



Defecating during Morning versus Afternoon: The Gut Microbiota of Zoo Rhesus Macaques (*Macaca mulatta*)

Jun-Dong Tian^{1,2}, Wei-Jie Guo^{1,2}, Han Yan¹, Jie Zhang¹, Shu-Liao Tian³ and Ji-Qi Lu^{1,2,*}

¹School of Life Sciences, Zhengzhou University, Zhengzhou 450001, China

²Institute of Biodiversity and Ecology, Zhengzhou University, Zhengzhou 450001, China

³Zhengzhou Zoo, Zhengzhou 450002, China

ABSTRACT

Animal gastrointestinal tracts host rich and diverse microorganisms, and the microbial community could be affected by many factors including activity rhythm which responds to the timing of feces defecation. To test the effect of activity rhythm on fecal microbiota, we used non-invasive sampling method to collect the feces defecated in the morning (AM group) and afternoon (PM group) from a zoo rhesus macaque population. Then 16S rRNA sequencing technology was adopted to assess the microbial communities. The results showed i) the dominant phyla were Firmicutes, Bacteroidetes, Proteobacteria and Spirochaetaceae accounting for over 86 % of the richness of whole microbiota, ii) Ruminococcaceae, Prevotellaceae, Lactobacillaceae and Spirochaetaceae were together taken over 60% at family level, iii) *Prevotella* was the dominant genus, iv) the community richness at OTU and family levels and both community evenness and community diversity at OTU level of the AM group were significantly higher than that of the PM group, v) though no analyses could significantly differentiate the two groups, there were significant differences in specific taxonomic groups including genus *Lactobacillus*, families Lactobacillaceae and Rhodospirillaceae, and phylum Tenericutes, and vi) the ratio of Firmicutes and Bacteroidetes in AM group was lower than that in PM group. The finding suggested that the gut microbiota in zoo rhesus macaques could be synchronized with the activity rhythm and also could be non-exclusively affected by human disturbance.

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Authors' Contribution

TJD and LJQ conceived and designed the study, and interpreted the data. GWJ and TSL collected fecal sample. GWJ, YH and ZJ extracted DNA from the samples. GWJ and TJD performed bioinformatics analyses. TJD and GWJ drafted the manuscript. LJQ helped in preparation of manuscript.

Key words

Gut microbiota, *Macaca mulatta*, Zoo, Activity rhythm, Firmicutes and Bacteroidetes ratio.

INTRODUCTION

Animal gastrointestinal tracts host rich and diverse microorganisms, which play critical role in food digestion and nutrients absorption, energy metabolism, immune function and behavioral responses. While, the factors, including phylogeny, physiological status, food items, activity rhythm, and sex and age, could affect the microbial community (Ding *et al.*, 2017; Amato *et al.*, 2018; Yi *et al.*, 2018; Hale *et al.*, 2018). Studies have demonstrated that the microbial community fluctuating with physiological rhythm could take up to 15 % of all the community, accounting for 60 % in mass, but changes in host physiological rhythm could disturb the diel rhythm of the intestinal microbiota (Paschos *et al.*, 2017; Liang and Fitzgerald, 2017).

Activity rhythm is an adaptive trait to environment, and many non-human primates evolve a diurnal pattern. For instance, study has indicated that Japanese macaque

(*Macaca fuscata*) could increase foraging time but decrease resting time when food is limited; while, they spent more time on resting and less time on moving when food is rich (Agetsuma, 1995; Hanya, 2004). However, silvery woolly monkey (*Lagothrix lagotricha poeppigii*) would increase resting time when food is rare (Fiore and Rodman, 2001). There are 4 peaks of foraging behavior and 4 periods for resting during the day in *M. thibetana huangshanensis* (Wang *et al.*, 2008). Studies on both black snub-nosed monkey (*Rhinopithecus bieti*) and rhesus macaque (*M. mulatta*) have shown forage peak in the morning and the afternoon but a resting period during noon (Li *et al.*, 2015; Tang *et al.*, 2011). However, activity rhythm could shape or be shaped by not only environmental conditions but also internal factors such as gastrointestinal tracts.

