

**A peer-reviewed version of this preprint was published in PeerJ on 22 December 2016.**

[View the peer-reviewed version](https://peerj.com/articles/2837) (peerj.com/articles/2837), which is the preferred citable publication unless you specifically need to cite this preprint.

Alcaraz LD, Hernández AM, Peimbert M. 2016. Exploring the cockatiel (*Nymphicus hollandicus*) fecal microbiome, bacterial inhabitants of a worldwide pet. PeerJ 4:e2837 <https://doi.org/10.7717/peerj.2837>

# Cockatiel (*Nymphicus hollandicus*) gut microbiomes, bacterial inhabitants of a worldwide distributed pet

Luis David Alcaraz<sup>1</sup>, Apolinar M. Hernández<sup>2</sup>, Mariana Peimbert<sup>Corresp.</sup><sup>2</sup>

<sup>1</sup> Laboratorio Nacional de Ciencias de la Sostenibilidad, Instituto de Ecología. Universidad Nacional Autónoma de México, Mexico city, Mexico

<sup>2</sup> Departamento de Ciencias Naturales. Unidad Cuajimalpa, Universidad Autónoma Metropolitana, Mexico city, Mexico

Corresponding Author: Mariana Peimbert  
Email address: marianapeimbert@gmail.com

**Background.** Cockatiels (*Nymphicus hollandicus*) were originally endemic to Australia, but they are nowadays popular worldwide pet birds. It is now possible to make detailed molecular studies on cultivable and uncultivable bacteria that are part of the intestinal microbiome of healthy animals, these studies showed that bacteria are an essential part of the capacity and metabolic status of animals. There are few studies of bird microbiomes and to date this is the first reported cockatiel microbiome work. **Methods.** In this paper we analyzed the gut microbiome of 3 healthy adult cockatiel birds by massive sequencing of 16S ribosomal gene. Additionally, we show a comparison with other poultry, and wild birds microbiomes and their taxa profiles **Results.** The vast majority of the Cockatiel's bacteria found were *Firmicutes*, while *Proteobacteria* and *Bacteroidetes* are poorly represented. 19,280 different OTUs were detected, of which 8,072 belong to the *Erysipelotrichaceae* family. **Discussion.** Cockatiels wide geographic distribution, and close human contact makes relevant to study their microbiomes, this study gives a baseline for their bacterial diversity. Cockatiels microbiomes diversity are dominated by *Firmicutes* of the *Erysipelotrichaceae* family. Cockatiels, and other wild birds are almost depleted of *Bacteroidetes* which happen to be abundant in poultry birds and this is probably related with the intensive human manipulation of poultry bird diets. Some pathogenic bacteria like *Clostridium colinum*, and *Serratia marcescens* are inhabitants of the cockatiel's microbiome while other pathogens are not elements of healthy cockatiel's microbiota, although the specimens collected were perfectly healthy at the time.

## Cockatiel (*Nymphicus hollandicus*) gut microbiomes, bacterial inhabitants of a worldwide distributed pet

### Author names and affiliations

Luis David Alcaraz<sup>a</sup>, Apolinar M. Hernández<sup>b</sup>, and Mariana Peimbert<sup>b,\*</sup>

5 <sup>a</sup>Laboratorio Nacional de Ciencias de la Sostenibilidad. Instituto de Ecología. Universidad Nacional Autónoma de México. AP 70-275, Ciudad Universitaria, UNAM, 04510, Cd. Mx., México.

<sup>b</sup>Departamento de Ciencias Naturales. Universidad Autónoma Metropolitana, Unidad Cuajimalpa. Av. Vasco de Quiroga 4871, Col. Santa Fe Cuajimalpa, 05348, Cd. Mx.,  
10 México.

### Corresponding author

Mariana Peimbert

E-mail address: [mpeimbert@correo.cua.uam.mx](mailto:mpeimbert@correo.cua.uam.mx)

Tel.: (5255) 5814 6500 ext. 3878

15 Departamento de Ciencias Naturales. Universidad Autónoma Metropolitana, Unidad Cuajimalpa. Av. Vasco de Quiroga 4871, Col. Santa Fé Cuajimalpa, 05348, Cd. Mx., México.

## Abstract

**Background.** Cockatiels (*Nymphicus hollandicus*) were originally endemic to Australia, but they are nowadays popular worldwide pet birds. It is now possible to make detailed molecular studies on cultivable and uncultivable bacteria that are part of the intestinal microbiome of healthy animals, these studies showed that bacteria are an essential part of the capacity and metabolic status of animals. There are few studies of bird microbiomes and to date this is the first reported cockatiel microbiome work.

**Methods.** In this paper we analyzed the gut microbiome of 3 healthy adult cockatiel birds by massive sequencing of 16S ribosomal gene. Additionally, we show a comparison with other poultry, and wild birds microbiomes and their taxa profiles

**Results.** The vast majority of the Cockatiel's bacteria found were *Firmicutes*, while *Proteobacteria* and *Bacteroidetes* are poorly represented. 19,280 different OTUs were detected, of which 8,072 belong to the *Erysipelotrichaceae* family.

**Discussion.** Cockatiels wide geographic distribution, and close human contact makes relevant to study their microbiomes, this study gives a baseline for their bacterial diversity. Cockatiels microbiomes diversity are dominated by *Firmicutes* of the *Erysipelotrichaceae* family. Cockatiels, and other wild birds are almost depleted of *Bacteroidetes* which happen to be abundant in poultry birds and this is probably related with the intensive human manipulation of poultry bird diets. Some pathogenic bacteria like *Clostridium colinum*, and *Serratia marcescens* are inhabitants of the cockatiel's microbiome while other pathogens are not elements of healthy cockatiel's microbiota, although the specimens collected were perfectly healthy at the time.

**Keywords:** Microbiome, Cockatiel, *Erysipelotrichaceae*, comparative bird microbiome

## Introduction

The study of microbiology is changing very rapidly thanks to the development of sequencing techniques more efficient and cheaper DNA, this has allowed the study of communities without the need to cultivate and isolate each colony. These technological  
45 developments have established that a healthy human contains 10–100 trillions of bacterial cells (Turnbaugh et al., 2007). The above has made us reanalyze the microorganisms associated to animals with a very different view of what we consider a healthy or a sick state.

There are relatively few studies on gut microbial diversity of birds by next generation  
50 sequencing (Waite and Taylor, 2014). The avian microbiome models that have been extensively studied are chickens and turkeys, poultry microbiome studies have focused mainly to improve the health and weight gain of birds without using antibiotics, as in mammals the presence of *Lactobacillus* has been of particular relevance (Stanley et al., 2014; Danzeisen et al., 2015). Studies on the vultures microbiomes, which are animals that  
55 feed on decomposing meat rich in toxins, and also with the penguins microbiomes shows an increased abundance of *Fusobacteria* (Dewar et al., 2013; Roggenbuck et al., 2014). Kakapo's microbes have been studied as part of its conservation program, they are parrots endemic to New Zealand that are critically endangered (Waite and Taylor, 2014 ). In addition, there are studies of some other bird species like the Hoatzin that has a similar  
60 microbiota to the rumen of cows, which is explained by the forage-based diet (Wright et al., 2009).

