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Characterization and comparison of gut microbiomes in nine species of parrots in captivity

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Abstract
Gut microbiomes have profound effects on the health of hosts. About 28% of extant parrot species are threatened with extinction. To inform conservation efforts, we characterized and compared the gut microbiomes in nine species of parrots in captivity. The core gut microbiome of parrots was dominated by three bacterial phyla: the Firmicutes, Proteobacteria, and Actinobacteria. This core gut microbiome is similar to that in other herbivorous birds. There were no statistical differences in the diversity of gut microbiomes among parrot species, but significant variations in richness were detected. Individuals of the same species had more similar gut microbial composition to each other than to other species, indicating the potential role of host ancestry on shaping gut microbiomes. However, gut microbiomes did not cluster according to host species. Furthermore, *Lactobacillus* might influence the gut microbial composition of parrots. These results can contribute to improving our understanding of the basic characteristics of parrot gut microbiomes and help with their conservation.

Keywords Captivity · Ex-situ conservation · Gut microbiome · Host phylogeny · *Lactobacillus* · Parrot species

1 Introduction

Bird diversity is an important component of biodiversity, which plays an important role in ecological balance (Sekercioglu 2006). Bird populations have been declining at the global scale due to human induced pressure (Simberloff 2001; Xu et al. 2016). According to BirdLife data in the 2018 IUCN red list, 13% of the known bird species are threatened (IUCN 2018). Many species of the order Psittaciformes (parrots) are threatened with extinction (Berg and Bennett 2010; Olah et al. 2016; Heinsohn et al. 2018).

Parrots are divided into three families (Psittacidae, Cacatuidae, Strigopidae), and are widely distributed in the tropical and subtropical habitats of 124 countries between latitudes 35 degrees north and 56 degrees south (Olah et al. 2016). Habitat loss and fragmentation driven by agriculture and urbanization have reduced the population sizes of parrots (Olah et al. 2016; Heinsohn et al. 2018). In addition, parrots are popular as pets due to their colorful plumage and capacity to mimic human speech (Berg and Bennett 2010; Bradbury and Balsby 2016), and they are the most common avians in the wildlife trade (Bush et al. 2014). With the confluence of these negative factors, about 28% of extant parrot species are critically endangered, endangered, or vulnerable (IUCN 2018). Fortunately, many parrot species have been placed in zoos due to their ornamental value and survival predicament (Rodríguez-López 2016). Understanding the common physiological condition of different parrot species in captivity is particularly important for their conservation.

Gut microbiomes are extremely important to the health of hosts (Andoh 2016; Wang et al. 2016). Gut microbiomes are involved in many key physiological and biochemical functions of hosts, such as nutrition metabolism, vitamin synthesis, and the maturation of the gut immune system (Andoh 2016; Wang et al. 2016). Gut microbial communities consist of stable and transient inhabitants that can assemble through purely stochastic processes associated with the environment or by interactions with hosts. If host-microbiome interactions impact assembly patterns, there can be concordance between host evolutionary histories and the ecological similarities of microbial community structures (Brooks et al. 2017; Kohl et al. 2018). Similarities in gut microbiomes among species

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are usually associated with host phylogenies (Sanders et al. 2014; Brooks et al. 2017; Kohl et al. 2018). Besides host phylogenies, several factors such as diet and health condition play important roles in shaping gut microbiomes (Rothschild et al. 2018). The gut microbiome of individuals in the same species and even in the same individual may vary dramatically with diet and health condition (Nelson et al. 2013; Waite et al. 2014; Kers et al. 2018).

The basic characteristics and dynamic change in the gut microbiomes of threatened birds have drawn increasing attention (Waite et al. 2012; Waite et al. 2014; Yang et al. 2016; Zhao et al. 2017). However, to our knowledge, there are few studies investigating the gut microbiomes of parrots and their dynamic changes, except the kakapo (*Strigops habroptila*) (Waite et al. 2012; Waite et al. 2014). The premise of understanding the potentially important roles of gut microbiomes in parrot physiological and biochemical functions is that the information on gut microbial communities of the parrots is collected and recorded systematically (Waite et al. 2012).

In this study, we used 16S rRNA high-throughput sequencing to investigate the microbial communities of nine species of parrots (seven species of Psittacidae and two species of Cacatuinae) in captivity. Then, we characterized core and unique gut microbiomes by comparing the different parrot species. This study contributes to our understanding of the basic characteristics of the gut microbiomes in parrots in captivity, and thereby promotes ex situ conservation of threatened parrot species.

2 Materials and methods

2.1 Subjects and sampling

One fecal sample was collected from 37 individuals from nine species of parrots. The parrot species belonged to eight genera in two families, including *Psittacus erithacus* ($n = 4$), *Ara ararauna* (n = 4), *Amazona aestiva* ($n = 5$), *Myiopsitta monachus* (n = 5), *Psittacula eupatria* ($n = 3$), *Psittacula derbiana* (n = 5), *Eclectus roratus* (n = 5), *Cacatua ducorpsii* (n = 3) and *Probosciger aterrimus* (n = 3). Conspecifics were raised together in cages in the Nanjing Hongshan Forest Zoo. The cages were arranged side-by-side. Little is known about sex, age, and relatedness of these parrots, but they were all healthy adults based on descriptions from the zookeeper and veterinarian. They were fed the same diet daily, which consisted of abundant commercial feed (Mazuri, USA) (which contained *Lactobacillus*), fruits, and vegetables. During sampling, the parrots ate in their regular cages, were then moved to individual cages, and samples were collected one to two hours after feeding. To ensure accuracy, cages were cleaned prior to sampling. The liquid components of the fecal samples were abandoned, and the solid components, specifically the interiors, were collected to prevent contamination by environmental microorganisms. All 37 fecal samples were collected immediately after excretion and stored in liquid nitrogen for laboratory experiments.

