# Sex Results in Divergence in Gut Bacterial Community between Female and Male Pardosa astrigera (Araneae: Lycosidae)

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**Abstract**

Sex is one of the important factors affecting gut microbiota. As key predators in agro-forestry ecosystem, many spider species show dramatically different activity habits and nutritional requirements between female and male. However, how sex affects gut microbiota of spiders is still unclear. Therefore, in this study, the compositions and diversities of gut bacteria, based on bacterial 16S rRNA gene sequencing, were compared between female and male Pardosa astrigera. We found that bacterial richness indices ($P < 0.05$) in female were significantly lower than male, meanwhile, β-diversity showed significantly different between female and male ($P < 0.05$). The relative abundance of Actinobacteriota and Rhodococcus (belongs to Actinobacteria) were significantly higher in female than male ($P < 0.05$). Whereas, the relative abundance of Firmicutes and Acinetobacter (belongs to Proteobacteria), Ruminococcus and Fusicatenibacter (all belong to Firmicutes), were significantly higher in male than female ($P < 0.05$). The results of PICRUSt2 showed that amino acid and lipid metabolisms were significantly higher in female than male ($P < 0.05$), whereas glycan biosynthesis and metabolism was significantly higher in male than female ($P < 0.05$). Our results imply that sexual variation is a crucial factor in shaping gut bacterial community in P. astrigera. Male P. astrigera dispersed more widely than the female hence the male had a higher bacterial diversity. While the distinct differences of bacterial composition mainly due to their different nutritional and energy requirements.

**Sex Results in Divergence in Gut Bacterial Community between Female and Male *Pardosa astrigera* (Araneae: Lycosidae)**

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Sex is one of the important factors affecting gut microbiota. As key predators in agro-forestry ecosystem, many spider species show dramatically different activity habits and nutritional requirements between female and male. However, how sex affects gut microbiota of spiders is still unclear. Therefore, in this study, the compositions and diversities of gut bacteria, based on bacterial 16S rRNA gene sequencing, were compared between female and male*Pardosa astrigera* . We found that bacterial richness indices ($P< 0.05$) in female were significantly lower than male, meanwhile, β-diversity showed significantly different between female and male ($P < 0.05$). The relative abundance of Actinobacteriota and *Rhodococcus* (belongs to Actinobacteria) were significantly higher in female than male ($P < 0.05$). Whereas, the relative abundance of Firmicutes and*Acinetobacter*(belongs to Proteobacteria),*Ruminococcus*and*Fusicatenibacter*(all belong to Firmicutes), were significantly higher in male than female ($P < 0.05$). The results of PICRUSt2 showed that amino acid

and lipid metabolisms were significantly higher in female than male ($P < 0.05$), whereas glycan biosynthesis and metabolism was significantly higher in male than female ($P< 0.05$). Our results imply that sexual variation is a crucial factor in shaping gut bacterial community in *P . astrigera* . Male *P . astrigera* dispersed more widely than the female hence the male had a higher bacterial diversity. While the distinct differences of bacterial composition mainly due to their different nutritional and energy requirements.