Both in the wild and in captivity Cockatiels (*Nymphicus hollandicus*) feed primarily on seeds but they also eat fruits and vegetables. Like all psittacine (parrots) they are characterized by not having ceca which has been attributed to a low fiber diet (DeGolier et al., 1999). Cockatiels are gregarious, small, elegantly colorful and their reproduction in captivity is relatively simple, making them a good choice as a pet. Cockatiel is the only member from the family Cacatuidae, these birds are naturally distributed in Australia with a global distribution as pet and ornamental bird. *N. hollandicus* shows social behaviors, in the wild they are grouped in flocks of 27 birds on average, however when there is shortage of food flocks increases their size up to 100 birds (Jones, 1987).

There are some studies on cultivable bacteria of Cockatiels, but very few of those were performed on healthy birds. One of the most comprehensive Cockatiel microbial studies is about bacterial diversity in skin, in which 37 colonies were isolated, 18 colonies correspond to *Staphylococcus* and 5 to *Corynebacterium* (Lamb et al., 2014). Given the wide distribution of Cockatiels as pets it is important to research on the biodiversity of bacteria associated with these birds. In this paper we describe the gut microbial diversity of healthy adult cockatiels by the use of next generation sequencing and analysis of ribosomal 16S gene, additionally Cockatiel's diversity is also compared with that observed in other herbivorous birds.

## 80 **Methods**

### **Sampling**

Fecal samples from three adult and healthy cockatiels (*Nymphicus hollandicus*) were obtained from two commercial breeders at the Sonora Market, Mexico City. First fecal deposition of the day were immediately recollected with cotton swabs, samples were stored  
85 at -80°C in resuspension buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA) until processed. No special permissions were required for this work, and the bird seller gave us allowance to sample stool, no birds were harmed in this study. *Nymphicus hollandicus* is listed as “Least Concern ver 3.1” in the Red List of the International Union for Conservation of Nature (IUCN; <http://www.iucnredlist.org/details/22684828/0>).

### 90 **DNA preparation**

DNA purification were performed by standard procedures (Sambrook et al., 1989) except that phenol extraction was performed at 55°C. DNA was further purified by High Pure PCR Template Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer instructions. Briefly, 30 µl of sample was resuspended in 150 µl of 50 mM glucose, 10 mM  
95 EDTA, 25 mM Tris-HCl pH 8.0, 0.1 mg of lysozyme was added and incubated for 5 min. Then, SDS was added to a final concentration of 2%, 200 µl of phenol were aggregated and incubated for 15 minutes at 55°C. The aqueous phase was separated and re extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). DNA was precipitated with sodium acetate and ethanol at -20 °C and resuspended in water.  
100 Later, binding buffer, which includes guanidine-HCl and proteinase K, was added; DNA was bound to a spin column of silica gel, the column was washed two times and finally DNA was eluted in 50 µl water.

### Amplification

Three PCR were performed for each sample. Primers MiSeq341F  
105 (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and  
MiSeq805R  
(GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC  
C) were used. The 3' end of primers amplifies for regions V3 and V4 of 16S gene  
(Herlemann et al., 2011), while 5' ends are adapter sequences for MiSeq™ (Illumina, San  
110 Diego, CA). The reactions were carried out in a final volume of 20 µl containing 250 µM  
dTNPs, 0.5 µM of each primer, 0.02 U Taq Platinum (Invitrogen, Carlsbad, CA), and 10x  
Taq Platinum buffer containing 1.5 mM MgCl<sub>2</sub>. The protocol used for PCR reactions was:  
initial denaturation at 95°C for 3 min, followed by 25 cycles consisting of denaturation at  
94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and a final  
115 extension step at 72°C for 5 min. PCR products were purified with High Pure PCR Product  
Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany).

### Sequencing

We used the National Autonomous University of Mexico's Massive DNA Sequencing  
Facility UUSMD services to build sequencing libraries and MiSeq™ 300bp paired ends,  
120 following Illumina® directions (Illumina, San Diego, CA).

### Data analysis

Raw reads were processed and quality filtered using FASTQC  
(<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and Fastx-Toolkit  
([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)). The reads were assembled and merged with



125 PANDASEQ (Masella et al., 2012), selecting a minimum length of 470 bp, minimum  
overlap of 15 bp and a quality cut-off for the assembly of 0.95, removing any ambiguous  
bases. Operational Taxonomic Units (OTUs) were picked with *cd-hit-est* using a 97%  
identity cut-off. OTU representative sequences were selected with *pick\_rep\_set.py* script  
from the QIIME pipeline (Caporaso et al., 2010). Taxonomy assignment of the  
130 representative OTUs was done using BLAST (e-value = 1e-10) against Greengenes DB (v  
13.8; DeSantis et al., 2006). Known contaminants were removed from the analysis  
(mitochondria, chloroplast), as well as chimeras. All statistical and diversity analyses were  
performed on R: phyloseq package (McMurdie and Holmes, 2013), plots were done using  
ggplot2 package and RColorBrewer library palettes.

135 We choose several available bird microbiomes to compare against cockatiel, all the  
sequences were downloaded from their papers declared repositories, all the sequences were  
processed with the same QC applied to cockatiel, as described before. The compared  
samples are: An endangered psittacine bird samples, three samples from the kakapo gut  
microbiome (Waite et al., 2012); three turkey gut microbiome samples (Danzeisen et al.,  
140 2015); three chicken gut microbiome samples (Stanley et al., 2013); one sample from wild  
duck's fecal samples microbiome (Strong et al., 2013); three samples from the emu caecal  
microbiome (Bennett et al., 2013); and finally we used three swine gut microbiome samples  
as an outgroup for comparative purposes (Looft et al., 2012). For the comparative birds  
microbiome dataset, each compared species was processed as stated before individually,  
145 then phyla abundances were calculated from the taxonomy assignments, transformed to  
relative frequency for each sample and plotted in an histogram. This was the alternative

procedure because the poor performance of *pick\_closed\_otus.py* reducing the overall diversity several magnitude orders in the cockatiels microbiomes.