2.2 DNA extraction, PCR amplification, and sequencing

Total DNA from fecal samples was extracted using the hexadecyltrimethylammonium bromide method. DNA templates were dissolved in 40 μL water. The concentrations and purities of the DNA extractions were measured using a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis. Then, DNA templates with concentrations between 29.60 to 47.20 ng/μL were used for PCR. The V3-V4 region of the 16S rRNA gene was amplified using the primers 341F (5'-CCTAYGGGRBGCASCAG-3′) and 806R (5'-GGACTACNNGGGTATCTAAT-3′). To ensure accuracy of the amplicons, all PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA). Reaction conditions were 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s. PCR products were mixed in equidensity ratios. Mixed PCR products ranging from 400 to 450 bp were recovered from the 2% agarose gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA). The libraries used for sequencing were generated using an Ion Plus Fragment Library Kit 48 rxns (Thermo Fisher Scientific, USA). The quality of the libraries was determined using a Qubit® 2.0 Fluorometer. The high-quality libraries were sequenced on an Ion S5™ XL platform (Thermo Fisher Scientific, USA).

2.3 Data analyses

Single-end reads were assigned to samples based on unique barcodes using Cutadapt v1.9.1 (Martin 2011). Quality filtering of the reads was performed according to the Cutadapt v1.9.1 quality controlled process (Martin 2011). By comparing reads with the reference database, chimeric sequences were detected and removed using the UCHIME algorithm (Edgar et al. 2011; Haas et al. 2011). After deleting invalid sequences, clean sequences were clustered into operational taxonomic units (OTUs) at a 97% identity level using Uparse v7.0.1001 (Edgar 2013). A representative sequence for each OTU was screened for further annotation. Annotation of each representative sequence was performed in Mothur v1.35.1 based on the SILVA rRNA gene database (Schloss et al. 2009; Quast et al. 2013). OTU abundance information was normalized using a standard sequence number corresponding to the

Table 1 The main quality control parameters of the 16S rRNA gene high-throughput sequencing

Parameters	Minimum	Maximum	Average ± SD	Total
Raw reads	69,847	98,178	85,096 ± 4226	3,148,536
Clean reads	65,283	95,316	80,134 ± 4088	2,964,954
Q20 values	79.06	88.77	84.75 ± 2.64	/
Efficiencies	90.70	97.95	94.19 ± 2.08	/

SD indicates standard deviation. Diagonal indicates no count

sample with the fewest sequences. Subsequent analyses were performed based on the normalized data.

Alpha diversity was used to describe community richness and diversity for each sample through four indices (Chao1, ACE, Shannon, and Simpson). All the indices were calculated with QIIME v1.7.0 (Caporaso et al. 2010). Variations in these indices among the different parrot species were assessed using ANOVAs in IBM SPSS statistics 19 (Field 2005). Differences in microbial communities were measured using weighted UniFrac distances, which indicate beta diversity, and were calculated using QIIME v1.7.0 (Caporaso et al. 2010). The statistical significance of the differences was estimated by Anosim in R v2.15.3 (Beck et al. 2011). To visualize the differences, UPGMA trees based on weighted UniFrac distance were generated in QIIME v1.7.0 (Caporaso et al. 2010). Linear discriminant analysis effect size (LEfSe) was performed using the network tool developed by Segata et al. (2011) to find the key OTUs causing differences. The relationships between *Lactobacillus* and the gut microbial compositions of parrots were estimated using Pearson correlation analysis in SPSS statistics 19 (Field 2005).

3 Results

3.1 Sequencing and metadata

We obtained 3,148,536 reads from the hyper-variable V3-V4 region of the 16S rRNA gene from the 37 fecal samples (Table 1). A total of 2,964,954 clean reads were identified after deleting the chimeric reads (Table 1). The average efficiency of sequencing was 94.19 ± 2.08% (average ± standard deviation), ranging from 90.7% to 97.95% (Table 1). The values of Q20 for each sample ranged from 79.06% to 88.77%, and the average was 84.75 ± 2.64% (Table 1). All clean reads were clustered into 2909 OTUs with ≥97% sequence similarity. The number of OTUs in each sample ranged from 109 to 722, and the average was 353 ± 160.

3.2 Core gut microbiomes

There were 1441 species-specific OTUs and 105 OTUs common in the gut microbiomes of the nine species of parrots (Fig. 1). Although common OTUs accounted for only 3.61% of total OTUs, they were dominant in the gut microbiomes (Figs. 1 & 2). To be more specific, the Firmicutes (55.45 ± 33.85%), Proteobacteria (27.03 ± 25.35%), Actinobacteria (10.68 ± 19.48%), Bacteroidetes (4.58 ± 5.71%), and Cyanobacteria (1.17 ± 3.16%) were the dominant phyla and were detected in all fecal samples (Fig. 2a). At the genus level, *Lactobacillus* (31.05 ± 35.84%), *Ralstonia* (12.11 ± 18.49%), *Clostridium* sensu stricto *1* (8.76 ± 17.77%), *Candidatus Arthromitus* (5.08 ± 14.86%), *Acinetobacter* (3.82 ± 7.36%), *Kocuria* (3.75 ± 12.69%), *Escherichia-Shigella* (3.29 ± 3.98%), *Planococcus* (3.21 ± 13.65%), *Rhodococcus* (2.48 ± 5.97%), and *Staphylococcus* (2.45 ± 3.35%) were the

