebacterium*, and *Bifidobacterium.* The dominance of *Fusobacterium* in fresh samples and *Actinobacteriota* in dry samples suggests an environmental influence on microbial composition. Fresh droppings provide a moist, nutrient rich environment favouring anerobic bacteria like *fusobacterium*, which thrive in oxygen deprived conditions. In contast, dry droppings, exposed to air and environmental stressors can promote growth of resilent taxa like *Actinobacteriota*, which are known for their ability to survive desiccation. These findings highlight the adaptablity of bacterial communities and their potential implications for urban microbial ecology and public health..

4. Discussion

The sampling size in this study is limited, the pooling of dry and fresh pigeon droppings from multiple locations provide a representative overview of the microbial communities in these environments. Metagenomic analysis revealed that *Kocuria, Galliscardovia ingluviei* in PY1 and *Fusobacterium gastrosuis* in PY2 were the most prominent species, commonly found in avian gut

Table 2. Summary of key bacterial findings in fresh and dry pigeon droppings.

Sample	Dominant bacterial genera (Phylum)	Relative abundance
Dry droppings (PY1)	*Kocuria* and *Galliscardovia* (Actinobacteriota) *Kordiimonas* (Proteobacteria)	12%
	Corynebacterium (Actinobacteriota)	8%
	Brevundimonas (Proteobacteria)	7%
	Cutibacterium (Actinobacteriota)	6%
	Methylobacterium (Proteobacteria)	5%
	Aeriscardovia (Actinobacteriota) *Nanosynbacter* (Patescibacteria)	4%
	Bifidobacterium and *Brachybacterium* (Actinobacteriota) *Brevibacterium* (Actinobacteriota)	3%
	Paracoccus and *Ensifer* (Proteobacteria) *Bowdeniella* (Actinobacteriota) UBA1020 (Patescibacteria)	2%
	Sphingomonas, *Bradyrizhobium* and *Acinetobacter (*Proteobacteria*)*	1%
Fresh droppings (PY2)	*Fusobacterium* (Fusobacteriota)	48%
	Streptococcus (Firmicutes)	22%
	Galliscardovia (Actinobacteriota)	13%
	Bifidobacterium (Actinobacteriota)	7%
	Corynebacterium (Actinobacteria)	3%
	Lactobacillus (Firmicutes) *Kocuria* (Actinobacteriota)	2%
	Liquorillactobacillus and *Romboutsia* (Firmicutes)	1%

microbiota. Several pathogenic and opportunistic bacteria were also identified, including *Acinetobacter pitti*, *Fusobacterium gastrosuis*, and *Peptostreptococcus canis*, highlighting potential public health risks.

Members of the genus *Bifidobacteria* are widely known for their presence in fecal matter and report in environmental water[12,13]. The detection of taxa belonging to the family Fusobacteriaceae, including the genus *Galliscardovia* as a dominant genus in pigeon droppings in this study suggest an underexplored microbial community in avian feces. While *Galliscardovia* has not been widely reported in similar studies, its presence in fresh pigeon droppings may warrant further investigation into its ecological role and potential implications for microbial interactions in urban environments. Similar findings of *Fusobacteria* dominance in fecal samples have been reported in studies of avian and mammalian microbiomes[14,15]. However its predominance in pigeon droppings remain less explored, highlighting the novelty of this observation.

Given the increasing contact between peridomestic animals like pigeons and humans, understanding the bacterial communities in pigeon droppings is crucial. Feral pigeons, thriving in urban environments, pose significant public health risks as carriers of zoonotic pathogens. Previous studies have identified pathogens such as *Salmonella typhimurium*, *Escherichia coli*, and *Streptococcus* spp. in pigeon populations. Our study adds to this body of knowledge by identifying new bacterial groups, emphasizing the value of 16S rRNA analysis in exploring microbial diversity in unexplored environments.

Previous studies have identified the potential threat posed by food contamination and close proximity to human habitats due to droppings from feral animals[16]. Fresh droppings contain a relatively low number of pathogenic species; however, as they dry, the samples harbor more virulent pathogens. These dry samples become a significant source of contamination, affecting food supplies and indoor air quality, which can lead to gastrointestinal infections and allergic reactions[17,18]. Multiple studies have shown microbial shifts resulting from interactions between humans and animals[19]. Elements of microbial communities can pass between humans and animals through close contact. Song *et al.*[20] found that cohabiting individuals who owned dogs shared more common skin bacteria than those without dogs, possibly due to the additional transmission vector provided by the pet. Similar trends have been observed in the exchange of microbes between humans and nearby livestock.

Our research indicates that droppings are rich in fungal species, which may contribute to the accumulation of aflatoxins, resulting in severe health conditions such as eye infections, liver cirrhosis and kidney failure. This study also underscores the critical importance of maintaining hygiene, particularly in rice storage facilities in Kerala, where rice is a staple food. Long-term storage of grains in unsanitary conditions poses serious health risks. Therefore, implementing effective control measures to prevent feral animals from accessing storage areas is strongly recommended to mitigate these risks.