### **Data accessibility**

150 Raw sequencing data is available on the NCBI under the project accession PRJNA320285, and the following Short Read Archive (SRA) accessions: SRR3473941, SRR3473942, and SRR3473943. OTU tables and their taxonomic assignments are available on figshare <https://dx.doi.org/10.6084/m9.figshare.3470555>

## Results

155 Gut microbiomes from three adult cockatiels were studied by sequence analysis of 16S  
ribosomal gene; specimens were healthy and lived in captivity. About 100,000 sequences  
were obtained per bird giving a total of 295,217 sequences which were clustered into  
19,280 Operational Taxonomic Units (OTUs) at a 97% identity cluster cut-off (Table 1).  
The vast majority (99.6%) of observed OTUs were taxonomically assigned with the  
160 Greengenes database which includes environmental samples. There are around 7,000  
different OTUs identified for each cockatiel, the calculated Chao1 diversity index indicates  
an expected richness from 32,000 to 44,000 OTUs, this implies that many other OTUs can  
be found. The Simpson index implies that for all three cases there are few predominant  
bacteria, so OTUs not found in this study are in very low proportions (Table 1). By doing a  
165 frequency analysis we observe that the vast majority of organisms were *Firmicutes*, 57% of  
the sequences corresponds to the *Erysipelotrichaceae* family, while 17 and 15% correspond  
to the *Clostridium* and *Lactobacillus* genus, respectively. *Proteobacteria* together stand for  
less than 1.5% of the observed bacteria, *Tenericutes* represent 6.3%, while *Bacteroidetes*  
are less than 0.05%. The groups that showed greater diversity were *Erysipelotrichaceae*  
170 (8,072), *Lactobacillus* (6,919) and *Mycoplasmataceae* (1,295). Observed *Cyanobacteria*  
correspond to the chloroplasts from the grain-based diet, just as some *Proteobacteria*  
identified as mitochondria grasses.

To better understand the distribution of bacteria in each animal we performed a bar plot  
graph which shows that there are dominant bacterial OTUs in each sample (Figure 1);  
175 *Erysipelotrichaceae*, *Clostridium* and *Lactobacillus* are the most abundant groups. It is  
interesting that although more than 8,000 different *Erysipelotrichaceae* OTUs were found,

only two OTUs were clearly dominant for the three samples we named these E1 and E2. Likewise, the *Lactobacillus* OTU L1 is the most common for all three birds and the same happens with *Clostridium colinum* OTU C1. We can also observe that 75% of the sample is made up of only 4 different bacteria in all cases. The Venn diagram shows that only 461 out of 19,279 OTUs are shared in all samples (Figure 2). However, these shared bacteria are predominant as correspond for 82, 83, and 85% of each bird microbiome. The 17,166 unique otus for each cockatiel are low frequency ones, for cockatiel 2, and 3 each exclusive OTU represents less than 0.1%; whereas Candidatus Division *Arthromitus* is exclusive for cockatiel 1 and its abundance is 1.9%.

We performed the standard QIIME's *pick\_closed\_otus.py* script strategy to compare with other birds microbiome datasets, which uses a closed reference DB and discard any non matching sequence for further analysis. When we tried to identify the sequences using the closed OTU database, which only includes type species, just 309 cockatiel's OTUs were designated (Table 1); this indicates that most bacteria from Cockatiels gut microbiome are not comprehended within the reference sequence models used by *pick\_closed\_otus.py* but they are common in other environments. This also points out that the *pick\_closed\_otus.py* is not the best way to analyze and compare microbiomes, although it is the recommended approach for comparing different microbiome studies using different 16S gene variable regions, sequencing coverages, and read lengths derived from independent experiments (Caporaso et al. 2010). To overcome the low number of *pick\_closed\_otus.py* assigned OTUs and perform the comparison, the raw datasets for the other bird microbiomes were downloaded and processed just as for cockatiels (see Methods) and comparisons were done using family taxonomic level.

## 200 Discussion

A graphical summary of the cockatiel's microbiome diversity is shown in Figure 3, where *Erysipelotrichaceae* family is highly dominant (57%) in the cockatiels gut microbiomes. This family is ubiquitous and most known strains are avirulent. They are Gram positive bacteria and there are both aerobic and anaerobic species. *Erysipelothrix rhusiopathiae* was first described in 1876 by Koch however many details of its physiology are unknown. The genus *Erysipelothrix* is aerobic, *E. rhusiopathiae* causes erysipelas disease in swines, poultry and also infects other animals including humans; treatment with penicillin is sufficient to treat erysipelas and in some countries it is common practice to vaccinate swines against the bacteria (Eamens et al., 2006). *E. tonsillarum*, unlike *E. rhusiopathiae*, can ferment sucrose and is not virulent yet the 16S ribosomal gene diverges only three bases (99.8% identical) (Kiuchi et al., 2000); the former prevents their differentiation in molecular studies such as this. About *Erysipelotrichaceae* anaerobic species very little is known, they are found in the gut and oral microbiome of healthy human and mouse, and some species have been associated with periodontitis and halitosis (Verbarq, et al., 2014). For all the OTUs phylotypes were assigned, but for most of them it was not possible to have a resolution up to gender or species levels, so we do not know if they are or not anaerobic nor can we relate them to some pathogenic species.

*Lactobacilli* are often in the gut microbiome of animals, they are used as probiotics for weight gain of the chickens as well as for protection against some enteric bacteria like *Salmonella* or *Campylobacter* (Patterson and Burkholder, 2003). In this analysis *Lactobacilli* represent on average 15% of the microbiome. The most common species for

cockatiels are *Lactobacillus coleohominis*, *L. reuteri* and *L. acidipiscis*, however many OTUs could not be homology assigned (phylotyped) at the species level.

The three cockatiels from two different farms presented *Clostridium colinum*

225 asymptotically with very different proportions 1.3, 37.1, and 11.3%, respectively. *C. colinum* is a Gram positive anaerobic bacterium that has been identified as a pathogen in poultry; it causes enteritis ulcerative, also called quail disease, symptoms includes liver and spleen injuries (Berkhoff, 1985). *C. colinum* infection can cause death within 2-3 days for bobwhite quail, while in other birds can cause anorexia, humped backs, and watery or  
230 bloody diarrhea. Mortality in chickens is relatively low (2-10%) and they usually recover in a couple of weeks. Ulcerative enteritis is associated with high population density and can be treated with streptomycin (Cooper et al., 2013). Our data indicate that *Clostridium colinum* is normally found in the cockatiel gut microbiome and only when out of control causes disease in cockatiels, also it could suggest that there are some low pathogenic OTUs.

235 The family *Mycoplasmataceae* constitutes 6.3% of the cockatiels' microbiome. In the Greengenes DB taxonomic classification they are part of Phylum *Tenericutes*, but many other classifications consider them part of Phylum *Firmicutes*. *Mycoplasmas* are bacteria without cell walls which are usually located in the gut. *Mycoplasmas* blooms are associated with diets rich in simple carbohydrates and thus are related to obesity in mice and humans,  
240 these blooms displace *Bacteroidetes* (Turnbaugh et al., 2006). The proportion observed in cockatiels is the same as healthy chickens, for *Clostridium perfringens* infected chickens this proportion increased 3.7 times (Stanley et al., 2013).