**KEYWORDS**

sex, gut microbiota, spider, Actinobacteriota, *Rhodococcus*

## 1 INTRODUCTION

Sex was found to be an important factor that influences gut microbiota (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012; Koren et al., 2012) that formed by long-term coevolution between host and microorganism (Matijasic et al., 2020). Female and male insects exhibit different ecological behaviors in terms of nutritional and dispersal capabilities (Minard, Mavingui & Moro, 2013; Rani, Sharma, Rajagopal, Adak & Bhatnagar, 2009), which lead to different gut microbiota community in host. For instance, Foster (1995) and Zouache et al. (2011) considered that male mosquitoes disperse less than female which could be a factor constraining bacterial diversity, Ng, Stat, Bunce, & Simmons (2018) proved that reduced exposure to diverse environmental microbiota could decrease in gut bacterial diversity of insect. Minard et al. (2013) found the different nutritional requirements between two sexes of mosquitos affect bacterial microbiota composition (e.g., higher relative abundance of Firmicutes and Firmicutes/Bacteroides (F/B) ratio, which contributed to nutritional efficiency (Wan et al., (2020). As a result, bacteria from genera*Bacillus* and *Staphylococcus* were detected in male mosquitos, whereas *Cryseobacterium* , *Pseudomonas* and*Serratia* were present exclusively in female mosquitos (Rani, Sharma, Rajagopal, Adak & Bhatnagar, 2009). Moreover, Wan et al. (2020) argued that higher gut bacterial diversity in female might contribute to the vertical transmission. Although many spider species show dramatically different activity habits between the two sexes, which results in different mobility and foraging opportunities (Aisenberg & Peretti, 2011), however, the gut microbiota of spiders influenced by sex was almost ignored.

Spiders are key predators in agro-forestry ecosystem (Nyffeler & Birkhofer, 2017). However, knowledge about the gut microbial community of spiders still limited. Hu et al. (2019) and Kumar et al. (2020) studied the diversity and composition of gut microbiota from a few spider species. Kennedy et al. (2020) suggested that gut microbiota in *Badumna longinqua* (Desidae) is dictated by the consumed prey, and the different prey taxa may remodel the gut microbiota in drastically different ways. Hu (2019) and Sheffer et al. (2019) compared the tissue- and population-level microbiota of some spiders. It is noteworthy that only Hu (2019) who studied gut microbiota influenced by sex. However, dissimilarity to previous results found from insects, Hu (2019) showed that bacterial microbiota has no significantly different between female and male of three spider species, including*Eriovixia cavaleriei* (Araneidae), *Larinioides cornutus*(Araneidae) and *Pardosa pseudoannulata* (Lycosidae), which catapulted the necessity of gathering gut microbiota data between two sexes of spiders.

The wolf spider*Pardosa astrigera* L. Koch 1878 is a wandering spider widely distributed throughout terrestrial environments, including agricultural lands in East Asia (World Spider Catalog, 2021). It is a very active ground-dwelling predator and dominant in most part of China. As a generalist predator, it plays a very important role in pest control in farmland ecosystem (e.g., it is an important natural enemy of *Plutella xylostella*(Plutellidae) on both cabbage and oilseed rape) (Quan, Wu, Zhou, Yun, & Jian, 2011). Great behavioral differences between female and male*P. astrigera* during the breeding period are reported, the female usually adopt a "sit and wait" strategy waiting for the male, to avoid the reduction of energy and being preyed by natural enemies, whereas the male are very active to look for female everywhere (Chen & Song, 1999). Thus, *P. astrigera* is a good agent while studying the effects of sex on gut microbiota. Therefore, we investigated the gut bacterial community of female and male *P . astrigera* by high-throughput sequencing. For this, in particular, we focused on two questions: (i) are diversity of gut bacteria in male *P .astrigera* higher than in the female; and (ii) are dominant gut microbiota related to their metabolic function

of energy demand differences between the two sexes?

## 2 MATERIALS AND METHODS

### 2.1 Spider and sample collection

Adult specimens of *P . astrigera* (n = 80; female, n = 40; male, n = 40) were randomly collected by pipe buckle method on 10 April, 2021 from corn field (41°56'N, 123deg22'E) aside by Puhe River, Northern Shenyang city, China. All spiders were transported to laboratory (Insect Ecology Laboratory, College of Life Science) and kept singly in plastic tubes (each 30 mm in diameter and 110 mm long) with moistened cotton at the bottom to maintain air humidity.

