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Original article

Insights into the gut bacterial communities of spider from wild with no evidence of phylosymbiosis

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ABSTRACT

In the present study, an effort has been made to elucidate the gut bacterial diversity of twelve species of the family Araneidae under three subfamilies collected from 5 states of India along with their predicted metabolic role in functional metabolism. Further, we also compared the host species phylogeny based on partial cytochrome c oxidase subunit I (COI) sequences with the gut bacteria composition dendrogram to decipher the phylosymbiotic relationships. Analysis revealed the presence of 22 bacterial phyla, 145 families, and 364 genera in the gut, with Proteobacteria, Firmicutes, Actinobacteria, and Deinococcus-Thermus as the highest abundant phyla. Moreover, phylum Bacteriodetes was dominated only in *Cyclosa mulmeinensis* and Chlamydiae in *Neoscona bengalensis*. At the genus level, *Bacillus*, *Acinetobacter*, *Cutibacterium*, *Pseudomonas*, and *Staphylococcus* were the most dominant genera. Furthermore, the genus *Prevotella* was observed only in *Cyclosa mulmeinensis,* and endosymbiont *Wolbachia* only in *Eriovixia laglaizei*. The differential abundance analysis (DeSeq2) revealed the 19 significant ASVs represented by the genera like *Acinetobacter*, *Vagococcus*, *Prevotella*, *Staphylococcus*, *Curvibacter*, *Corynebacterium*, *Paracoccus*, *Streptococcus*, *Microbacterium*, and *Pseudocitrobacter*. The inter- and intra-subfamilies comparison based on diversity indices (alpha and beta diversity) revealed that the subfamily Araneinae have high richness and diversity than Argiopinae and Gasteracanthinae. The phylosymbiotic analysis revealed that there is no congruence between the gut bacteria composition dendrogram with their host phylogeny.

1. Introduction

The vital gut communities, bacteria in arthropods can provide their host with essential and beneficial functions like nutrient production, digestion, energy metabolism, and regulation of the immune system (Warnecke et al. 2007, Engel et al. 2012, Engel and Moran 2013, Gaio et al. 2011, Hu et al. 2019). The correlation between the host species phylogeny with their gut bacteria phylogeny have been studied in various groups like ants (Sanders et al. 2014), *Drosophila* (Chandler et al. 2011, Wong et al. 2013), and isopterans (Dietrich et al. 2014) etc.

Spiders (Order Araneae) are known as generalist predators (Bristowe 1941). All the species are not useful against a particular pest, but few of them are used as a biological controlling agent on agricultural pests (Marc et al., 1999), such as for the control of cotton pests in China (Zhao 1993), apple orchard pests in Israel and Europe (Mansour 1980, Riechert and Lockley 1984, Marc 1993). They are also used as ecological indicator species for environment monitoring (Clausen 1986; Maelfait & Hendrickx 1997; Churchill 1997). The endosymbiont *Wolbachia* in spiders is well studied (Duron et al., 2008; Goodacre et al., 2006; Zhang et al., 2018), but the studies on the abundance of *Wolbachia* in gut and their impact on the abundance of other bacteria taxa is poorly understood (Hu et al. 2019). Like other arthropods, the endosymbionts in the gut of spiders is responsible for the host nutrition and sex ratio alterations (Gunnarsson et al., 2009; Vanthournout, Swaegers & Hendrickx 2011; Vanthournout, Vandomme, & Hendrickx 2014; Vanthournout & Hendrickx, 2015).

Spiders exhibit extra oral digestion (EOD), immobilize their prey by injecting venom and regurgitating digestive fluid onto (or into) their prey and then sucking back again the resulting liquefied tissue (Foelix, 2011, Kennedy et al., 2020). This type of feeding

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behaviour also makes spiders an interesting model to study the composition and function of their gut microbial communities. However, the gut bacterial diversity of this most diverse group is poorly explored.