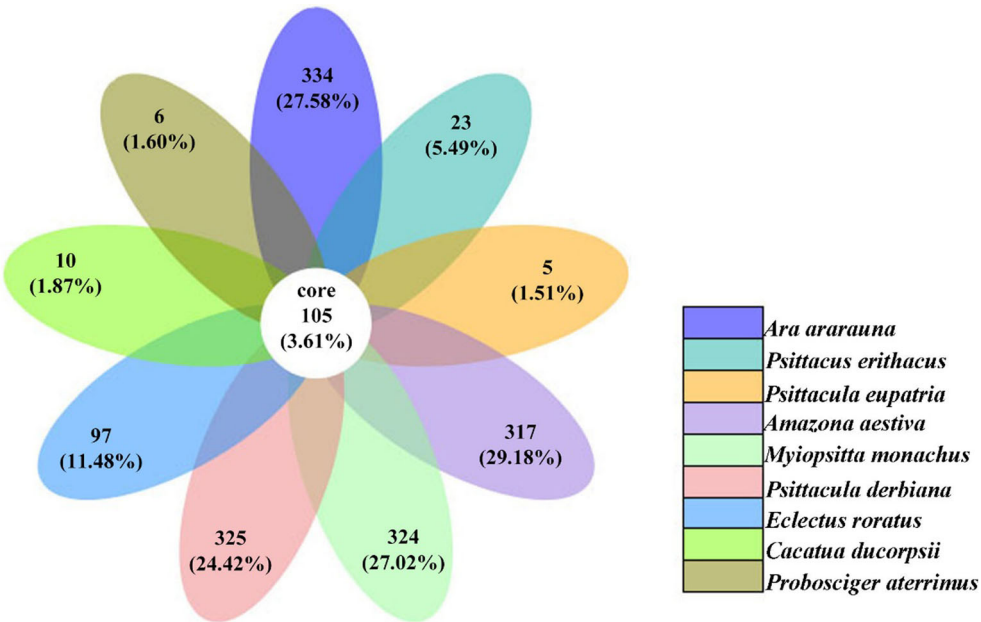


Fig. 1 Flower diagram showing the common OTUs in the gut microbiomes of the nine parrot species

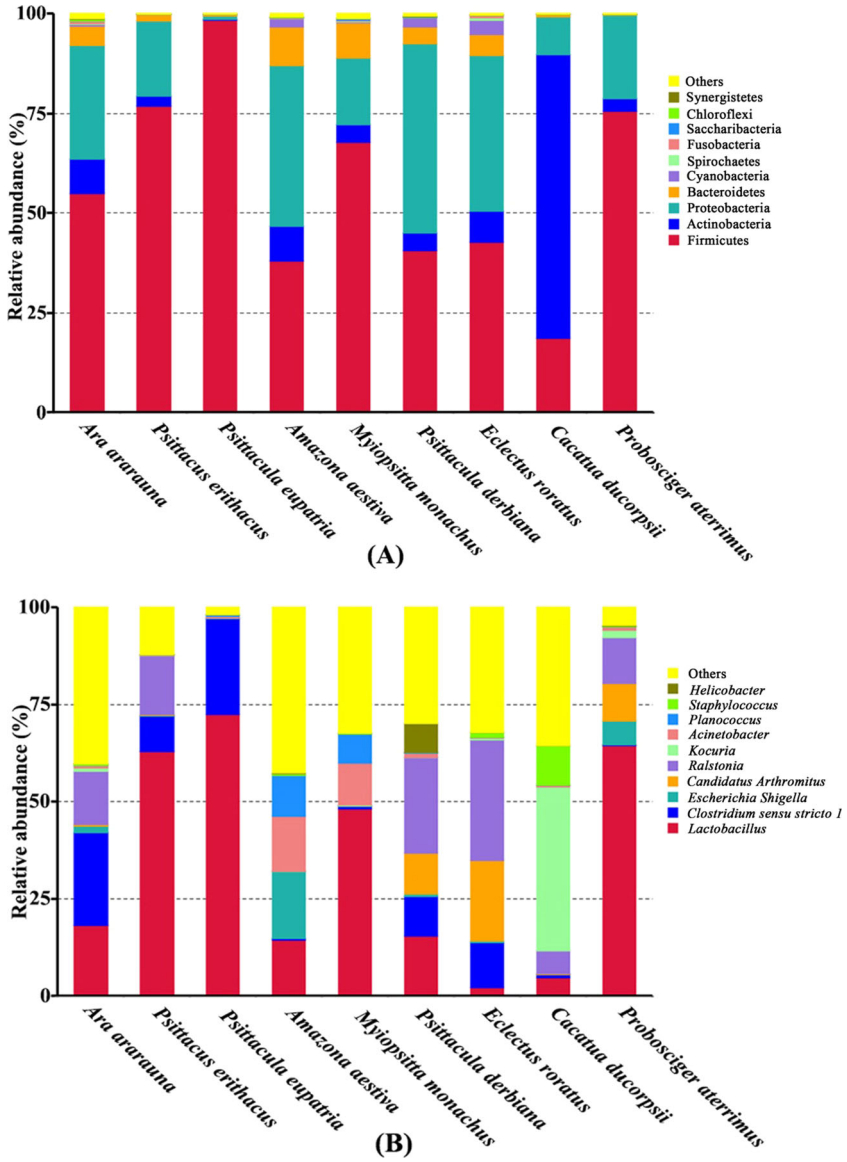


Fig. 2 Average relative abundance of OTUs at the phylum (**a**) and genus (**b**) level in the gut microbiomes of the nine parrot species

dominant components of the gut microbiomes in these parrots (Fig. 2b). Although there were dominant bacteria in the guts of these parrots, the relative abundance of dominant bacteria varied among parrot species (Fig. 2).