Rhesus macaques not only are key laboratory animal but also play an important role in animal/wildlife eco-tourism. Study on captive rhesus macaques has demonstrated that the fecal microbiome highly correlated with that in colonic lumen and mucosa and the differences in microbial community related to functional adaptation (Yasuda *et al.*, 2015). Given the difference in the timing of feces producing and being defecated in the morning

* Corresponding author: lujq@zzu.edu.cn

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and afternoon, it is reasonable to argue that the defecating period affect the gastrointestinal microbiome in rhesus macaques. In the present study, we investigated the fecal microbiome of zoo rhesus macaques in order to test the above-mentioned predictions.

MATERIALS AND METHODS

Fecal collection, gDNA extraction and sequencing

On January 10th 2017, non-invasive sampling was adopted to collect fresh feces defecated in the morning (AM group) and during the afternoon (PM group) by rhesus macaques housed in Zhengzhou Zoo, Zhengzhou, Henan, China. These macaques are food-provisioned two times each day at 10:00 and 15:00, respectively. The dietary are principally composed of dried yellow corn, wheat seeds, and steamed bread made of bean pulp. In addition, other food items such as carrot, apple, cucumber, sweet pepper, other vegetables and fruits could be offered according to seasonality. Following feces defecation, part of the feces was harvested, as soon as possible (in general < 2 min), in sterilized 5 mL EP tubes and stored within dry ice box, and then these samples were transported to the lab in Zhengzhou University, Zhengzhou, Henan, China.

Total genomic DNA (gDNA) was extracted from fecal samples using the PowerFecal® DNA Isolation Kit (MO BIO laboratories, Inc.) following the manual provided by the manufacturer. The quantity and quality of the extracted total gDNA for each sample were assessed, and finally 10 samples for each group were available for further analyses.

Based on the extracted fecal gDNA samples, 16S ribosomal RNA (rRNA) gene sequence libraries were generated using the V3-V4 (341F: CCTAYGGGRBGCASCAG; 806R: GGACTACNNGGTATCTAAT) primer region. The reaction procedure comprised an initial denaturation step at 94°C for 2 min, followed by 44 cycles of 94°C for 20 sec, 56°C for 30 sec, and 68°C for 40 sec; a final elongation at 68°C for 5 min. The PCR products were assessed and all the 20 samples were available for further analyses.

Bioinformatics and statistical analyses

The generated gene library was sequenced on the Illumina HiSeq 2500 platform (Illumina, Inc.) at Mega Genomics Corporation (Beijing, China) with read type PE250. After quality filtering and the removal of barcode and primer sequences, the two reads had been jointed through overlap using QIIME via fastq-join method with > 10 bp overlap and < 20 % overlap mismatch rate (Caporaso *et al.*, 2010; Aronesty, 2011). Raw tags characterized with N-rich or low-quality bases were removed and then the clean tags were obtained. The reads were screened for chimeras with UCHIME (Edgar *et al.*,

2011), and chimeras were excluded accordingly to get the effective tags for further analysis. Then effective tags were clustered into operational taxonomic units (OTUs) at a 97 % similarity level using USEARCH (<http://www.drive5.com/usearch/>). OTUs were taxonomically annotated using the UCLUST algorithm (Edgar, 2010), and OTU identities were assigned using the Ribosomal Database Project Classifier (Cole *et al.*, 2014) with the Silva (Release128, <http://www.arb-silva.de>). The Majorbio I-Sanger Cloud Platform (<https://www.i-sanger.com/>; Shanghai Majorbio Bio-pharm Technology Co., Ltd) was used to help analyze the data.

The community composition of each sample was counted at the phylum, family, genus, and OTU levels. The alpha diversity including community richness, evenness and diversity at the four levels were estimated by observed richness (Sobs), Shannon index-based measure of evenness (Shannon-even) and the Shannon index (Shannon). For statistical analysis, non-parametric Wilcoxon test was used to evaluate the difference in above-mentioned parameters between two groups. Principal component analysis was employed to diagnose which component(s) could differentiate the two groups. ANOSIM and Adonis analyses were used to test the similarity between the two groups. Significant level was set as 0.05 with two-tailed.