Till date, gut bacterial diversity of only thirteen spider species have been reported (Kennedy et al., 2020; Hu et al., 2019; Rivera et al., 2017; Sheffer et al., 2020, Kumar et al., 2020). Kennedy et al., studied the impact of different prey on the gut microbiome of the spider species *Badumna longinqua*, and revealed a clear correlation between the prey insects and the gut microbiome (Kennedy et al., 2020). Hu et al., 2019, documented the gut microbial profiling and observed that the phylum Proteobacteria was the most dominant, including Tenericutes, Actinobacteria, Firmicutes, Acidobacteria and Bacteroidetes. Furthermore, Rivera et al., 2017 studied the microbial profiling using body swaps and excreta samples of *Rabidosa rabida*. The culture studies revealed the presence of the genus *Staphylococcus* sp. in body swaps and *Staphylococcus aureus* in excreta samples. Furthermore, the species *Staphylococcus aureus* is also responsible for the staph infection in humans (Foster 1996) and it will open the opportunity for researchers to explore that the spiders may be the potential vector of this pathogen or not. Sheffer et al., 2020 reported the presence of novel bacterial symbiont affiliated to Tenericutes in the wasp spider i.e. *Argiope bruennichi*, which they named as DUSA (Dominant unknown symbiont of *Argiope bruennichi*) (Sheffer et al., 2020). Kumar et al. studied the gut bacterial diversity of 7 species belonging to the family Thomisidae and Oxyoopidae from wild populations. Further, the subfamily Araneinae of family Araneidae has been recovered as a paraphyletic group (Scharff et al. 2020) and it would be interesting to study the phylosymbiotic relationship by correlating the gut bacterial divergence with their host phylogeny.

In the present study, an effort has been made to explore gut bacterial composition of twelve species of the family Araneidae under three subfamilies Araneinae, Argiopinae and Gasteracanthinae through 16 s rRNA amplicon sequencing along with inter- and intra-subfamily comparison. In addition to this, phylosymbiotic relationships, and the predicted functional metabolism analysis were also investigated.

2. Materials and methods

2.1. Sample collection

In this study, we have collected specimens of all the twelve spider species from the five states of India (Arunachal Pradesh, Assam, Gujarat, Odisha and West Bengal) (Table 1). The specimens were collected from the field by the following collection methods: hand picking, sweep net, and yellow pan trap method. All the specimens are sorted and kept individually in a separate empty vial. After 8 h of collection, each specimen is transferred in 100% alcohol and stored in 4 °C. Subsequently, each specimen was washed thrice with PBS solution to remove the contamination. After washing, DNA is extracted from a single specimen of each species. The spiders used in this study were non-endangered and non-protected species. The taxonomic identification of these specimens was done by Priya Prasad on a NIKON SMZ25 stereo microscope using available literature (Table 1).

2.2. DNA Isolation, amplification and sequencing

The DNA of a single specimen of each spider species was extracted using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. The quantification of the DNA was checked by Qubit 2.0 Fluorometer (Q32866, Thermofisher), and the quality was checked using agarose gel electrophoresis (Cell BioScience Alphalmager MINI). DNA of 10 specimens of each species was pooled for amplification and sequencing. The extracted DNA was amplified using the primer sets of V3-V4 hypervariable regions of the 16S rRNA 341F (5′- ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Total 25 µl of mixture was prepared for the PCR, including 1 µl of each primer, 0.5 µl of Taq DNA polymerase (Takara), 1 µl of dNTPs, 2.5 µl of 10 × buffer, 50 ng of template, and Milli-Q water. The PCR cycle involved denaturation for 5 min at 98 °C followed by 35 cycles for 30 s at 98 °C, annealing for 45 s at 53 °C, and elongation at 72 °C for 45 s, and final extension of 7 min at 72 °C. The PCR products were visualized using agarose gels for high-throughput sequencing of microbial diversity. The sequencing of the targeted gene region of 16S rRNA was carried out on the Illumina HiSeq platform (Illumina Hiseq2500). The qualified constructed Nextra library was sequenced using PE300bp (Illumina Hiseq2500 RC V2 Kit) with rapid mode. The National Center for Biotechnology Information (NCBI) GenBank Portal has been used for the submission of the generated raw reads to under the BioProject ID PRJNA638522.

2.3. Bioinformatics and statistical analyses

The generated paired end raw reads of twelve spider species were merged into single reads in QIIME2 (ver. 2019.10) (Bolyen et al., 2019) using demultiplexing. These reads were processed by DADA2 (Callahan et al., 2016) pipeline in QIIME2 for quality filtering, trimming, de-noising and merging (Table S1). The chimeric reads were filtered and the non-chimeric reads were assigned into Amplicon Sequence Variants (ASVs). These ASVs were further classified based on SILVA 99% similarity database (version 132) using QIIME2 q2-feature-classifier plugin. The generated taxonomy and

Table 1
Detail of the studied species included in this study.