Of the bacteria found in just one individual cockatiel “*Candidatus* division *Arthromitus*” with a frequency of almost 2%. *Ca. Arthromitus* is a segmented filamentous non culturable Gram positive bacterium; the filaments are anchored to the intestinal epithelium end they are important for development of the mice immune system (Talham et al., 1999). The fully sequenced *Ca. Arthromitus*’ genome shows a reduced genome suggesting a close and lasting relationship with their host (Bolotin et al., 2014). In turkeys, they have been described as part of normal bacterial succession that gets established around week 6; *Ca. Arthromitus* has also been linked to weight gain because they displace some types of *Lactobacilli* (Danzeisen et al., 2013). In the cockatiel, we do not observe a clear displacement of *Lactobacilli*; however, as in the aforementioned paper not all the birds show this bacterium.

Cockatiel’s most abundant *Proteobacteria* was *Serratia marcescens* which is usually located in water and foods, but it is also a nosocomial pathogen which can cause respiratory and urinary infections, meningitis, endocarditis, etc (Hejazi and Falkiner, 1997). Its frequency is not negligible because it represents 1% of the observed bacteria.

We specifically look for some pathogenic bacteria in the cockatiels microbiomes. *Escherichia*, *Shigella*, *Mycobacterium*, *Chlamydia*, *Mycoplasma* and *Pasteurella* were not detected; while *Salmonella*, *Helicobacter*, *Campylobacter*, *Klebsiella*, *Staphylococcus*, *Aeromonas*, *Proteus*, *Listeria* and *Enterococcus* were found in just an animal to a lesser extent of  $1 \times 10^{-5}$ . *Pseudomonas* species that were found are not pathogenic to animals, we also detected *Streptococcus* in the cockatiel 2 with a 0.1% frequency. The above indicates

that these pathogenic bacteria are not part of the normal gut microbiome as they were not  
265 found or detected only in one bird.

Because of the importance of the type of diet for the development of microbiome, cockatiel  
microbiome data were compared with other grain-eating birds microbiomes (kakapo, emu,  
duck, turkey, chicken), and with swine gut microbiomes as a mammal outgroups (Figure 4).  
The comparison shows that the most abundant phyla averages for Cockatiels are:  
270 *Firmicutes* (91%), followed by *Tenericutes* (5.9%), *Spirochaetes* (1.4%), and  
*Proteobacteria* (1.3%). *Firmicutes* are in high relative abundances (>50%) in turkeys, and  
chickens as well but within this farm birds the second most abundant Phylum is  
*Bacteroidetes* which is neglectable for cockatiels. Cockatiels and chickens have unusual  
higher average (cockatiels = 5.9%; chickens = 13%) amounts of *Tenericutes* when  
275 comparing with the rest of the analyzed bird species (3%). *Bacteroidetes* are broadly  
distributed (15.62%), but in all the cockatiels (0.3%), one kakapo, and from the wild duck  
with barely detectable amount of this phylum while turkey, chicken and pork have a high  
presence of *Bacteroidetes*, these three cases are overcrowded and extremely sedentary  
animals. The cockatiel comparison with the kakapo is the most obvious as they are also  
280 parakeets; kakapo are free-living birds that are in a conservation program, the microbiome  
of the kakapo is composed mostly by *Proteobacteria* (79.61%). Duck and emu have a high  
abundance of *Fusobacteria* (duck=57%, emus=24.67%), this has also been observed in  
birds with a carnivorous diet as penguins and vulture. When comparing the swine  
microbiomes to the birds we can observe that the first abundance places is dominated by  
285 *Bacteroidetes* (50.25%), followed by *Proteobacteria* and *Firmicutes* in equivalent amounts  
both have an average ~23%.



By means of a Non-metric Dimensional Scaling (NMDS) bird samples were clustered accordingly to their microbiome taxonomy profiles (Figure 5). The clustering was done using family taxa (N = 203), because each individual dataset was taxonomically assigned independently. Poultry bird species cluster closer between them in the center with 290 microbiomes dominated by *Firmicutes*, *Actinobacteria*, *Tenericutes* for chickens and turkeys; emu microbiome hosts a middle ground between *Firmicutes* and *Proteobacteria* in the center of the clustering; Kakapos microbiomes clusters apart from every other bird and is dominated by *Proteobacteria* species; wild duck microbiome is mainly to 295 *Actinobacteria*, *Firmicutes*, and *Proteobacteria*; cockatiels microbiomes cluster to the lower right quadrant and are dominated by *Firmicutes*, *Tenericutes*, *Spirochaetes*, and some *Proteobacteria*. Swines are used as a comparative outgroup and their microbiomes hosts large amounts of *Bacteroidetes*.

It seems like the *Bacteroidetes* are not major players in at least three bird species analyzed 300 here: cockatiels, kakapos, and wild ducks. The *Bacteroidetes* could be an addition to birds microbiomes due to poultry management, but they seem reduced in wild birds and parrots, which are not being selected for rapid weight gain. In mammals like the mouse, the increase of ~50% *Firmicutes*, and the corresponding decrease of *Bacteroidetes* abundance is connected to an obese mice phenotype, and the rise of *Firmicutes* in obese mice is 305 connected to increased capabilities to harvest energy from the diet (Turnbaugh et al., 2006). The *Firmicutes* richness in wild birds and the cockatiels microbiomes could be also be connected to a more efficient energy harvesting capabilities, as the increase in the *Bacteroidetes* frequencies in the poultry birds could be directed by the intensive human

manipulation. However, further work is needed to be further compare the phyla abundances  
310 in a wider set of poultry raised, and wild living birds.

## Conclusions

Cockatiel's microbiome gives a baseline bacterial diversity for this petting species which is distributed across all continents. We were able to estimate a total of 19,280 unique OTUs in all the sampled cockatiels, which is a huge amount of potential bacterial species, but this  
315 species diversity is not taxonomically widespread as cockatiel microbiome is clearly dominated by *Firmicutes*, especially *Erysipelotrichaceae* family. Some pathogenic bacteria like *Clostridium colinum*, and *Serratia marcescens* are part of normal intestinal microbiota while other pathogenic bacteria are not found regularly in cockatiels. Finally, when comparing other birds microbiomes Cockatiels' microbiome is hosting the most *Firmicutes*  
320 dominated microbiome, with *Proteobacteria* abundances similar to chicken and turkey microbiomes, although virtually depleted of some widespread phyla in several birds like *Bacteroidetes*. However, bacteria found in the cockatiel gut microbiome are also found in other animals.

## Conflict of interest

325 The authors declare no conflict of interest-

## Acknowledgements

We thank Ricardo Grande for his technical assistance.