Spiders were starved for 10 days before dissection to remove the non-native microorganisms in the gut (Hu et al., 2019). To ensure the sterile condition during dissection, the aseptic table was wiped with 75% ethanol for 3 times and irradiated with ultraviolet lamp for 60 min. Before dissection, each spider was sterilized by 75% ethanol for 5 min while rinsing 3 times by sterile water before and after sterilization to remove contaminants on its body surface. Then the residual water on surface of spider was sucked dry by sterilized filter paper. The gut was dissected in sterile phosphate-buffered saline (PBS) solution with a sterilized scissor under a microscope and washed with sterile water, placed into 1.5 ml microcentrifuge tube and temporarily stored in refrigerator (Haier BCD-252WBCS, Qingdao, China) at -20. The dissection was done on ice. Ten guts were added in a tube as 1 sample and instantly quick- freezed in liquid nitrogen, finally stored in a -80degC freezer (AUCMA DW-86L500, Qingdao, China) until DNA extraction. Each female and male has four biological replicates.

### 2.2 DNA extraction and 16S rRNA gene amplicon sequencing

The total DNA of each pooled sample was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) following the manufacturer's protocol. The quality and integrity of the collected DNA were assessed by 1% agarose gel electrophoresis, its concentrations and purities were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

The DNA was amplified using 16S rRNA gene V3-V4 regions primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Kumar et al., 2020). The Polymerase Chain Reaction (PCR) amplification containing 4 μL 5 × buffer, 2 μL dNTPs (2.5 mM), 0.8 μL forward primer (5 μM), 0.8 μL reverse primer (5 μM), 0.4 μL DNA polymerase, 10 ng template DNA, and finally dd$H_2O$ up to 20 μL. The PCR reaction under the following conditions: initial denaturation at 95 for 3 min, and 29 cycles of denaturation at 95 for 30 s, annealing at 53 for 30 s and extension at 72 for 45 s, and a final extension at 72 for 10 min. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer's instructions and quantified using Quantus Fluorometer (Promega, USA). Sequencing was carried out on an Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

### 2.3 Bioinformatics, sequence analysis and statistical analysis

In order to obtain more reliable and high-quality sequencing results (valid reads), the following pre-procedures were performed on the raw reads from the Illumina MiSeq platform: raw reads were demultiplexed, quality-filtered by FASTP (version 0.19.6; Chen, Zhou, Chen, & Gu, 2018) and merged by FLASH (version 1.2.11; Magoc & Salzberg, 2011), and high-quality reads were clustered as an operational taxonomic unit (OTU) by UPARSE (version 7.0) when the sets of sequences shared at least 97% identity (Edgar, 2013), and chimeric sequences were identified and removed. All OTUs with totaling reads more than 50 were used. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (version 2.11; Wang, Garrity, Tiedje, & Cole, 2007) against the Silva 16S rRNA database (version 138) using confidence threshold of 70% (Quast et al., 2013).

Mothur software (version 1.30.2) was employed to calculate α-diversity including Sobs, Chao1, Shannon,

Simpson, and Coverage, and Student's$t$ -test was performed to compare α-diversity estimates and$P$ -value less than 0.05 was considered statistically significant. β-diversity analysis was performed and visualized with principal coordinates analysis (PCoA) were determined by Bray-Curtis distances, based on OTU compositions and Adonis test (with 999 permutations) was conducted to show differentiation in microbial structures of different sexes.

Taxa abundances in two sexes at the phylum and genus levels were compared by Wilcoxon rank-sum test and a two-tailed $P$ -value less than 0.05 was considered significant (with bootstrap values 95%). The different biomarkers associated with sex were characterized by linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011). Microbial functions were predicted by using phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2) based on high-quality sequences, and an Independent sample $t$ -test was performed to measure whether the difference between the two sexes is significant (SPSS, version 26.0), and the diagrams were finished by Origin (version 2019).

## 3 RESULTS

### 3.1 Bacterial 16S rRNA sequence data

A total of 335455 raw reads were yielded from 8 samples. After quality filtering, 299149 valid reads were obtained, with an average of 37393 valid reads per sample remaining. All estimated Coverage values were over 99%, which indicated the current sequences sufficiently covered the diversity of the sample of bacterial communities (Table 1). Retained 268 OTUs, clustered at 97% sequence similarity, were detected in samples. Among them, 155 OTUs were shared between both sexes, 110 OTUs were specific in male, whereas only three OTUs were specific in female.