RESULTS

Abundances and diversity of different taxonomic units

The taxonomic units analyzed in the following process included four levels, OTU, genus, family and phylum. The abundances of OTU level were 775 in total. At OTU level, there were 28 OTUs only found in fecal samples defecated in the morning but only 6 OTUs in fecal samples defecated in the afternoon; however, these OTUs were rare ones which presented either in AM group (mean $\pm$ SD: 2.2 $\pm$ 1.2 samples, range from 1 to 5) or PM group (mean: 1 sample) with a low proportion. For genus level, there were 162 in total, and 1 of them only found in the morning feces but with a low prevalence (1/10 % for OTU328 and 4/10 for OTU372). There were no obvious differences in abundances of family (57) and phylum (13) levels between feces defecated in the morning or afternoon.

The co-occurrence diagram directly displayed the community richness between groups (Fig. 1). At phylum level, the microbiota of the two groups was dominated by Firmicutes and Bacteroidetes, followed by Proteobacteria and Spirochaetaceae, and all these 4 phyla accounted for over 86 % of the whole microbial community (Fig. 1A). At family level, Ruminococcaceae, Prevotellaceae, Lactobacillaceae and Spirochaetaceae were together taken over 60 % (Fig. 1B). Moreover, *Prevotella* was the core genus (Fig. 1C).

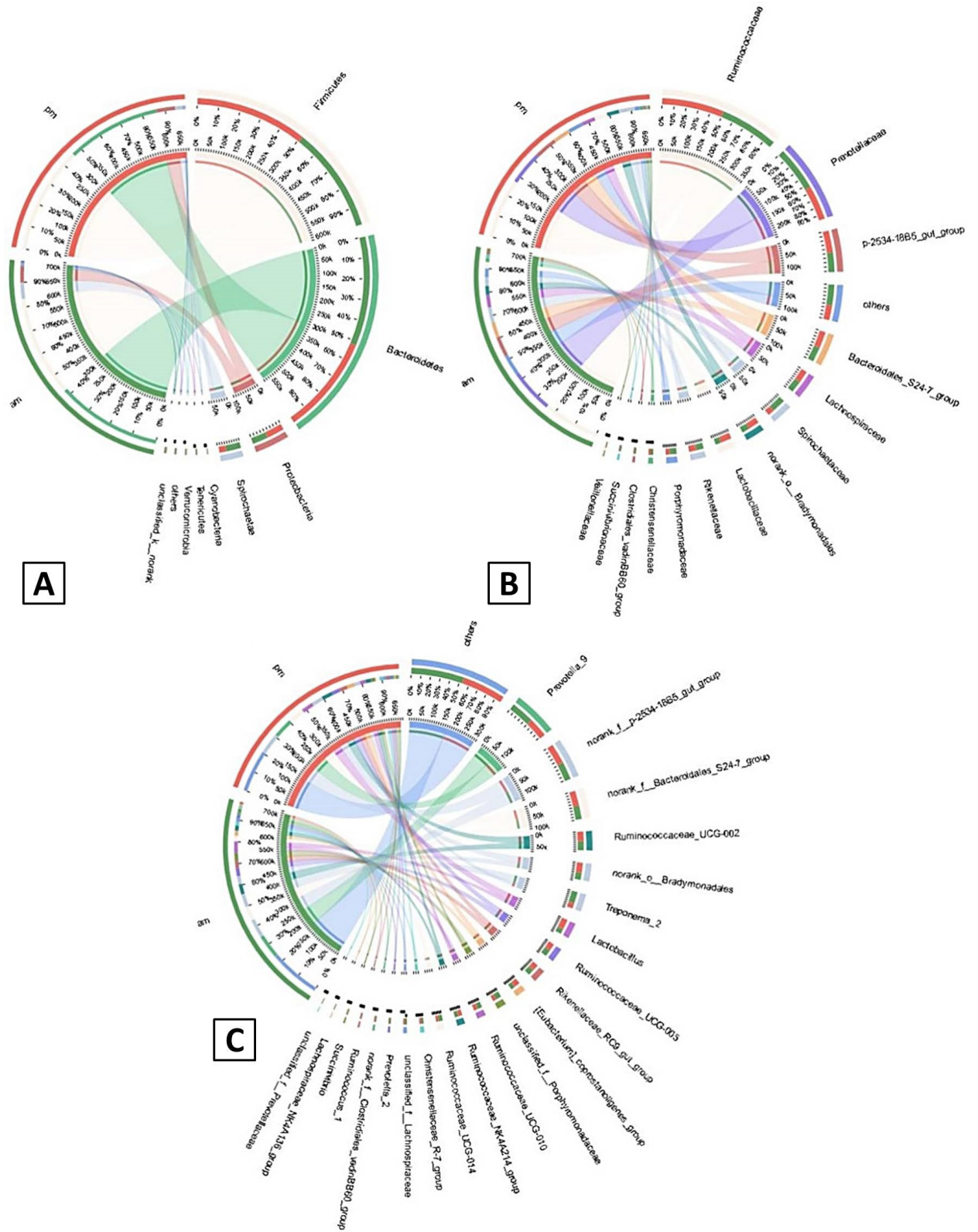


Fig. 1. Circos graphs at different taxonomic levels. A, Phylum; B, family; C, genus; AM, morning group; PM, afternoon group.