3.3 Differences in gut microbiomes among parrot species

The average Chao 1, Ace, Shannon, and Simpson values for all nine species of parrots were 386.281 ± 134.974, 389.982 ± 118.395, 3.630 ± 0.939, and 0.736 ± 0.113, respectively (Fig. 3). The richness of gut microbiomes differed sharply among parrot species. The Chao1 and Ace values for *P. erithacus*, *P. eupatria*, and *P. aterrimus* were significantly lower than those of the other six species (P<0.05, Fig. 3). While the Shannon and Simpson values varied with parrot species, these differences were not statistically significant

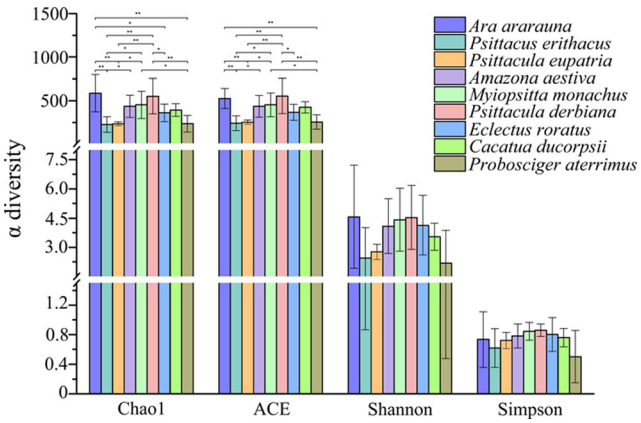


Fig. 3 Comparisons of the richness and diversity of gut microbiomes among nine parrot species. Significance differences are indicated as "**" ($P < 0.01$), "*" ($P < 0.05$)

Table 2 Differences in gut microbial compositions of nine parrot species

	A. ararauna	*P. erithacus*	*P. eupatria*	*A. aestiva*	*M. monachus*	*P. derbiana*	*E. roratus*	*C. ducorpsii*	*P. aterrimus*
A. ararauna		–	–	–	–	–	–	–	–
P. erithacus	0.125		–	–	–	–	–	–	–
P. eupatria	0.352	0.037		–	–	–	+	–	–
A. aestiva	0.100	0.275	0.518		–	–	+	+	–
M. monachus	0.269	0.075	0.159	0.080		+	+	+	–
P. derbiana	−0.138	0.175	0.415	0.152	0.364		–	–	–
E. roratus	0.000	0.306	0.569	0.268	0.588	−0.168		+	–
C. ducorpsii	0.519	0.333	1.000	0.446	0.713	0.456	0.559		–
P. aterrimus	0.037	−0.074	0.333	0.180	0.026	0.169	0.333	0.630	

R values are below the diagonal, the significance of their differences is above. When R > 0, the difference between species is greater. When R < 0, the difference within species is greater. Significance of difference is indicated as "+" ($P < 0.05$) and "−" ($P > 0.05$)

(P>0.05, Fig. 3). In addition, there was no evidence of similarity in the richness and diversity of the gut microbiomes of closely related parrots (Fig. 3).

Gut microbial compositions were similar within species, based on a global R value of 0.254 ($P = 0.04$), and almost all R values were greater than zero (Table 2). However, differences in gut microbial composition among species did not reflect host phylogenies, as the R values between some closely related parrots were greater than those between distantly related parrots (Table 2). For instance, the R value between *P. eupatria* and *P. derbiana* in the same genus was not minimum (Table 2). To visualize the differences, UPGMA trees based on weighted