5. Conclusions

The metagenomic analysis of pigeon droppings by targeting the V3-V4 region of 16S rRNA helps in uncovering the bacterial diversity within the pigeon droppings. This study revealed unique bacterial community compositions from the samples. The differences in microbial composition between fresh and dry droppings emphasize the role of environmental factors in shaping the bacterial communities. The diverse bacterial communities identified, including potential pathogens, highlight the need for continued monitoring and research to better understand the ecological dynamics of pigeon droppings and their impact on public health and offers valuable insights for public health strategic planning and disease control.

While there are broader studies on microbial contamination from pigeons, research focused on the unique conditions and ecological contexts of Kerala is limited. By targeting this specific region, the study offers valuable data on how local environmental factors and pigeon behavior may impact the spread of microbes, especially in areas like food storage godowns that are critical for food safety.

This study demonstrates the power of metagenomic analysis in uncovering microbial diversity and provides a foundation for future investigations into the interactions between pigeons and human populations. The actual taxonomic composition may vary depending on factors such as diet, environmental condition and geographic location metagenome analysis provides a powerful tool for studying microbial communities and its diversity in various environments including pigeon dropping.

Furthermore, the analysis revealed differences in microbial composition between fresh and dry pigeon droppings, which could be linked to environmental factors such as moisture levels and microbial adaptation to the avian gut. This novel finding emphasizes the importance of understanding microbial dynamics in urban ecosystems, where pigeons often congregate in food storage areas. Future investigations into the potential health risks and microbial interactions in such habitats could lead to more effective strategies for managing microbial contamination in food storage environments.

Conflict of interest statement

The authors claim there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

Anisha K, Tajo A conceptualized the study, designed the experimental framework, collected and analyzed the data and drafted the manuscript. Tajo A supervised the project, designed the experiment framework, and provided critical review and approval. Both authors read and approved the final manuscript.