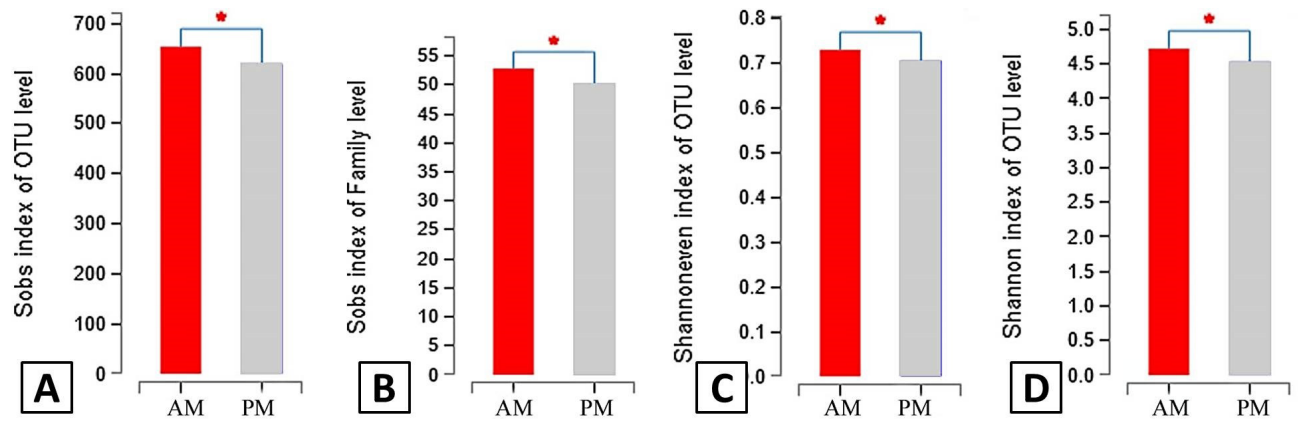


Fig. 2. The indices of the diversity of the microbial community at OTU and family levels. AM, morning group; PM, afternoon group.