### 3.2 Bacterial diversity between the two sexes

Bacterial community richness and diversity varied between female and male of $P$ . *astrigera* (Table 1). The results of Student's$t$ -test showed that bacterial α-diversity of Sobs ($P< 0.05$), Chao1 ($P < 0.05$) and Shannon index ($P < 0.001$) in female were significantly lower than male, whereas, Simpson index in female was significantly higher than male ($P < 0.05$). Meanwhile, male showed a much larger standard deviation in Sobs and Chao1 indices than female.

The PCoA results of β-diversity illustrated that bacterial community of the two different sex groups clustered independently. The results of Adonis test showed significant difference between female and male ($P < 0.05$,$R^2 = 0.48$; Figure 1) proving that the community composition and their relative abundance were distinct differences between the two sex groups, and the variation range between male is much greater than that of female.

### 3.3 Bacterial compositionsbetween the two sexes

The relative abundances of dominant ($> 1\%$) gut bacteria showed apparent differences between two sexes at different taxon levels (Figure 2). At phylum level, a total of 21 phyla were identified across all data, among which, Actinobacteriota, Firmicutes and Proteobacteria were the dominant phyla in both two sexes, in addition, Cyanobacteria and Bacteroidetes were dominant in male (Figure 2a). The results of Wilcoxon rank-sum test indicated that female had a significantly higher relative abundance of Actinobacteriota and a significantly lower relative abundance of Firmicutes relative to male ($P < 0.05$; Figure 3a). Other phyla were all higher in male than female, though no significant difference. At genus level, a total of 168 genera were found across all data, among which 5 dominant genera belong to female, whereas 12 dominant genera belong to male (Figure 2b). The results of Wilcoxon rank-sum test showed that female had a significantly higher relative abundance of *Rhodococcus* and significantly lower relative abundance of *Acinetobacter* ,*Ruminococcus* and *Fusicatenibacter* relative to male ($P < 0.05$; Figure 3b). All other genera had no significant difference between the two sexes.

In agreement with composition analysis, noteworthy changes in bacterial community were found from the result of LEfSe analysis between female and male, based on relative abundance of biomarkers of bacteria

(LDA Score > 4, $P < 0.05$; Figure 4a, b). In female, two groups of bacteria were significantly enriched, namely*Rhodococcus* (from phylum to genus),*norank_f_norank_o_0319-6G20* (from class to genus). In male, two groups of bacteria were significantly enriched, namely *Blautia* (from phylum to genus),*Lactobacillus*(the phylum and family to genus).

### 3.4 Functional predictions with PICRUSt2

A total of 11 level-2 pathways that associated with key metabolic functions were contained in the result of functional predictions using PICRUSt2 analysis. Among these, amino acid metabolism, xenobiotics biodegradation and metabolism, lipid metabolism, metabolism of terpenoids and polyketides in female were significantly higher than male ($P< 0.05$), and glycan biosynthesis and metabolism was significantly lower than male ($P < 0.05$; Figure 5).

## 4 DISCUSSION

This study compared the gut bacterial community between female and male*P. astrigera* . Although all individual spiders using in the present study were collected at early spring season from a highly homogeneous cornfield in very small range, however, dramatic divergences in gut bacterial diversity and composition were found between the two sexes. Despite Hu (2019) observed that bacterial diversity indices and relative abundance of dominant bacteria have no significant difference between female and male spiders, our results proved that sexual variation is a crucial factor in shaping the gut bacterial community in spider.