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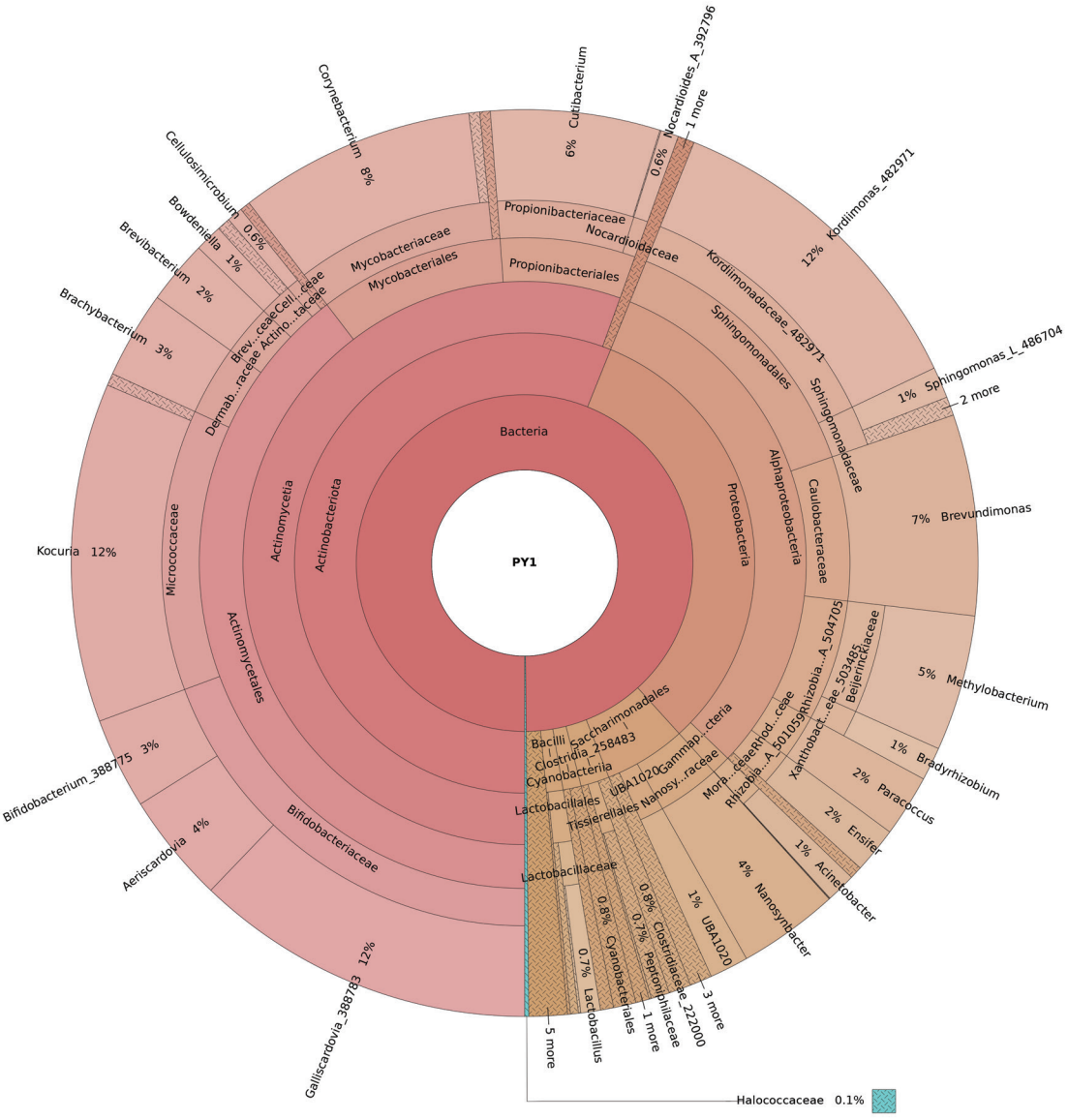
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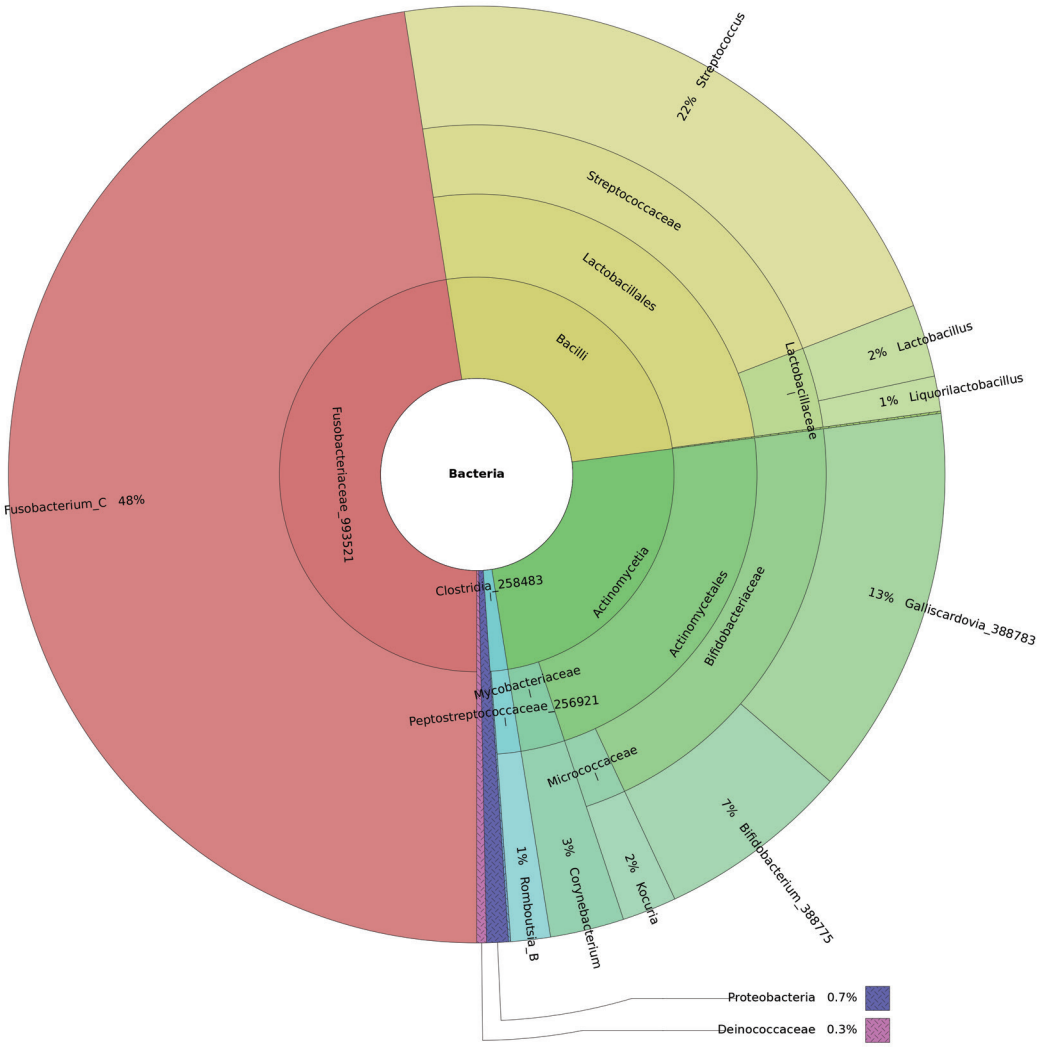
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Supplementary Figure 1. Krona plot showing the representative of bacterial distribution from fresh pigeon droppings represented as PY 1.



Supplementary Figure 2. Krona plot showing the representative of bacterial distribution from fresh pigeon droppings represented as PY 2.