## References

- 330 Bennett, D.C., Min, H., Eun, J., Leung, F.C., Cheng, K.M., 2013. Characterization of  
cecal microbiota of the emu ( *Dromaius novaehollandiae* ). *Vet. Microbiol.* 166, 304–  
310. doi:10.1016/j.vetmic.2013.05.018
- Berkhoff, H.A., 1985. *Clostridium colinum* sp. nov., nom. rev., the Causative Agent  
of Ulcerative Enteritis (Quail Disease) in Quail, Chickens, and Pheasants. *Int. J. Syst.  
Bacteriology* 35, 155–159.
- 335 Bolotin, A., Wouters, T. De, Schnupf, P., Bouchier, C., Loux, V., Rhimi, M., Jamet, A.,  
Dervyn, R., Boudebouze, S., Blottière, H.M., Sorokin, A., Snel, J., Cerf-Bensussan,  
N., Gaboriau-Routhiau, V., van de Guchte, M., Maguin, E., 2014. Genome Sequence  
of “*Candidatus Arthromitus*” sp. Strain SFB- Mouse-NL, a Commensal Bacterium  
with a Key Role in Postnatal Maturation of Gut Immune Functions. *Genome Announc.*  
340 2, e00705–14. doi:10.1128/genomeA.00705-14.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello,  
E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T.,  
Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D.,  
Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J.,  
345 Yatsunenko, T., Zaneveld, J., Knight, R., 2010. correspondence QIIME allows analysis

of high-throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nature* 7, 335–336. doi:10.1038/nmeth0510-335

Cooper, K.K., Bueschel, D.M., Songer, J.G., 2013. Anaerobe Presence of *Clostridium perfringens* in retail chicken livers. *Anaerobe* 21, 67–68.

350 doi:10.1016/j.anaerobe.2013.03.013

Danzeisen, J.L., Calvert, A.J., Noll, S.L., McComb, B., Sherwood, J.S., Logue, C.M., Johnson, T.J., 2013. Succession of the turkey gastrointestinal bacterial microbiome related to weight gain. *PeerJ* 1, e237. doi:10.7717/peerj.237

355 Danzeisen, J.L., Clayton, J.B., Huang, H., Knights, D., McComb, B., Hayer, S.S., Johnson, T.J., 2015. Temporal Relationships Exist Between Cecum, Ileum, and Litter Bacterial Microbiomes in a Commercial Turkey Flock, and Subtherapeutic Penicillin Treatment Impacts Ileum Bacterial Community Establishment. *Front. Vet. Sci.* 2, 56. doi:10.3389/fvets.2015.00056

360 DeGolier, T. F., Mahoney, S. A., Duke, G. E., 1999. Relationships of avian cecal lengths to food habits, taxonomic position, and intestinal lengths. *Condor* 622-634.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi:10.1128/AEM.03006-05

365 Dewar, M.L., Arnould, J.P.Y., Dann, P., Trathan, P., Groscolas, R., Smith, S., 2013. Interspecific variations in the gastrointestinal microbiota in penguins. *Microbiologyopen* 2, 195–204. doi:10.1002/mbo3.66

- Eamens, G.J., Forbes, W.A., Djordjevic, S.P., 2006. Characterisation of *Erysipelothrix rhusiopathiae* isolates from pigs associated with vaccine breakdowns. *Vet. Microbiol.* 115, 329–338. doi:10.1016/j.vetmic.2006.02.015
- 370
- Hejazi, A., Falkiner, F. R., 1997. *Serratia marcescens*. *Journal of Medical Microbiology* 46, 903-912.
- Herlemann, D.P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F., 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5, 1571–9. doi:10.1038/ismej.2011.41
- 375
- Jones, D., 1987. Feeding Ecology of the Cockatiel, *Nymphicus hollandicus*, in a Grain-Growing Area. *Aust. Wildl. Res.* 14, 105–15.
- Kiuchi, A., Hara, M., Pham, H., Takikawa, K., 2000. Phylogenetic Analysis of the *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum* Based Upon 16s rRNA. *DNA Seq.* 11, 257–260. doi:10.3109/10425170009033239
- 380
- Lamb, S., Sobczynski, A., Starks, D., Sitinas, N., 2014. Bacteria Isolated From the Skin of Congo African Grey Parrots ( *Psittacus erithacus* ), Budgerigars ( *Melopsittacus undulatus* ), and Cockatiels ( *Nymphicus hollandicus* ). *J. Avian Med. Surg.* 28, 275–279. doi:10.1647/1082-6742-28.4.275
- 385
- Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., 2012. In-feed antibiotic effects on the swine intestinal microbiome. *PNAS* 109, 1691–1696. doi:10.1073/pnas.1120238109
- Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G., Neufeld, J.D., 2012. PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13, 1–
- 390 7. doi:10.1186/1471-2105-13-31

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8, e61217. doi:10.1371/journal.pone.0061217

395 Patterson, J. A., Burkholder, K. M., 2003. Application of prebiotics and probiotics in poultry production. Poultry Science 82, 627-631.

Roggenbuck, M., Bærholm Schnell, I., Blom, N., Bælum, J., Bertelsen, M.F., Pontén, T.S., Sørensen, S.J., Gilbert, M.T.P., Graves, G.R., Hansen, L.H., 2014. The microbiome of New World vultures. Nat. Commun. 5, 5498. doi:10.1038/ncomms6498

400 Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning. Cold spring harbor laboratory press, New York.

Stanley, D., Geier, M.S., Hughes, R.J., Denman, S.E., Moore, R.J., 2013. Highly variable microbiota development in the chicken gastrointestinal tract. PLoS One 8, 6–12. doi:10.1371/journal.pone.0084290

405 Stanley, D., Hughes, R. J., Moore, R. J., 2014. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Applied Microbiology and Biotechnology 98, 4301-4310.

Strong, T., Dowd, S., Gutierrez, A.F., Coffman, J., 2013. Amplicon pyrosequencing of wild duck eubacterial microbiome from a fecal sample reveals numerous species linked to human and animal diseases. F1000Research 1–18. doi:10.12688/f1000research.2-224.v1

410 Talham, G.L., Jiang, H., Bos, N.A., Cebra, J., 1999. Segmented Filamentous Bacteria Are Potent Stimuli of a Physiologically Normal State of the Murine Gut Mucosal

Immune System Segmented Filamentous Bacteria Are Potent Stimuli of a  
415 Physiologically Normal State of the Murine Gut Mucosal Immune System. *Infect.  
Immun.* 67, 1992.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., Gordon, J.I.,  
2006. An obesity-associated gut microbiome with increased capacity for energy  
harvest. *Nature* 444, 1027–31. doi:10.1038/nature05414

420 Turnbaugh, P.J., Turnbaugh, P.J., Ley, R.E., Ley, R.E., Hamady, M., Hamady, M.,  
Fraser-Liggett, C.M., Fraser-Liggett, C.M., Knight, R., Knight, R., Gordon, J.I.,  
Gordon, J.I., 2007. The human microbiome project. *Nature* 449, 804–10.  
doi:10.1038/nature06244

Verbarg, S., Göker, M., Scheuner, C., Schumann, P., Stackebrandt, E., 2014. The  
425 Families Erysipelotrichaceae emend., Coprobacillaceae fam. nov., and  
Turicibacteraceae fam. nov. In *The prokaryotes* (pp. 79-105). Springer Berlin  
Heidelberg.