Sl. No.	Specimen code	Species	Collection Locality	Lat Long and Elevation	References used for Identification
Araneinae					
	AA 2217	*Araneus mitificus*	Odisha	N20.50 E85.96; 19 m	Kim & Lee 2012
	AA 787	*Cyclosa spirifera*	Assam	N 27.47 E94.91; 98 m	Keswani 2013
	AA 795	*Cyclosa mulmeiensis*	Assam	N 27.66 E95.36; 102 m	Yin et al. 2012
	AA 598	*Cyclosa bianchoria*	Arunachal Pradesh	N 28.60 E95.49; 1909 m	Yin et al. 2012
	AA 2116	*Eriovixia excelsa*	Odisha	N18.78 E82.70; 887 m	Tso & Tanikawa 2000
	AA 1438	*Eriovixia laglaizei*	Odisha	N20.4 E85.82; 31 m	Han & Zhu 2010
	AA 1873	*Neoscona bengalensis*	West Bengal	N22.57 E88.31; 10 m	Tikader 1982
	AA 397	*Neoscona nautica*	Assam	N26.67 E92.85; 63 m	Yin et al. 2012
Argiopinae					
	AA 136	*Argiope pulchella*	West Bengal	N22.16 E88.82; 8 m	Jäger 2009
	AA 29	*Cyrtophora cicatrosa*	West Bengal	N23.41 E87.11; 100 m	Yin et al. 1997
Gasteracanthinae					
	AA 154	*Gasteracantha kuhli*	Gujarat	N20.77 E 73.67; 415 m	Tan et al. 2019
	AA 1164	*Gasteracantha hasselti*	Assam	N 27.29 E 95.51; 203 m	Tan et al. 2019

feature tables along with metadata file were processed for the downstream analysis. A web-based tool, MicrobiomeAnalyst (Dhariwal et al., 2017) using Marker Data Profiling (MDP) module was used for bacterial diversity analysis. A total of 1326 ASVs were recovered out of 5967 ASVs after removing the singletons. These ASVs were further filtered based on low abundance features with prevalence 10 and low variance features with a default inter quantile range and resulting a total of 1175 ASVs. To test the ASVs differential abundance analysis based on filtered data between three subfamilies of family Araneidae, we used DEseq2 (Love et al. 2014) package in MicrobiomeAnalyst to identify the ASVs that differ between the groups (Table S2).

The Alpha-diversity was analysed using T-test/ANOVA statistical methods with observed, Chao1, Shannon and Simpson as diversity measures. The PERMANOVA based statistical method for Bray Curtis and Ward's linkage-based method for Unweighted UniFrac distance measure were used for analyzing the beta diversity. Further, an online tool jvenn (Bardou et al., 2014) (http://jvenn.toulouse.inra.fr) was used for the construction of the Venn diagram, while the R-based (R core team 2020), Metacoder software (Foster et al., 2017) was used for the picturing of the heat tree. We used UpSetR software in R to generate the plots of each subfamily ASVs (Conway et al., 2017). The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (Langille et al., 2018) (PICRUSt2) was used for predicting the functional metabolic pathways. The predicted metabolic pathways were characterized through **K**yoto **E**ncyclopedia of **G**enes and **G**enomes (KEGG) (Kanehisa et al, 2000; Kanehisa et al., 2019; Kanehisa, 2019) database and accuracy evaluated by the Nearest Sequenced Taxon Index (NSTI) values. Further, the generated file along with the metadata were loaded on the MicrobiomeAnalyst web server for the diversity association between the predicted pathways and ASVs. Further, to test the correlation between the host phylogeny and bacterial composition based phylogeny, Mantel test was implemented with 9999 permutations to compare the distance matrices of host species COI sequences and gut bacteria.