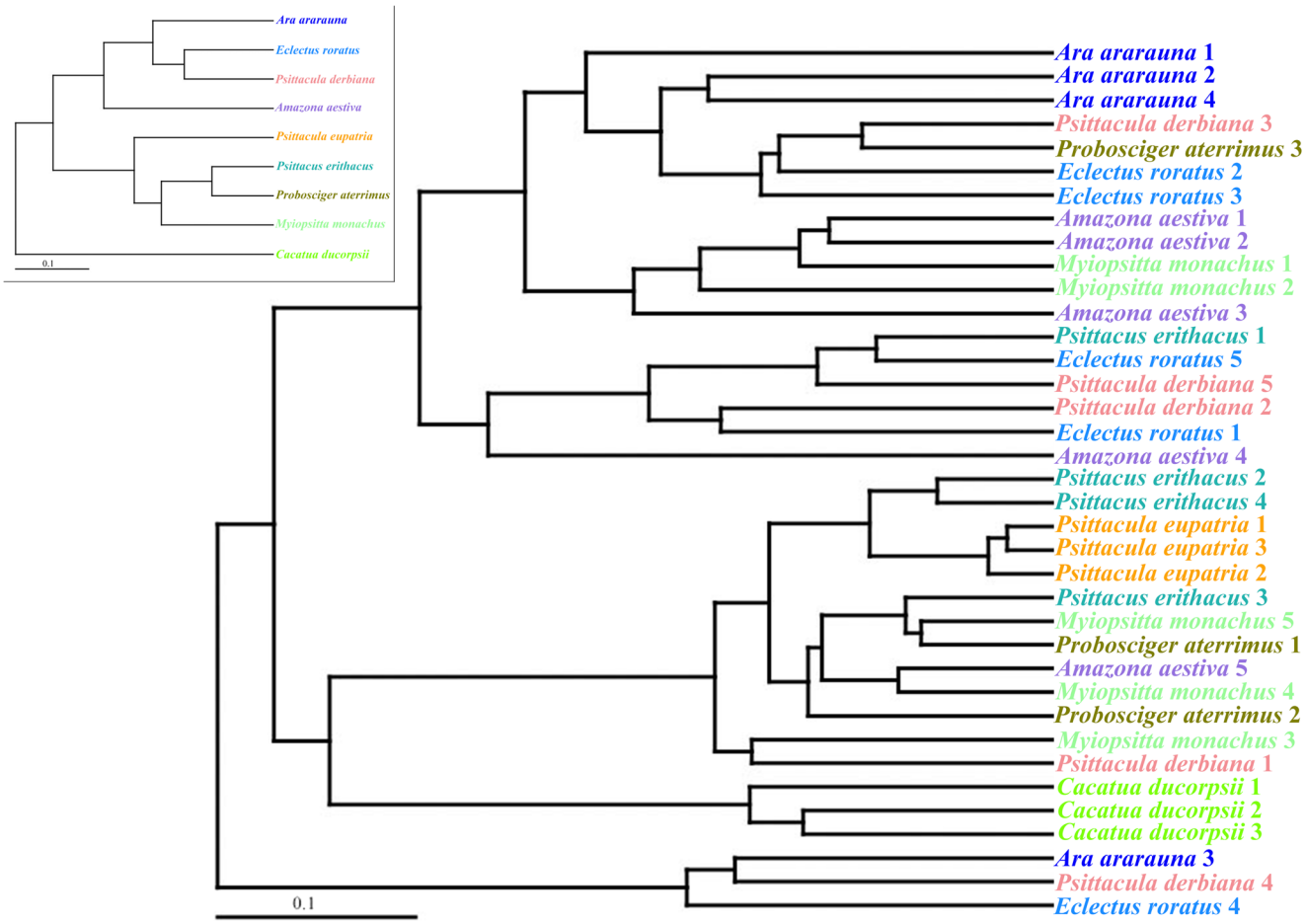
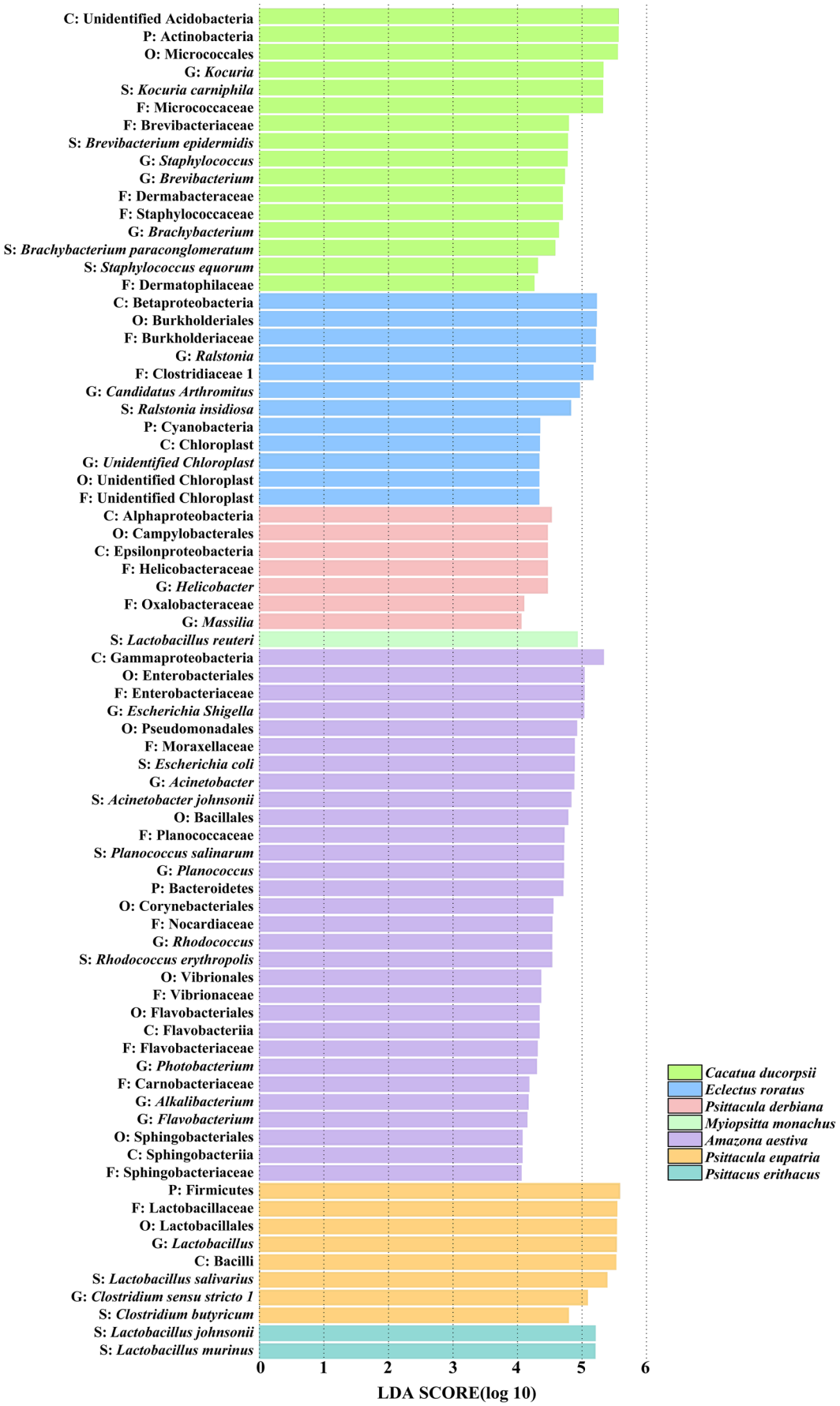