Waite, D.W., Deines, P., Taylor, M.W., 2012. Gut microbiome of the critically  
endangered New Zealand parrot, the kakapo (*Strigops habroptilus*). *PLoS One* 7.  
430 doi:10.1371/journal.pone.0035803

Waite, D.W., Taylor, M.W., 2014. Characterizing the avian gut microbiota:  
Membership, driving influences, and potential function. *Front. Microbiol.* 5, 1–12.  
doi:10.3389/fmicb.2014.00223

Wright, A.-D.G., Northwood, K.S., Obispo, N.E., 2009. Rumen-like methanogens  
435 identified from the crop of the folivorous South American bird, the hoatzin  
(*Opisthocomus hoazin*). *ISME J.* 3, 1120–1126. doi:10.1038/ismej.2009.41

## **Tables titles**

**Table 1.** Cockatiel's microbiome basic statistics. Number of reads, OTUs and diversity indexes.

440 **Table 2.** Cockatiel's microbiome assigned phylotypes and OTUs abundances.

\*A total of 295,217 sequencing reads were clustered into 19,280 non-redundant OTUs using a 97% identity threshold. The Frequency column was calculated using the total amount of sequencing reads assigned to a phylotype or an OTU for each described level.

## **Figures legends**

445 **Figure 1.** Frequency of OTUs for each cockatiel. The three cockatiels are clearly dominated by *Firmicutes*. *Clostridium* OTU C1 overrepresentation is accompanied by a decrease of *Erysipelotrichaceae* E1 and E2 and *Lactobacillus* L1 increase. Most OTUs are at very low frequencies (<0.04).

**Figure 2.** Shared and unique OTUs in cockatiel individuals. Most observed OTUs are  
450 unique for each cockatiel, however shared otus are present at a higher frequency and correspond to more than 80% of the sequences.

**Figure 3.** Cockatiel's microbiome summary. *Firmicutes* dominate the microbiome diversity with *Erysipelotrichaceae*, *Clostridium*, *Lactobacillus*, and Ca. *Arthromitus*. In a lesser scale the *Tenericutes* phylum with *Mycoplasmataceae* as the most abundant family. Other phyla



455 like *Spirochaetes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* are barely detectable  
in this study.

**Figure 4.** Relative frequency of bacteria in intestinal microbiome of granivores. The most  
common phyla in grain-eating birds are *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. In  
cockatiels only *Firmicutes* are dominant. *Tenericutes* are common in cockatiels and  
460 chickens, while *Fusobacteria* are in greater proportion in emu and duck.

**Figure 5.** Non-metric Dimensional Scaling (NMDS) biplot analysis for the bird  
microbiomes. A total of 203 taxa of the Family level were included to this analysis. Swine  
microbiomes are used for outgroup purposes only, and they are clustered apart by their  
*Bacteroidetes*. Interestingly the ordination has most of *Proteobacteria* on the left, and  
465 *Firmicutes* on the right quadrants. There are clusters of poultry related species like is the  
case for turkey, chicken, and emu. Kakapo clusters apart from every other species on the  
left quadrants and they are clearly being clustered by its *Proteobacteria* abundances. The  
wild duck clusters apart due to its particular microbiome configuration with *Actinobacteria*,  
*Fusobacteria*, and *Proteobacteria*. Finally, cockatiels are in the bottom right quadrant being  
470 clustered apart by their *Firmicutes*, *Tenericutes*, *Spirochaetes*, and some *Proteobacteria*  
families.

**Table 1** (on next page)

Table 1. Cockatiel's microbiome basic statistics.

Number of reads, OTUs and diversity indexes.

1  
2  
3  
4  
5**Table 1.**

	<b>Cockatiel 1</b>	<b>Cockatiel 2</b>	<b>Cockatiel 3</b>	<b>Total</b>
Total sequences (paired end)	90,520	97,161	107,536	295,217
Observed OTUs	6,957	7,566	7,154	19,280
Assigned phylotypes (Greengenes DB)	6,932	7,537	7,129	19,206
Assigned phylotypes (Closed OTUs DB)	109	206	115	309
<b>OTUs Diversity</b>				
Chao1	32,790±1,260	36,659±1,364	42,278±1,822	
Shannon	3.2	3.47	3.27	
Simpson	0.779	0.841	0.845	

6  
7  
8  
9

**Table 2** (on next page)

Table 2. Cockatiel's microbiome assigned phylotypes and OTUs abundances.

\*A total of 295,217 sequencing reads were clustered into 19,280 non-redundant OTUs using a 97% identity threshold. The Frequency column was calculated using the total amount of sequencing reads assigned to a phylotype or an OTU for each described level.