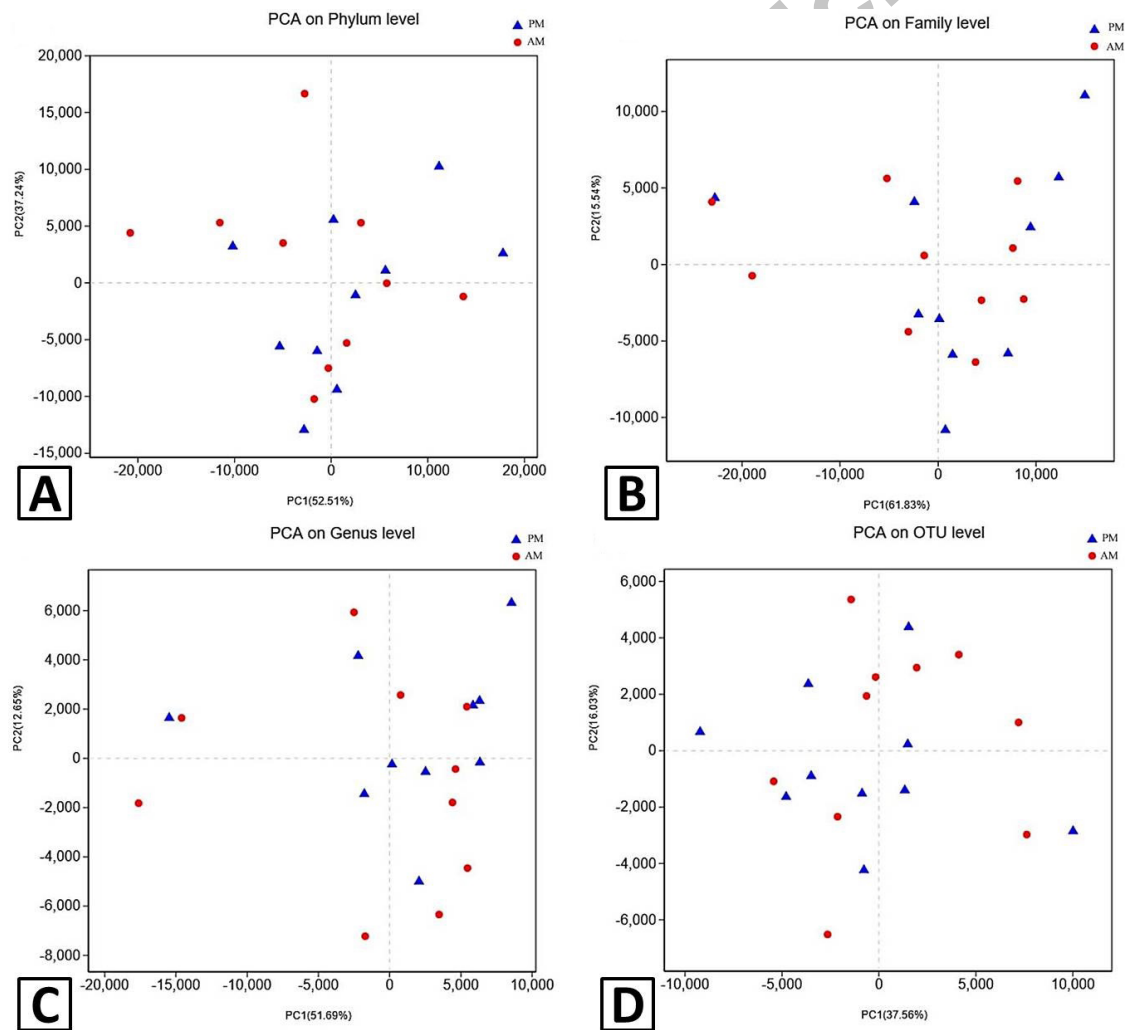


Fig. 3. Principal component analysis of the microbial community at phylum (A), family (B), genus (C) and OTU (D) levels. AM, morning group; PM, afternoon group.

Moreover, indices of the diversity of the microbial community at the 4 taxonomic levels were analyzed based on the two groups. The community richness sobs (Wilcoxon's test, P -value = 0.0449), community evenness shannoneven (Wilcoxon's test, P -value = 0.0312) and community diversity shannon (Wilcoxon's test, P -value = 0.0452) at OTU level of morning group were significantly higher than that of the afternoon group, and the community richness sobs (Wilcoxon's test, P -value = 0.0449) at family level of the morning group was significantly higher than that of the afternoon group (Wilcoxon's test, P -value = 0.018) (Fig. 2). However, there were no significant

differences for these indices at genus, family or phylum levels (Wilcoxon's test, all P -values > 0.05).

Characteristics of the microbiota between two groups

By the analyses of the indices estimating beta diversity, no principal component(s) could lead to any significant signal to differentiate the two groups (Fig. 3). These findings were also consistent with the analyses of the grouping that revealed by ANOSIM (ANOSIM, $R = -0.0164 < 1$, P -value = 0.581, Permutation number = 999) and Adonis analyses (Permutational MANOVA, $R^2 = 0.04639 < 1$, P -value = 0.563).