Fig. 4 UPGMA trees of gut microbiomes of the nine parrot species based on weighted UniFrac distance. The main and inset trees show the phylogenies at the individual and species levels, respectively


C: Unidentified Acidobacteria
P: Actinobacteria
O: Micrococcales
G: Kocuria
S: Kocuria carniphila
F: Micrococcaceae
F: Brevibacteriaceae
S: Brevibacterium epidermidis
G: Staphylococcus
G: Brevibacterium
F: Dermabacteraceae
F: Staphylococcaceae
G: Brachybacterium
S: Brachybacterium paraconglomeratum
S: Staphylococcus equorum
F: Dermatophilaceae
C: Betaproteobacteria
O: Burkholderiales
F: Burkholderiaceae
G: Ralstonia
F: Clostridiaceae 1
G: Candidatus Arthromitus
S: Ralstonia insidiosa
P: Cyanobacteria
C: Chloroplast
G: Unidentified Chloroplast
O: Unidentified Chloroplast
F: Unidentified Chloroplast
C: Alphaproteobacteria
O: Campylobacterales
C: Epsilonproteobacteria
F: Helicobacteraceae
G: Helicobacter
F: Oxalobacteraceae
G: Massilia
S: Lactobacillus reuteri
C: Gammaproteobacteria
O: Enterobacteriales
F: Enterobacteriaceae
G: Escherichia Shigella
O: Pseudomonadales
F: Moraxellaceae
S: Escherichia coli
G: Acinetobacter
S: Acinetobacter johnsonii
O: Bacillales
F: Planococcaceae
S: Planococcus salinarum
G: Planococcus
P: Bacteroidetes
O: Corynebacteriales
F: Nocardiaceae
G: Rhodococcus
S: Rhodococcus erythropolis
O: Vibrionales
F: Vibrionaceae
O: Flavobacteriales
C: Flavobacteriia
F: Flavobacteriaceae
G: Photobacterium
F: Carnobacteriaceae
G: Alkalibacterium
G: Flavobacterium
O: Sphingobacteriales
C: Sphingobacteriia
F: Sphingobacteriaceae
P: Firmicutes
F: Lactobacillaceae
O: Lactobacillales
G: Lactobacillus
C: Bacilli
S: Lactobacillus salivarius
G: Clostridium sensu stricto 1
S: Clostridium butyricum
S: Lactobacillus johnsonii
S: Lactobacillus murinus
0
1
2
3
4
5
6
LDA SCORE(log 10)
Cacatua ducorpsii
Eclectus roratus
Psittacula derbiana
Myiopsitta monachus
Amazona aestiva
Psittacula eupatria
Psittacus erithacus

◀ **Fig. 5** The key OTUs causing differences in gut microbial composition of the nine parrot species. These OTUs had the highest differences in abundance among the nine parrot species, but they were not unique to some parrots. Phylum, class, order, family, genus, and species are indicated by P, C, O, F, G, and S, respectively

UniFrac distance were constructed. Gut microbiomes did not cluster according to host species (Fig. 4). Except for *C. ducorpsii* and *P. eupatria*, the gut microbiomes of individual parrots mixed desultorily (Fig. 4). At the species level, the clustering of gut microbiomes was also inconsistent with host phylogenies. For example, the gut microbiomes of *P. eupatria* and *P. derbiana* were separated by the gut microbiome of *P. aterrimus*, which belong to Cacatuinae (Fig. 4).

LEfSe was performed to find the key OTUs causing the differences in gut microbial composition among the nine species of parrots. The relatively high abundance of 76 OTUs in specific hosts caused the observed differences (Fig. 5). These OTUs were mainly distributed in *A. aestiva*, *C. ducorpsii*, and *E. roratus*, while not one was found in *A. ararauna* and *P. aterrimus* (Fig. 5). The key OTUs at the genus level were *Kocuria*, *Staphylococcus*, *Brevibacterium*, *Brachybacterium*, *Ralstonia*, *Candidatus Arthromitus*, *Unidentified Chloroplast*, *Helicobacter*, *Massilia*, *Escherichia Shigella*, *Acinetobacter*, *Planococcus*, *Rhodococcus*, *Photobacterium*, *Alkalibacterium*, *Flavobacterium*, *Lactobacillus*, and *Clostridium* sensu stricto *1* (Fig. 5).

3.4 Effects of *Lactobacillus* on gut microbial composition of parrots

Considering the high abundance of *Lactobacillus* and its variability among parrot species, the effects of these bacteria on gut microbial composition were estimated. The α diversity values decreased sharply with increases in the relative abundance of *Lactobacillus*, indicating that the relative abundance of *Lactobacillus* was negatively correlated with the richness and diversity of gut microbiomes ($-0.619 \le r \le -0.489$, Fig. 6). The relative abundance of *Lactobacillus* was also inversely related with eight other bacterial genera, especially *Ralstonia* and *Rhodococcus* ($-0.6 < r < -0.2$, Fig. 7). Overall, *Lactobacillus* might alter the gut microbial composition of parrots.