1  
2  
3  
4  
5  
6**Table 2.**

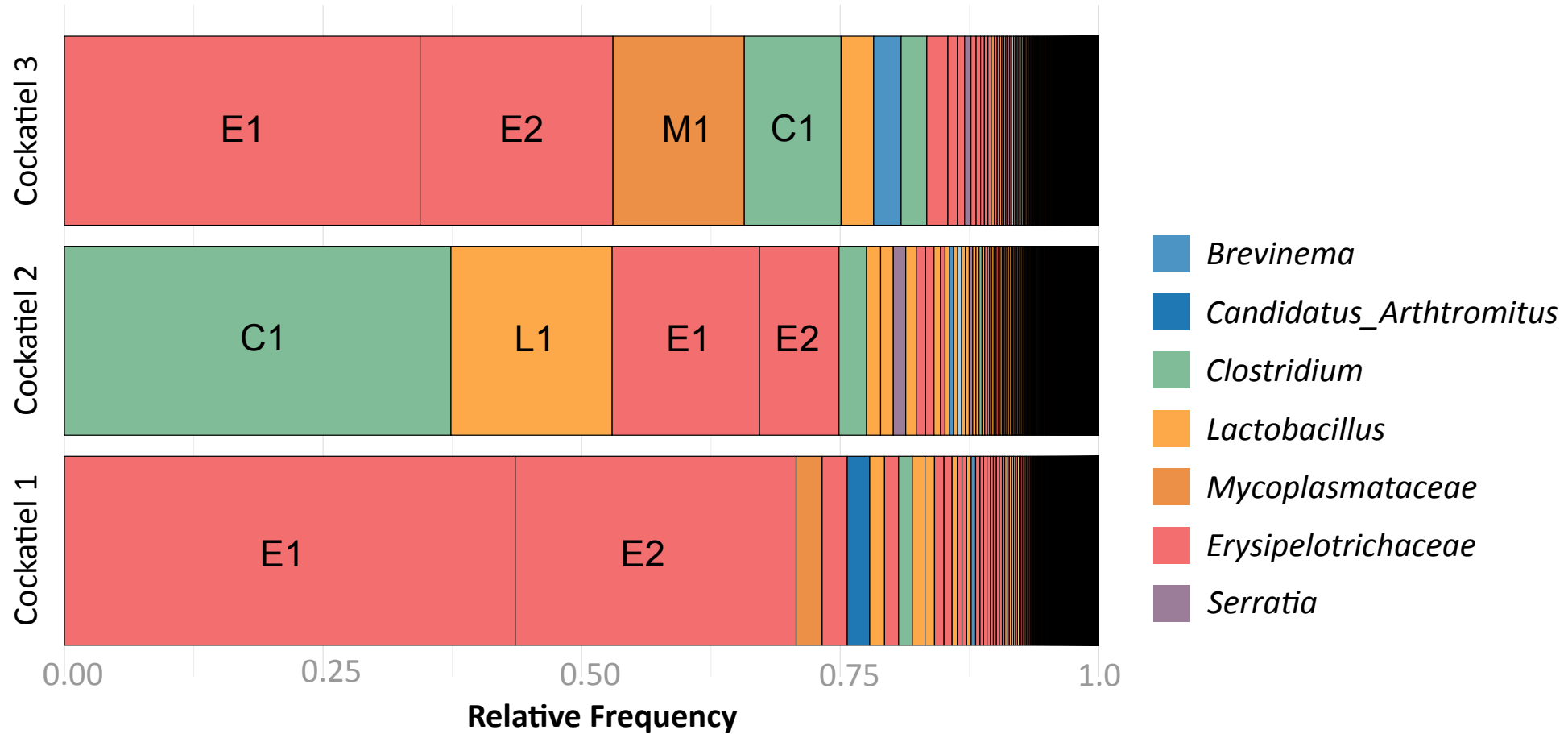
Phylum	Class	Order	Family	Genus	OTUs Number	Frequency* (%)
Firmicutes					16,287	90.5
	<i>Erysipelotrichi</i>	<i>Erysipelotrichales</i>	<i>Erysipelotrichaceae</i>		8,072	57.4
	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Lachnospiraceae</i>	<i>Clostridium</i>	759	17.1
	<i>Bacilli</i>	<i>Lactobacillales</i>	<i>Lactobacillaceae</i>	<i>Lactobacillus</i>	6,919	15.0
	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Ca. Arthromitus</i>	73	0.67
Tenericutes					1,300	6.3
	<i>Mollicutes</i>	<i>Mycoplasmatales</i>	<i>Mycoplasmataceae</i>		1,295	6.3
Spirochaetes					754	1.5
	<i>Brevinematae</i>	<i>Brevinematales</i>	<i>Brevinemataceae</i>	<i>Brevinema</i>	753	1.5
Proteobacteria					732	1.4
	$\gamma$ -Proteobacteria	<i>Enterobacteriales</i>	<i>Enterobacteriaceae</i>	<i>Serratia</i>	359	1.0
Cyanobacteria					41	0.191
Bacteroidetes					60	0.036
Actinobacteria					22	0.024
Other					83	0.051
<b>Total</b>					<b>19279</b>	<b>100%</b>

7  
8

**Figure 1** (on next page)

Figure 1. Frequency of OTUs for each cockatiel.

The three cockatiels are clearly dominated by *Firmicutes*. *Clostridium* OTU C1 overrepresentation is accompanied by a decrease of *Erysipelotrichaceae* E1 and E2 and *Lactobacillus* L1 increase. Most OTUs are at very low frequencies (<0.04).



**Figure 2** (on next page)

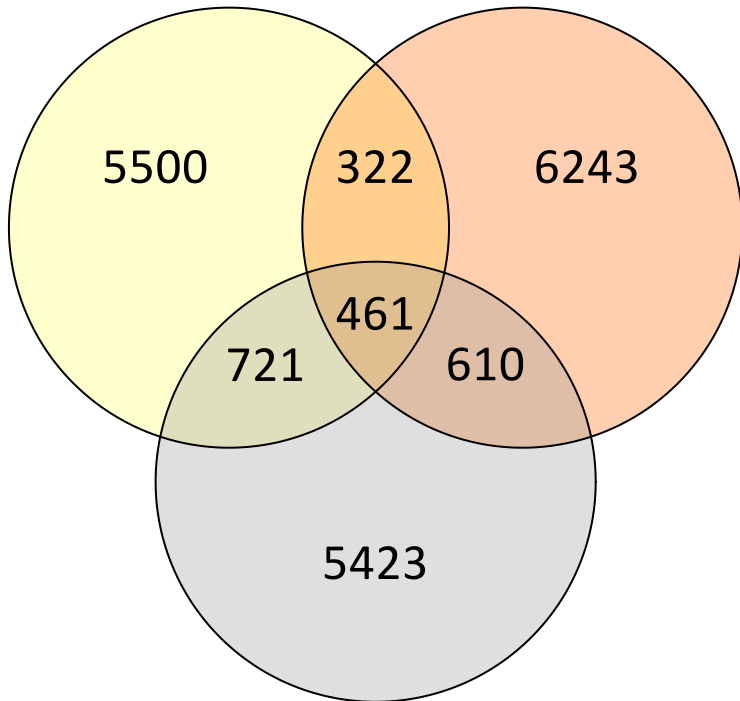
Figure 2. Shared and unique OTUs in cockatiel individuals.

Most observed OTUs are unique for each cockatiel, however shared otus are present at a higher frequency and correspond to more than 80% of the sequences.