Contrary to the previous results with high gut bacterial diversity were found in female insects (Han et al., 2017; Mason et al., 2019; Wan et al., 2020; Xu et al., 2016), however, we found male spiders had significantly higher gut bacterial richness than female. Environmental factors have been proved to be an important role in gut microbiota assembly in arthropods (Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011; Wong, Chaston, & Douglas, 2013), for insects can obtain microbiota from their surrounding environments (Douglas, 2011). For the reason that female *P. astrigera* takes a "sit and wait" strategy during breeding period, so the male has to wandering around to find female, thus they have chances to face more diverse environment and different preys in the meantime. The big deviation of Sobs and Chao1 indices in male also matched the judgment that environmental microbiota has an important impact on gut microbiota. Consistent with our first hypothesis, this sex-related behavior of male *P . astrigera* results in a significantly higher bacterial richness than female. This finding corresponds with those of Foster (1995) and Zouache et al. (2011) who suggested that less dispersal and tending to remain close to breeding sites could be a factor constraining bacterial diversity of male mosquitoes. Similarly, Ng et al. (2013) considered that reduced constant exposure to diverse environmental microbiota could result in decrease in gut bacterial diversity in crickets. The results of bacterial β-diversity showed that female and male distributed on the two side of the biplot, with more dispersed male' points, which confirmed the importance of dispersal capabilities for diversity of spider gut bacteria.

Our results showed dramatic shifts in gut bacterial community composition between female and male *P. astrigera* , the relative abundance of dominant bacteria between two sexes differed in different taxon level. Furthermore, the divergences of gut bacterial community composition are mainly reflected from the different bacterial groups involved in metabolic activities, which is due to the different nutritional needs caused by sex differences. Female spiders try to avoid the reduction of energy and accumulate substantial nutrients for spawning simultaneously. As a result, very high Actinobacteriota and *Rhodococcus* (belongs to Actinobacteriota) found in female probably due to Actinobacteriota appears to supplement nutrition and are required for normal growth (Salem, Kreutzer, Sudakaran, & Kaltenpoth, 2013). On the contrary, we found that male have higher Firmicutes and F/B ratio than female, which contribute to the decomposition of complex carbohydrates, fatty acids, polysaccharides (Flint, Bayer, Rincon, Lamed, & White, 2008) and conduce to energy harvest (Ng, Stat, Bunce, & Simmons, 2018; Turnbaugh et al., 2006; Yu, Shen, Li, Yao, & Yin, 2020). Therefore, high Firmicutes and F/B ratio might meet the energy needs of male spiders that have to walk around in the breeding period to find their partners. We also note simultaneously that bacterial genera not related to sex were rather similar between female and male *P. astrigera* . For example, fenithrotion-

resistant*Burkholderia* has ability to hydrolyze the compound, thus protecting its host (Kikuchi, Hosokawa, & Fukatsu, 2011).

Our results also affirmed that the same conclusion of effects of sex on gut bacteria found in insects, that is *P. astrigera* spider has significant differences in gut bacteria due to different behavior and physiological needs. Male *P. astrigera*has to wandering around to find female, thus has a significantly higher gut bacterial richness than a "sit and wait" female. Moreover, the female has a high relative abundance of Actinobacteriota to meet their need of spawning and reproduction, by contrary, the male has a high relative abundance of Firmicutes and F/B ratio due to their energy demand of looking for partners. For the reason that sexual dimorphism is a very common phenomenon in spiders, the potential importance of sex on gut bacteria should not be ignored in future research in spider.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

Ying Gao and Guo Zheng designed the experiments, performed the data analysis and wrote initial draft of the manuscript, Ying Gao performed laboratory work, Pengfeng Wu, Shuyan Cui and Abid Ali helped to revise the manuscript. All authors approved the final version of manuscript for submission.

## DATA AVAIL ABILIT Y S TATEMENT

The original data of the gut microbiota relative abundance in spiders are available from the NCBI Sequence Read Archive (SRA) database (Accession number: SUB10677882).