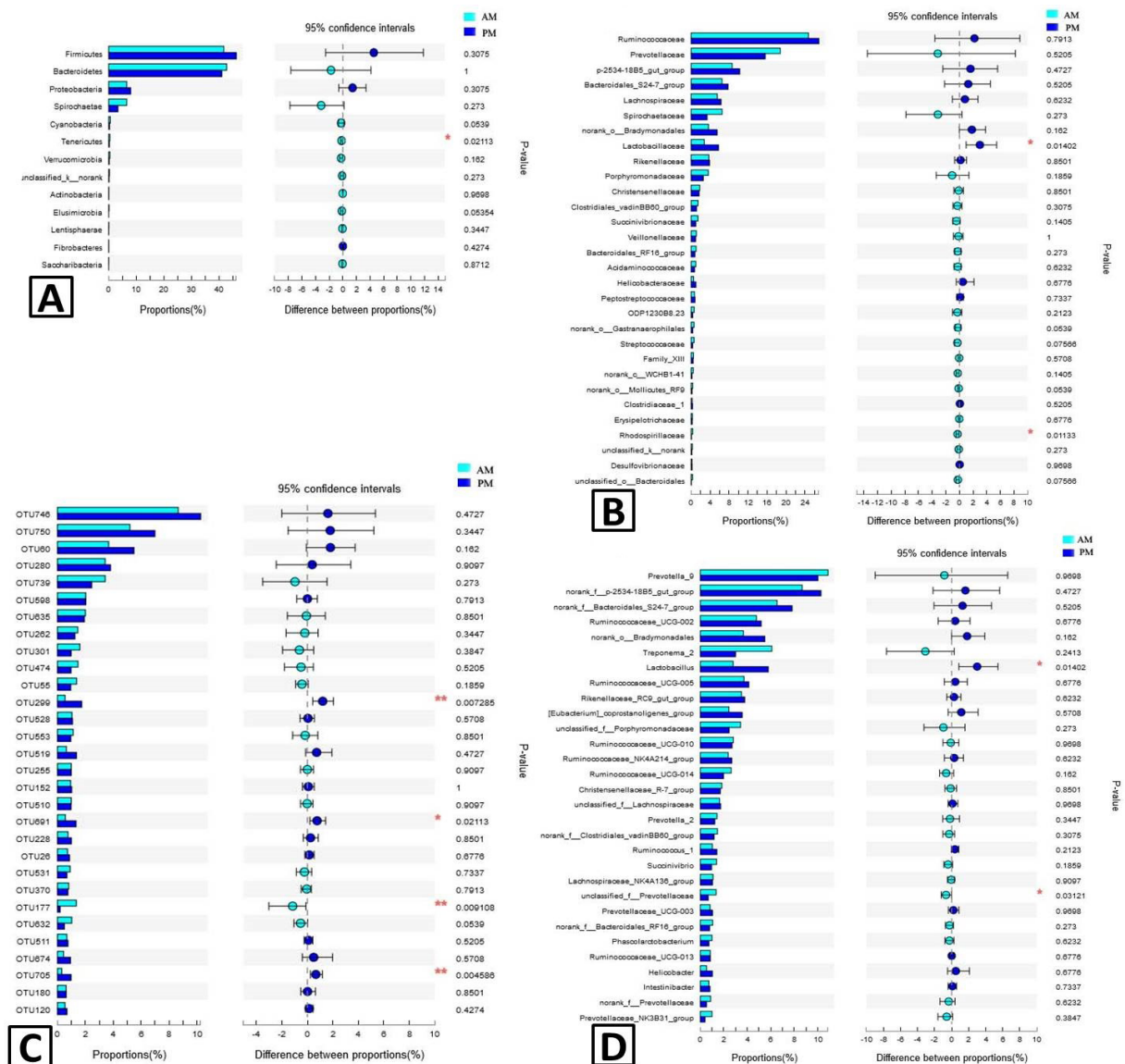


Fig. 4. Bar plot for comparison of microbial communities between two groups at phylum (A), family (B), genus (C) and OTU (D) levels. AM, morning group; PM, afternoon group.

However, differences in the community richness subs of some of the taxon including the OTU, genus, family and phylum levels could be observed, especially for the first thirty ones (all the taxon for the phylum level) according to their mean richness (Fig. 4). At OTU level, the richness of OTU299, 691 and 705 (all belong to genus *Lactobacillus*) in afternoon group were significantly higher than that of the morning group (Wilcoxon's test, all P -values < 0.0212); however, OTU177 (belong to family Spirochaetaceae and genus *Treponema_2*) was opposite. At genus level, *Lactobacillus* was significantly higher in afternoon group (Wilcoxon's test, P -value = 0.014), but *unclassified_f_Prevotellaceae* was significantly higher in morning group (Wilcoxon's test, P -value = 0.0312). At family level, Lactobacillaceae was significantly higher in afternoon group (Wilcoxon's test, P -value = 0.0140), but Rhodospirillaceae was significantly higher in morning group (Wilcoxon's test, P -value = 0.0113). At phylum level, the differences in the richness slightly varied for the first 4 phyla; however, the richness of Tenericutes was significantly higher than that of the afternoon group (Wilcoxon's test, P -value = 0.0211) (Fig. 4). Moreover, the ratio of Firmicutes and Bacteroidetes in morning group (0.9764, 41.80/42.81) was lower than that in afternoon group (1.1283, 46.35/41.08) (Fig. 4).