4 Discussion

4.1 Common gut microbiomes shared by parrots and other herbivorous birds

We observed a core gut microbiome common to all nine species of parrots (Figs. 1 & 2). The core gut microbiome at the phylum level was mainly composed of the Firmicutes, Proteobacteria, and Actinobacteria (Fig. 2). These bacterial phyla are also dominant in the guts of kakapo and other herbivorous birds (Waite et al. 2012; Waite et al. 2014; Yang et al. 2016; Zhao et al. 2017). However, the dominant bacterial genera can vary among bird species (Waite et al. 2014; Yang et al. 2016; Zhao et al. 2017). Even in the same host species, there were often changes in the relative abundance of the common bacterial genera. There are obvious differences in the bacterial genera of the gut microbiome of juvenile and adult kakapo in different populations (Waite et al. 2014). Even so, *Clostridium* and *Lactobacillus* dominate the gut microbiomes of most herbivorous birds, as shown here and in previous studies (Waite et al. 2014; Yang et al. 2016; Zhao et al. 2017).

The compositions of gut microbiomes in herbivorous birds might be related to their diets. For instance, the high relative abundance of *Clostridium* may reflect the ability of this genus to effectively catabolize the high-fiber diets of herbivorous avian hosts (Zhao et al. 2017). In addition, the relative abundance of *Lactobacillus* in the nine species of parrots was much higher than previous studies, which might be related to the commercial feed used in this zoo, which contained *Lactobacillus*.

4.2 Gut microbiomes of parrots shaped by both host and environmental factors

Although the parrots were away from their natural habitats and living in a zoo, the gut microbial compositions individuals of the same species were more similar to each other than to those of other species (Global R = 0.254, $P = 0.04$, Table 2). The differences in gut microbiomes among parrot species might have been caused by 76 OTUs (Fig. 5). These OTUs might be related to differences in food preference and physiological activities. The parrots could choose food because abundant commercial feed, fruits, and vegetables were provided daily. Many *Chloroplast* were found in the fecal samples of

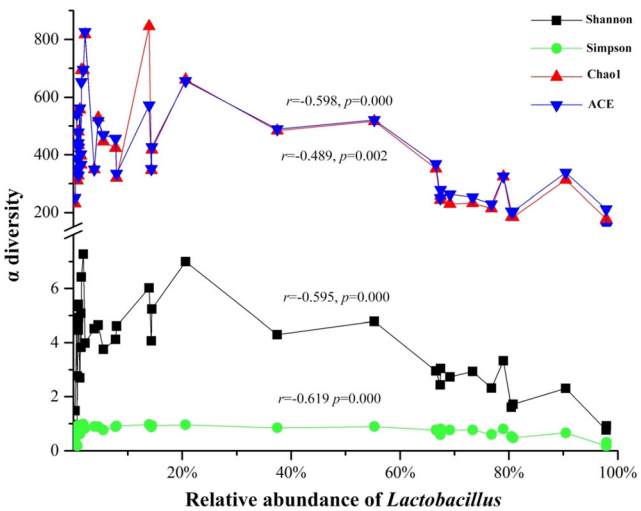


Fig. 6 Line chart showing the relationships between the relative abundance of *Lactobacillus* and α diversity

E. roratus, which might indicate these parrots preferred fruits and vegetables. However, plenty of *Chloroplast* also might be caused by poor digestive capacity. Abundant *Lactobacillus* were found in the fecal samples of *M. monachus*, *P. erithacus,* and *P. eupatria*, which might indicate that these parrots ate more commercial feed. Some bacteria might interact with hosts and might be transmitted from generation to generation (Uenishi et al. 2007; Brooks et al. 2017; Kohl et al. 2018).

In addition to stable bacteria, transient bacteria can inhabit host guts through stochastic processes associated with the environment (Brooks et al. 2017; Kohl et al. 2018). In our study, we observed three negative R values in Anosim (Table 2), which may be related to transient bacteria. The UPGMA trees of gut microbiomes at individual and species levels were not in accord with host phylogenies (Fig. 4), also suggesting the presence of transient bacteria. Similar diets and host genetics might lead to intricate relationships in the gut microbiomes of the nine species of parrots (Nelson et al. 2013). Taken together, gut microbiomes of parrots appear to have been shaped by both host and environmental factors.

4.3 Potential effects of *Lactobacillus* on the gut microbiomes of parrots

Lactobacillus might benefit hosts by protecting the hosts against potential invasions by pernicious bacteria and promoting the absorption of protein, monosaccharides, calcium and magnesium (Hemarajata and Versalovic 2013; Valeriano et al. 2017). *Lactobacillus* has become the most common probiotic in commercial feed (Bai et al. 2013; Phuoc and Jamikorn 2017). Adding *Lactobacillus* into basic animal diets has