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TABLE 1 The α-diversity indices (Mean ± SD) of bacterial communities of*Pardosa astrigera* . The differences based on Student's*t* -test

| | Sobs | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|
| Female | 91.25 ± 15.63 | 92.28 ± 17.09 | 0.96 ± 0.27 | 0.71 ± 0.09 | 0.9999 |
| Male | 153.75 ± 39.98 | 159.03 ± 40.98 | 2.96 ± 0.38 | 0.16 ± 0.03 | 0.9998 |

| | Sobs | Chao1 | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|
| *P*-value | 0.0269 | 0.0238 | 0.0001 | 0.0000 | 0.1135 |

Note: $P < 0.05$ indicates significantly difference.

FIGURE LEGENDS

FIGURE 1 Beta diversity difference in gut bacteria within sex. Principal coordinates analysis (PCoA) based on Bray-Curtis distances and Adonis test (with 999 permutations) to show differentiation in microbial structures of different sexes

FIGURE 2 Gut bacterial compositions at the level of phylum and genus. Taxa with less than 1% membership in samples of each group are grouped within "Others"

FIGURE 3 The gut bacterial composition and difference at the level of phylum and genus in sex. The difference based on Wilcoxon rank-sum test and a two-tailed $P$ -value less than 0.05 was considered significant (with bootstrap values 95%). *: $P < 0.05$

FIGURE 4 LEfSe analysis for remarking the significantly abundant bacterial community. (a) Cladogram showing the relationship among taxa (from the inner to outer rings, phylum, class, order, family, and genus). (b) The bar plot showing the different taxa with a LDA score $> 4$, $P < 0.05$

FIGURE 5 Compared of predicted function of gut bacteria in *Pardosa astrigera* . The difference based on Independent sample $t$ -test. *:$P< 0.05$