DISCUSSION

Gastrointestinal microbiome play critical role on food digestion and nutrition ingestion, and the microbiome functions rhythmically (Liang and Fitzgerald, 2017; Page, 2019). However, the finding of the current study showed a weak difference in microbial community of the fecal samples defecated in the morning and afternoon, especially at the OTU level.

Evolutionary constraints could shape the microbial communities in vertebrate animals, and therefore core gut microbiota could be characterized to limited species or vertebrate taxonomic groups (Amato *et al.*, 2018; Yi *et al.*, 2018; Hale *et al.*, 2018). In the current study, two groups shared amounts of microbial communities at genus, family and phylum levels (Fig. 1), which could be considered as the core microbiota. This finding was consistent with previous study conducted with the DGGE and q-PCR technique (Zhao *et al.*, 2013), and also consistent with study on laboratory rhesus macaques investigated via 16S sequencing technique (Yasuda *et al.*, 2015). These characteristics in dominant microbial communities were considered as to response to their dietary. For instance, phyla Firmicutes and Bacteroidetes in general could help the host digest and utilize plant resources (Ley *et al.*, 2008; Hale *et al.*, 2018). Previous study suggests that

Lachnospiraceae and Ruminococcaceae are beneficial to offer the host obtain complex plant resources (Biddle *et al.*, 2013), which is consistent with studies on some mammalian species (McLellan *et al.*, 2013; Bian *et al.*, 2013). In addition, the dominant genus of gut microbiota of these zoo rhesus macaques was *Prevotella*, therefore the enterotype could be assigned as Type II (Costea *et al.*, 2018). Genus *Prevotella* is characterized as being involved in mucin oligosaccharide degradation, which responses to plant-dominant food resources in rhesus macaques (Wright *et al.*, 2000; Flint *et al.*, 2008; Thierry, 2011). The daily diet for these zoo rhesus macaques are mainly dried yellow corn, wheat seeds and steamed bread made principally of bean pulp, and seasonal vegetables and fruits also would be offered. These food items are rich in oligosaccharide, which favor the gut microbiota such as *Prevotella*.

However, the gut microbiota also could show changes in composition based on differences in many scenarios such as geographical variation, seasonality, age and sex, social relationship, behaviors, diet, and physiological status (Sun *et al.*, 2016; Yi *et al.*, 2018; Amato *et al.*, 2018). In the present study, weak differences in microbial communities were detected, especially at OTU and genus levels (Fig. 4). These could be considered as the flexibility of rhesus macaque gut microbiota responding to the timing of feces producing, human disturbance, or the active/rest status. For instance, the difference in community richness of Tenericutes between two groups could be due to the timing of feces producing affected differentially by human disturbance and physiological status. In the zoo rhesus macaques, feces defecated in the morning would be produced mainly during the previous night with little human disturbance, while feces defecated in the afternoon could be produced mainly in the morning with strong human disturbance. This also could cause that the ratio of Firmicutes and Bacteroidetes in the morning group was lower than that in afternoon group. Study has indicated that decrease in the Firmicutes and Bacteroidetes ratio in obese human individuals is correlated with weight loss over time, and it increases with weight loss on low-calorie diet (Ley *et al.*, 2006). In addition, the community richness of dominant both Lactobacillaceae family and *Lactobacillus* genus in afternoon group was significantly higher than that of the morning group. The difference could be mainly resulted from the activity rhythm which could also affect the gut microbiota. Previous study on activity budget of these rhesus macaques has exhibited two active peaks at 10:00 and 16:00, respectively (Wang, 2014). Activity during daytime could force the gastrointestinal movement, therefore stimulates the functioning of these microbial groups (Thaiss *et al.*, 2014).

CONCLUSIONS

The present study investigated the effect of defecating timing on gut microbiota in zoo rhesus macaques. The results demonstrated that there were weak differences in gut microbial communities between feces defecated in the morning or afternoon, which could mainly respond to the activity rhythm of the rhesus macaques, and also could be non-exclusively induced by human disturbance.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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