2.4. Host species phylogeny

The phylogeny of the host species was constructed using the partial Cytochrome *c* oxidase subunit I (COI) sequences. These sequences of the host species were taken from the earlier study (Tyagi et al. 2019) except *Eriovixia laglaizei* (Accession number: MK392634, MK392659, MK392665, MK392676, MK392691, MK392701, MK392733, MK392761, MK392773, MK393097). The COI sequence of *Eriovixia laglaizei* was generated and submitted to BOLD (Barcode of Life Data Systems) under the project "Spiders from India" (BIN AEC1993). The sequence of *Neoscona bengalensis* is not available in GenBank or BOLD and mite sequence (BOLDMSACA57112_OG_Acari) was used as an out-group (Astrin et al. 2016). The Bayesian inference was constructed in CIPRES Portal (http://www.phylo.org/; Miller et al. 2010) using Mr. Bayes 3.2 (Ronquist et al. 2012) with nst = 6, four (one cold and three hot) metropolis-coupled Markov Chain Monte Carlo (MCMC), for 50,000,000 generations with 25% burn in and trees saving at every 100 generations. Further, to decipher the phylosymbiotic relationship between the host phylogeny and bacterial composition based phylogeny, Unweighted UniFrac dendrogram was constructed.

3. Results

3.1. Rarefaction and ASVs distribution

A total of 1,077,166 sequences of 16S rRNA (V3-V4) was identified after pre-processing steps i.e. merging, quality filtering, chimera removal, etc. which ranges from 69,573 to 106,136 (average of 89,763 reads per sample) and assigned into 5967 ASVs (Tables S1). Rarefaction curve analysis using Good Coverage reached the saturation which indicated that the sufficient sequencing depth has been achieved to decipher the bacterial community structure. Further, Venn analysis revealed that out of 5967 ASVs, 65% were unique for Araneinae, 7% for Argiopinae and 11% for Gasteracanthinae, while only 4.5% ASVs were shared as core microbiome in the family Araneidae (Fig. 1a, 1b). Moreover, we had carried out the analysis to decipher the percentage contribution of phylum from each subfamily i.e. Araneinae, Argiopinae, and Gasteracanthinae towards the core microbiome. From results, it was observed that all the sub families contributed almost equally in term of phylum Proteobacteria (23–25%), Bacteroidetes (22%), and Firmicutes (22–25%) while significant differences was observed in the abundance of Deinococcus-Thermus (10–22%) and Actinobacteria (11–22%) (Fig. 1c). Further for downstream analysis, 5967 ASVs were subjected to removal of singletons (4641 ASVs), low variance (20 ASVs) and low abundance features (131 ASVs) and resulted into 1175 ASVs. Further, we have also rarefy the data in normalization step in MicrobiomeAnalyst with Total Sum Scaling (TSS) and minimum library size option (Weiss et al. 2017). To elucidate the bacterial abundance, diversity indices and differential abundance analysis, we used the normalized data.

3.2. Structure and composition of bacterial diversity

The taxonomic classification of the annotated sequences revealed the presence of 22 bacterial phyla and 364 bacterial genera in all. The phylum Proteobacteria was predominant with an abundance of around 49–75%, followed by the phylum Firmicutes with an abundance of 14–35%, were detected in the gut of all spider species. Other bacterial taxa like Actinobacteria (1–14%), Bacteroidetes (1–10%), and Deinococcus-Thermus (2–6%) were detected in the gut of eleven spider species of the family Araneidae. The major contribution of the phylum Actinobacteria comes from two species of the subfamily Araneinae, i.e. *Cyclosa spirifera* and *Eriovixia laglazei*, while the phylum Bacteriodetes was most strongly represented in *Cyclosa mulmeinensis* (Araneinae). The microbiome of the species *Araneus mitificus* was constituted by only two phyla i.e. Proteobacteria and Firmicutes. In addition to this, the phylum Chlamydiae was observed only in one species of the subfamily Araneinae, i.e. *Neoscona bengalensis* (Fig. 2)

A total of 81 orders were detected in the current dataset, among them the orders that majorly contribute to the total bacterial diversity in subfamily Araneinae were *Pseudomonadales, Enterobacteriales*, *Bacilliales*, *Lactobacilliales*, *Micrococcales*, *Corynebacteriales*, *Propionibacteriales*, *Flavobacteriales*, *Bacteriodales*, *Rickettsiales, Thermales* (Figure S1). Similar trends were observed in subfamily Argiopinae and Gasteracanthinae with considerable changes in the abundance of order *Bacilliales, Bacteriodales* and *Rickettsiales* in Argiopinae (Figure S2), and the abundance of orders *Enterobacteriales*, *Lactobacilliales*, *Siphingomonadales*, *Thermales, Bacteriodales* in Gasteracanthinae (Figure S3).