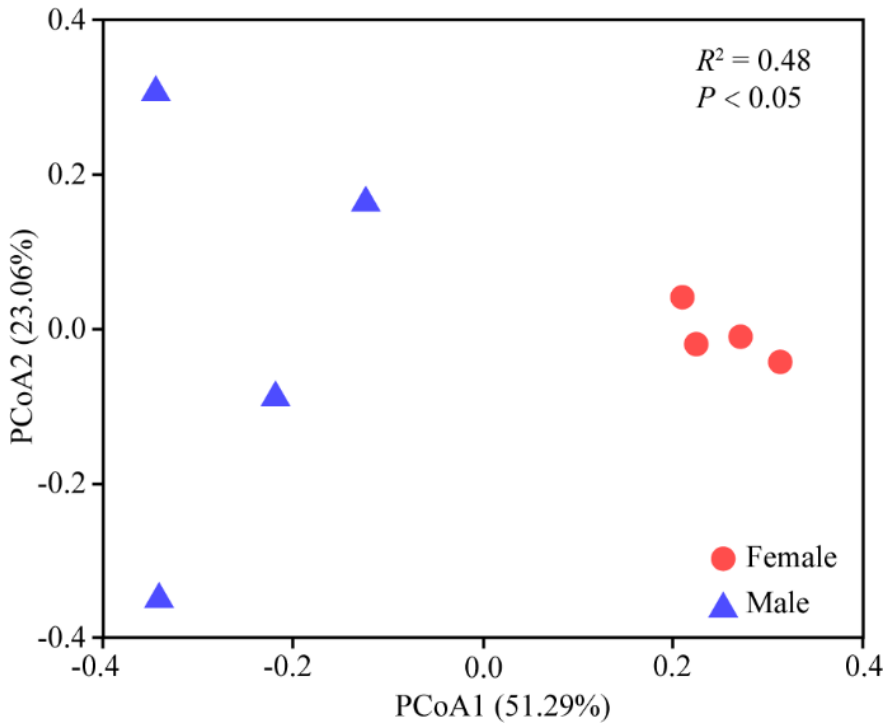



FIGURE 1

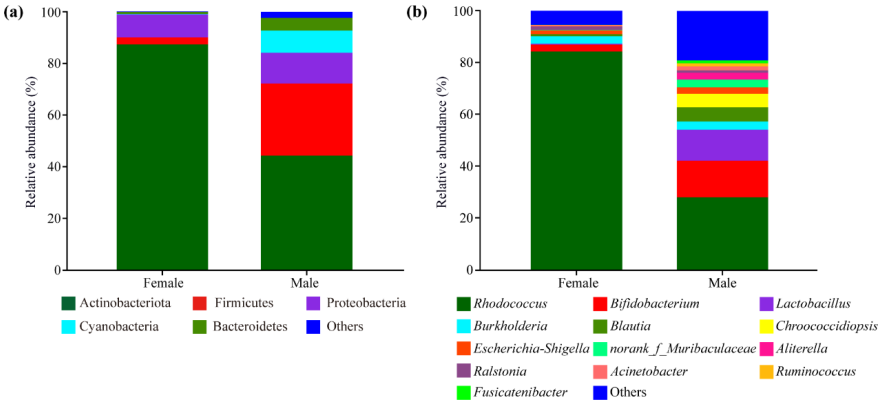



FIGURE 2

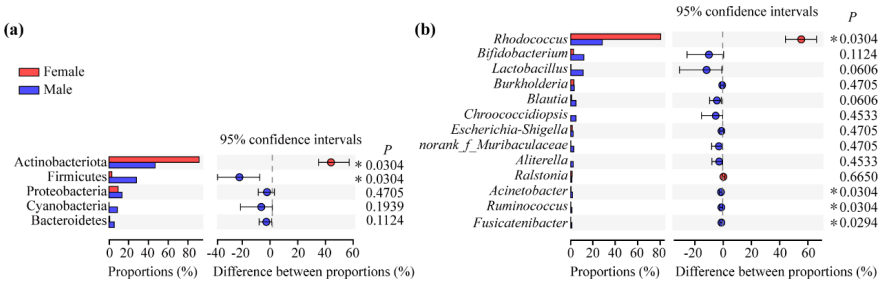
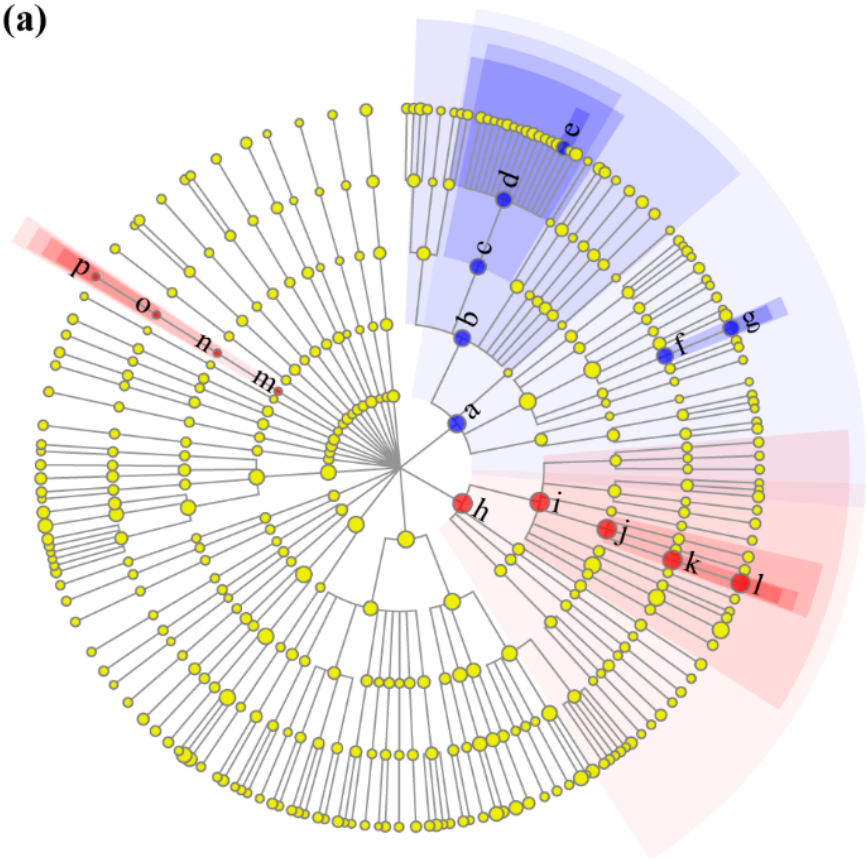