cockatiel 1

cockatiel 2

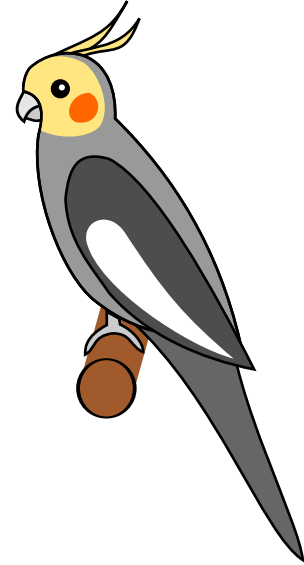
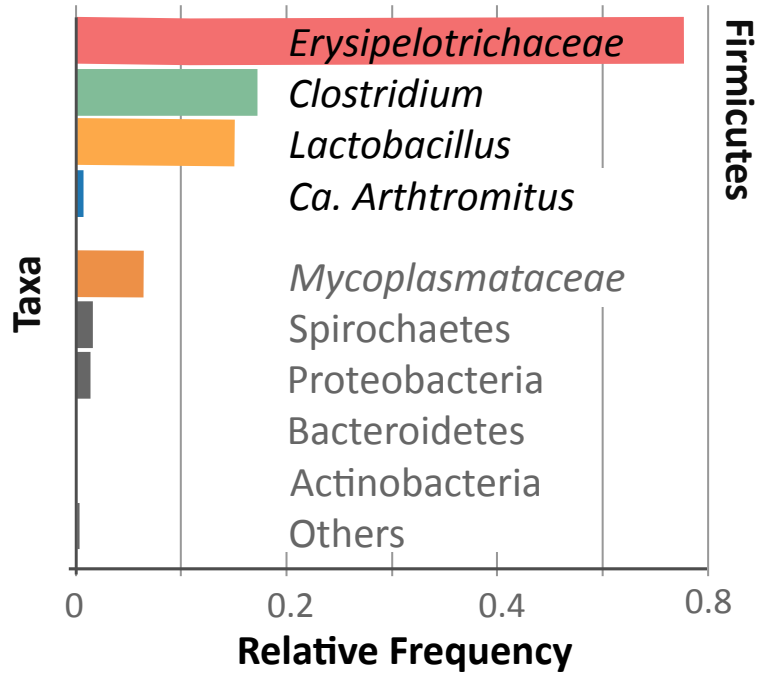


cockatiel 3

**Figure 3**(on next page)

Figure 3. Cockatiel's microbiome summary.

*Firmicutes* dominate the microbiome diversity with *Erysipelotrichaceae*, *Clostridium*, *Lactobacillus*, and *Ca. Arthromitus*. In a lesser scale the *Tenericutes* phylum with *Mycoplasmataceae* as the most abundant family. Other phyla like *Spirochaetes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* are barely detectable in this study.

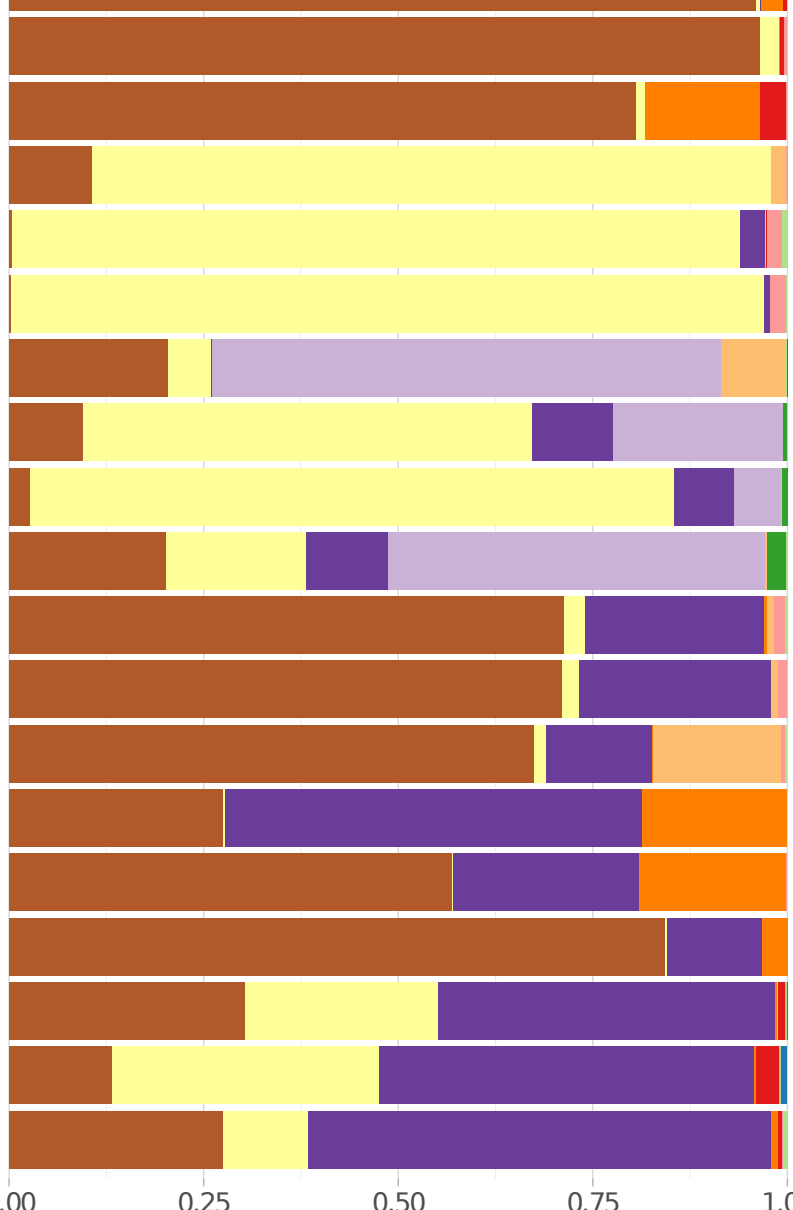


**Figure 4**(on next page)

Figure 4. Relative frequency of bacteria in intestinal microbiome of granivores.

The most common phyla in grain-eating birds are *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. In cockatiels only *Firmicutes* are dominant. *Tenericutes* are common in cockatiels and chickens, while *Fusobacteria* are in greater proportion in emu and duck.

Cockatiel 1  
 Cockatiel 2  
 Cockatiel 3  
 Kakapo 1  
 Kakapo 2  
 Kakapo 3  
 Duck  
 Emu 1  
 Emu 2  
 Emu 3  
 Turkey 1  
 Turkey 2  
 Turkey 3  
 Chicken 1  
 Chicken 2  
 Chicken 3  
 Swine 1  
 Swine 2  
 Swine 3



**Taxa**

- Firmicutes
- Proteobacteria
- Bacteroidetes
- Fusobacteria
- Tenericutes
- Actinobacteria
- Spirochaetes
- Cyanobacteria
- Synergistetes
- Fibrobacteres
- Others

0.00 0.25 0.50 0.75 1.00

**Figure 5**(on next page)

Figure 5. Non-metric Dimensional Scaling (NMDS) biplot analysis for the bird microbiome.

A total of 203 taxa of the Family level were included to this analysis. Swine microbiomes are used for outgroup purposes only, and they are clustered apart by their *Bacteroidetes*. Interestingly the ordination has most of *Proteobacteria* on the left, and *Firmicutes* on the right quadrants. There are clusters of poultry related species like is the case for turkey, chicken, and emu. Kakapo clusters apart from every other species on the left quadrants and they are clearly being clustered by its *Proteobacteria* abundances. The wild duck clusters apart due to its particular microbiome configuration with *Actinobacteria*, *Fusobacteria*, and *Proteobacteria*. Finally, cockatiels are in the bottom right quadrant being clustered apart by their *Firmicutes*, *Tenericutes*, *Spirochaetes*, and some *Proteobacteria* families.