At the family level, Moraxellaceae (20%), Enterobacteriaceae (13%), Bacillaceae (9%), Pseudomonadaceae (6%), Burkholderiaceae (5%), were observed in all three subfamilies of spiders. The remaining 47% diversity was contributed by other families, including Prevotellaceae, Staphylococcaceae, Propionibacteriaceae, Corynebacteriaceae, Micrococcaceae, Thermaceae etc. Two families, Burkholderiaceae and Pseudomonadaceae, were not observed in *Araneus mitificus* (Araneinae). The major contribution of the family Prevotellaceae was reflected in only *Cyclosa mulmeinensis* (Araneinae).

At the genus level, 364 genera were observed in the current dataset of spiders. The genera *Acinetobacter* (5–34%), *Bacillus* (0–

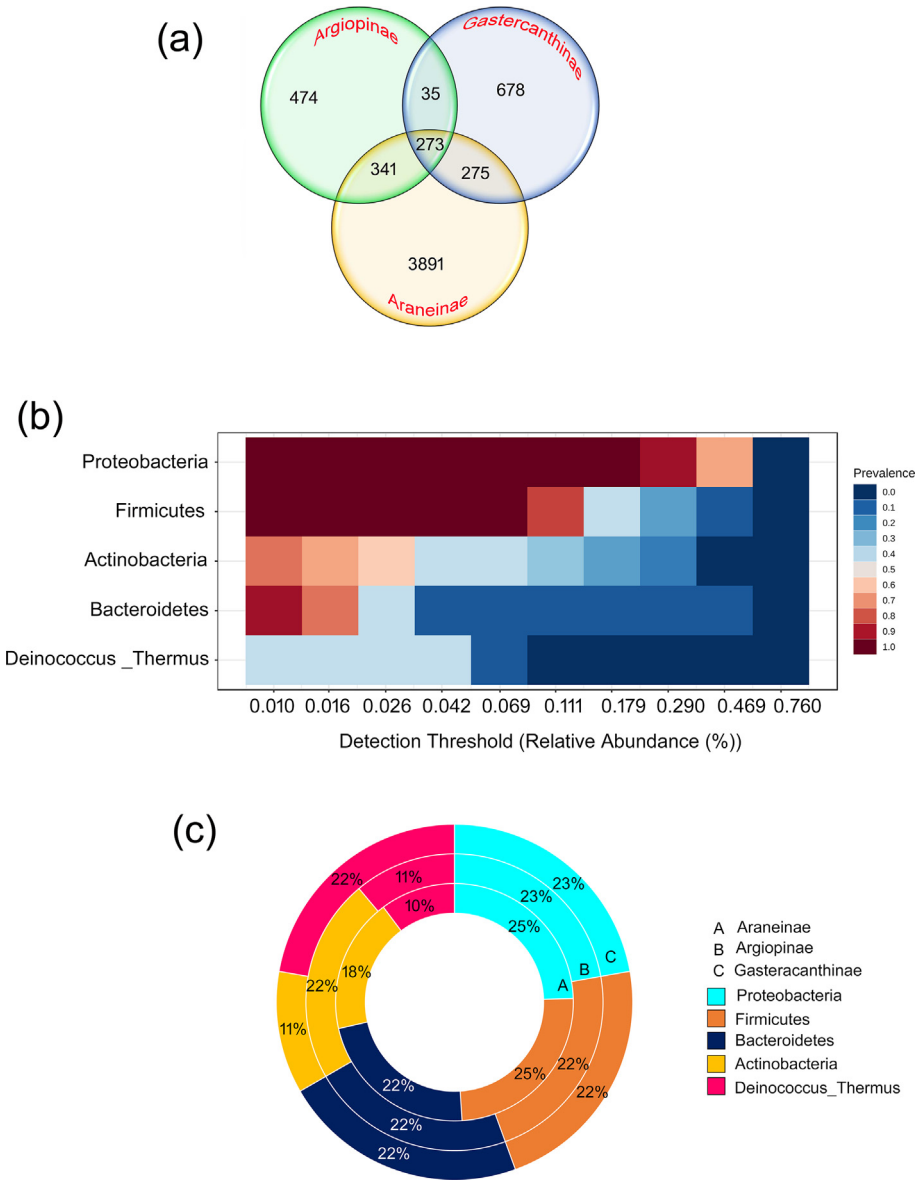

Fig. 1. (a) Venn diagram (b) Percentage contribution of core microbiome of family Araneidae (c) Percentage contribution of phylum from each subfamilies i.e. Araneinae, Argiopinae, and Gasteracanthinae towards the core microbiome.