FIGURE 3

**(a)**




a: p_Firmicutes
b: c_Clostridia
c: o_Lachnospirales
d: f_Lachnospiraceae
e: g_Blautia
f: f_Lactobacillaceae
g: g_Lactobacillus
h: p_Actinobacteriota
i: c_Actinobacteria
j: o_Corynebacteriales
k: f_Nocardiaceae
l: g_Rhodococcus
m: c_Oligoflexia
n: o_0319-6G20
o: f_norank_o_0319-6G20
p: g_norank_f_norank_o_0319-6G20

**(b)**

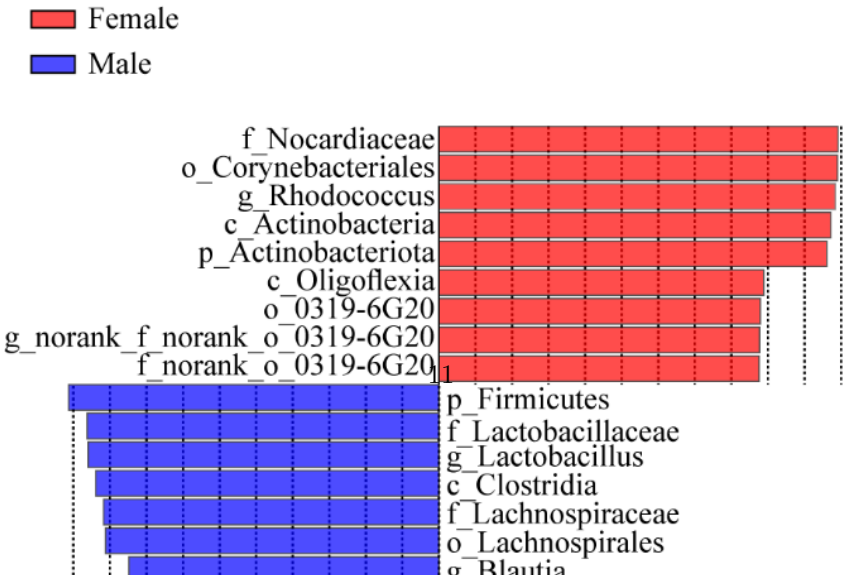


FIGURE 4

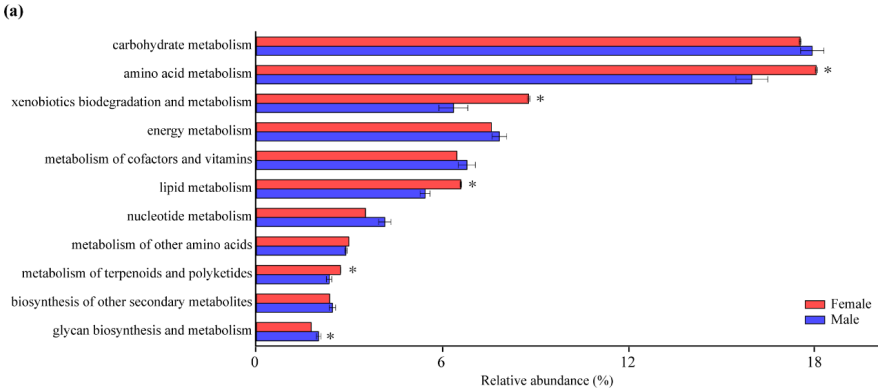



FIGURE 5