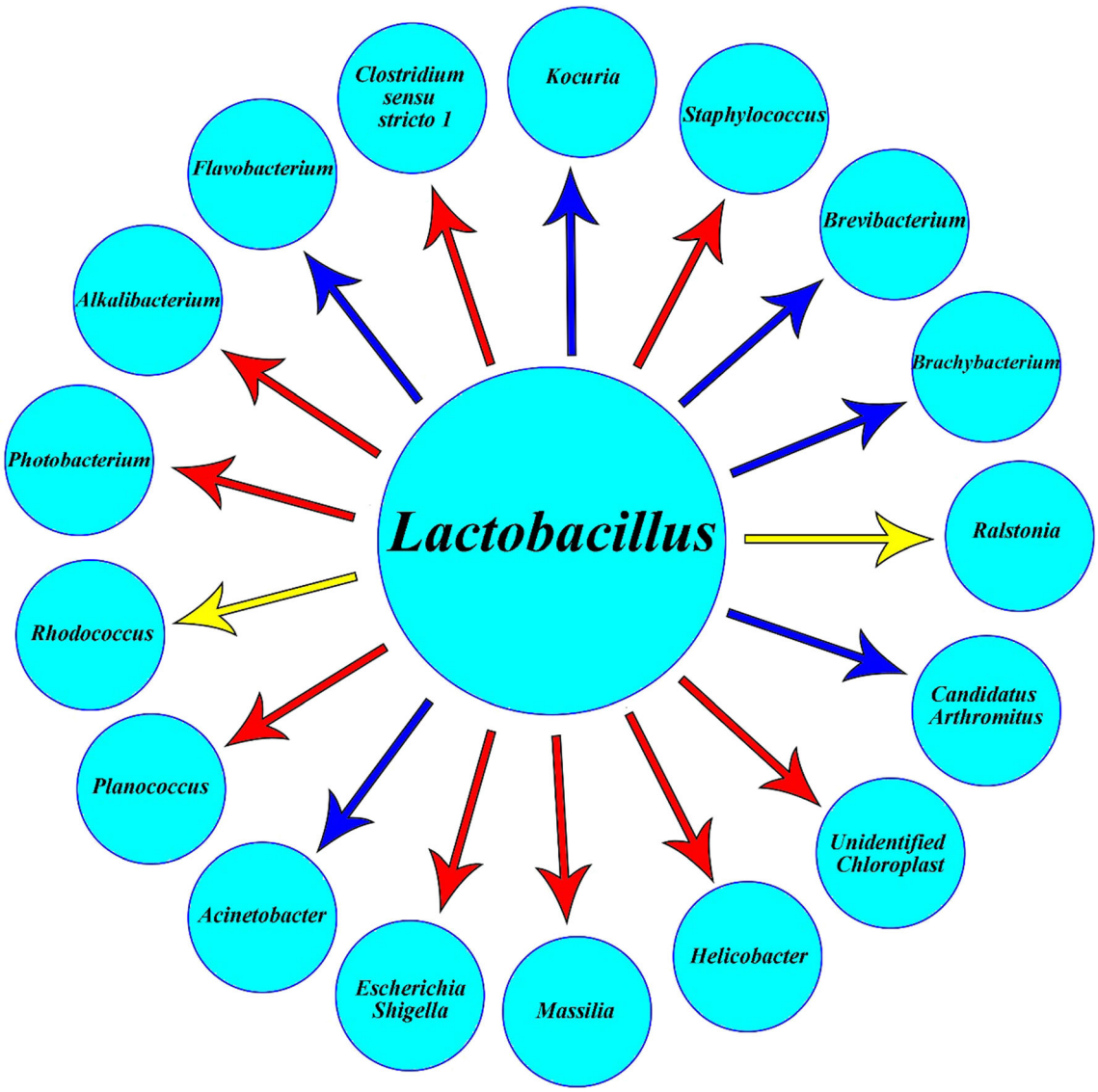


Fig. 7 The relationships between the relative abundance of *Lactobacillus* and other bacteria. Red, blue and yellow arrows indicate little to no correlations ($0<|r|<0.2$), weak negative correlations ($0.2<|r|<0.4$) and moderate negative correlations ($0.4<|r|<0.6$), respectively

become popular (De Angelis et al. 2006). A commercial feed with *Lactobacillus* was used in the zoo, but we did not see evidence that it could effectively withhold maleficent bacteria (such as *Staphylococcus*, *Helicobacter,* and *Escherichia Shigella*) in parrots (Figs. 6 & 7). Indeed, we found that *Lactobacillus* might significantly lessen the richness and diversity of gut microbiomes in parrots (Figs. 6 & 7); however, we did not characterize the effects on physiological activities of hosts. Therefore, more research is needed to clarify the influence of *Lactobacillus* on the gut microbiomes of parrots, which will offer guidance for adjusting parrot diets.

4.4 Limitations of this study

Parrots are divided into three families, and a total of around 400 species of parrots exist in the world (IUCN 2018). Subjects for this study only included nine species from two families. Moreover, all the parrots were kept in the same zoo. This study may not exactly reflect the gut microbiomes of all parrots in natural habitats. Many host factors (such as gender, actual age, and health condition) and environmental factors (such as diet composition and microbiology in the environment) can influence the gut microbiomes of parrots. In a study on the influence of bird age and hand rearing on the fecal microbiome of the kakapo, the microbial community structure of juvenile and adult kakapos changed over time, and the abundance of key OTUs was related to antibiotic treatment and captivity (Waite et al. 2014).

Understanding the differences in gut microbiomes among parrots living in different conditions would contribute to finding the key bacteria that affect parrots' physiological activities. However, the dynamic changes in gut microbiomes were not studied here. In addition, the study of gut microbiomes is susceptible to environmental contamination, which can be mitigated using controls, but no controls were used in this study. Improved experimental protocols would lead to more impactful studies of gut microbiomes to better conserve parrots.

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