24%), V4 (3–23%), *Cutibacterium* (0–16%), *Pseudomonas* (0–9%), and *Staphylococcus* (0–12%), *Coryneobacteria*_1 (1–11%) constitute towards the gut microbiome of Indian Spiders. In addition to this, the genus *Prevotella* (60%) was observed only in *Cyclosa mulmeinensis* while endosymbiont *Wolbachia* (28%) was detected only in *Eriovixia laglaizei* (Araneinae) (Fig. 3).

3.3. Inter- and Intra-subfamilies gut bacteria comparison in family Araneidae

To identify inter- and intra-subfamilies core bacterial taxa, the shared and unique ASVs were examined. Inter-subfamilies comparison revealed that 4.5% ASVs were shared, and 65% unique for Araneinae, 7% for Argiopinae and 11% for Gasteracanthinae. On the other hand, intra-subfamilies comparison in subfamily Araneinae, eight species shared a relatively low 0.06% (3) of total ASVs, while 0.5% (24 ASVs) unique for *Araneus mitificus*, 2.5% (122) for *Cyclosa mulmeinensis*, 7% (339) *Cyclosa bianchoria*, 7.7% (371) *Eriovixia laglaizei*, 14% (677) *Neoscona nautica*, 14.5% (696) *Cyclosa spirifera*, 16.4% (787) *Eriovixia excelsa*, 17.9% (858) *Neoscona bengalensis*. The maximum ASVs (164 ASVs) shared between *Neoscona nautica* and *Neoscona bengalensis* while minimum (1 ASV) shared by *Araneus mitificus* with four species (*Neoscona bengalensis*, *Cyclosa spirifera*, *Cyclosa mulmeinensis*, *Eriovixia laglaizei*) (Fig. 4a). In case of Argiopinae, both the species shared 6.1% ASVs, and 37.6% unique for *Argiope pulchella*, 56.1% for *Cyrtophora cicatrosa* (Fig. 4b). In case of Gasteracanthinae, both the species shared 18.4%, while 43.2% unique for *Gasteracantha hasselti*, 38.3% for *Gasteracantha kuhli* (Fig. 4c).

Further, the DEseq2 is carried out to identify which gut bacterial taxa differed between these three subfamilies (Table S2). This analysis identified the 19 significant ASVs with a p value cut off 0.05 and indicated the presence of the following genera *Acinetobacter*, *Vagococcus*, *Prevotella*_9, *Staphylococcus*, *Curvibacter*, *Corynebacterium*_1, *Paracoccus*, *Streptococcus*, *Microbacterium*, and *Pseudocitrobacter* as significant. The taxa of the family Araneidae gut

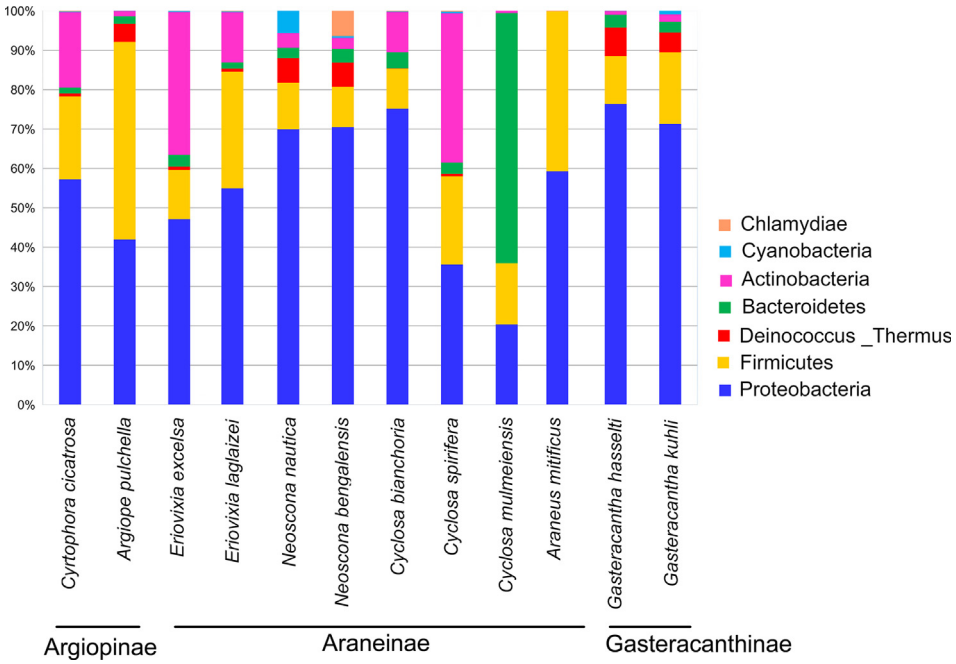

Fig. 2. Abundance of gut bacterial diversity at the phylum level of twelve spider species under three subfamilies, Araneinae, Argiopinae, and Gasteracanthinae.

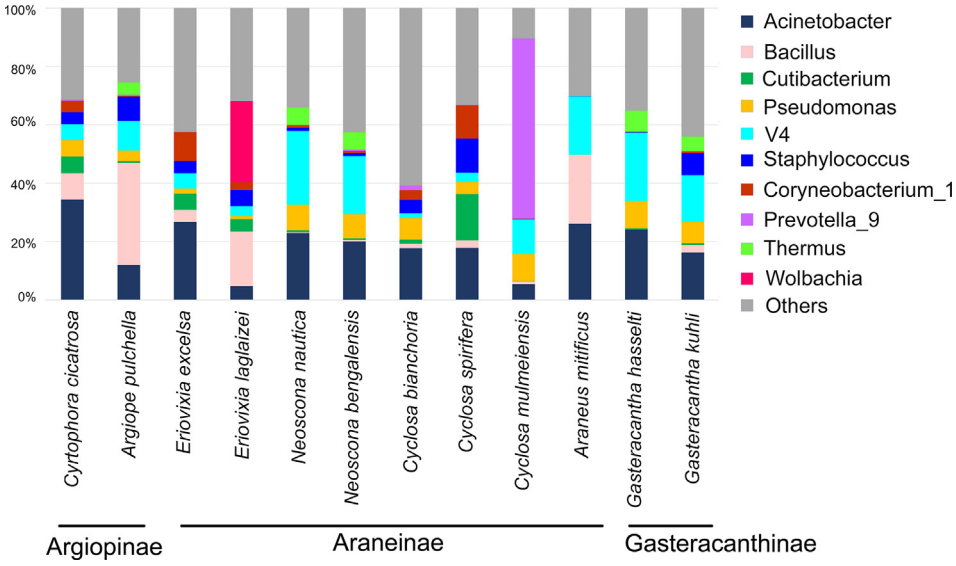

Fig. 3. Abundance of gut bacterial diversity at the genus level of twelve spider species under three subfamilies, Araneinae, Argiopinae, and Gasteracanthinae.

bacterial community, Moraxellaceae; Acinetobacter, was found to be significantly higher abundance in the Argiopinae as compared with Araneinae and Gasteracanthinae (Fig. 5).

Moreover, another taxa of the Araneidae gut community, Enterobacteriaceae; *Cosenzaea*, were significantly higher in abundance in the Argiopinae and Gasteracanthinae as compared with Araneinae. The bacterial taxa Enterococcaceae; *Vagococcus* were also significantly higher abundance in the Argiopinae as compared with Araneinae and Gasteracanthinae. The bacterial taxa in the family Araneidae gut community, Prevotellaceae; *Prevotella*_9 was abundant in Araneinae as compared with Argiopinae and Gasteracanthinae. The bacterial taxa of Staphylococcaceae; *Staphylococcus* was more abundant in Araneinae and Argiopinae as compared to Gasteracanthinae. The bacterial taxa, Burkholderiaceae; *Curvibacter*, were more abundant in the Argiopinae as compared with Araneinae and Gasteracanthinae. Other low abundance taxa were also found to be different in abundance between these three subfamilies.

3.4. Diversity metrics for comparison of gut microbiota

To decipher the bacterial community richness, α- and β-diversity analyses were carried out. The diversity measurements of Chao1, Observed, Shannon and Simpson were used for the α-diversity analysis, while Bray-Curtis and Unweighted Unifrac diversity measures for β-diversity analysis. The α-diversity for three spider subfamilies (Araneinae, Argiopinae, and Gasteracanthinae) of the family Araneidae lies in the range of 39–559 (Chao1, Observed). The changes observed in the community richness of three subfamilies were non-significant ($p > 0.05$) (Table S3).

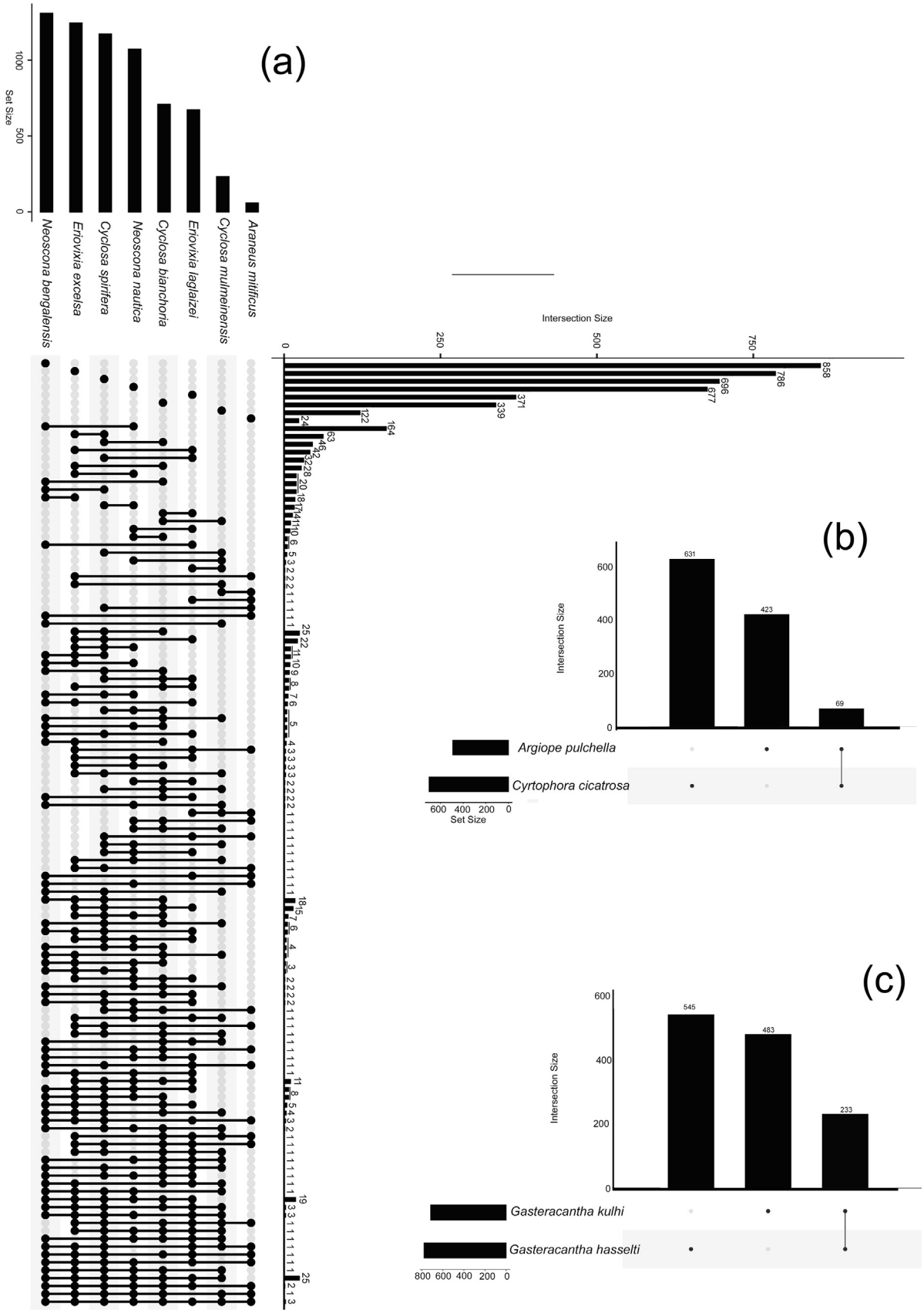

Fig. 4. An Upset plot unique and shared ASVs between the species of subfamilies (a) Araneinae (b) Argiopinae (c) Gasteracanthinae. Set size bar representing the ASVs of host species, dark circle indicated the unique ASVs of the host species, dark circle connecting bar indicated the sharing between the species.

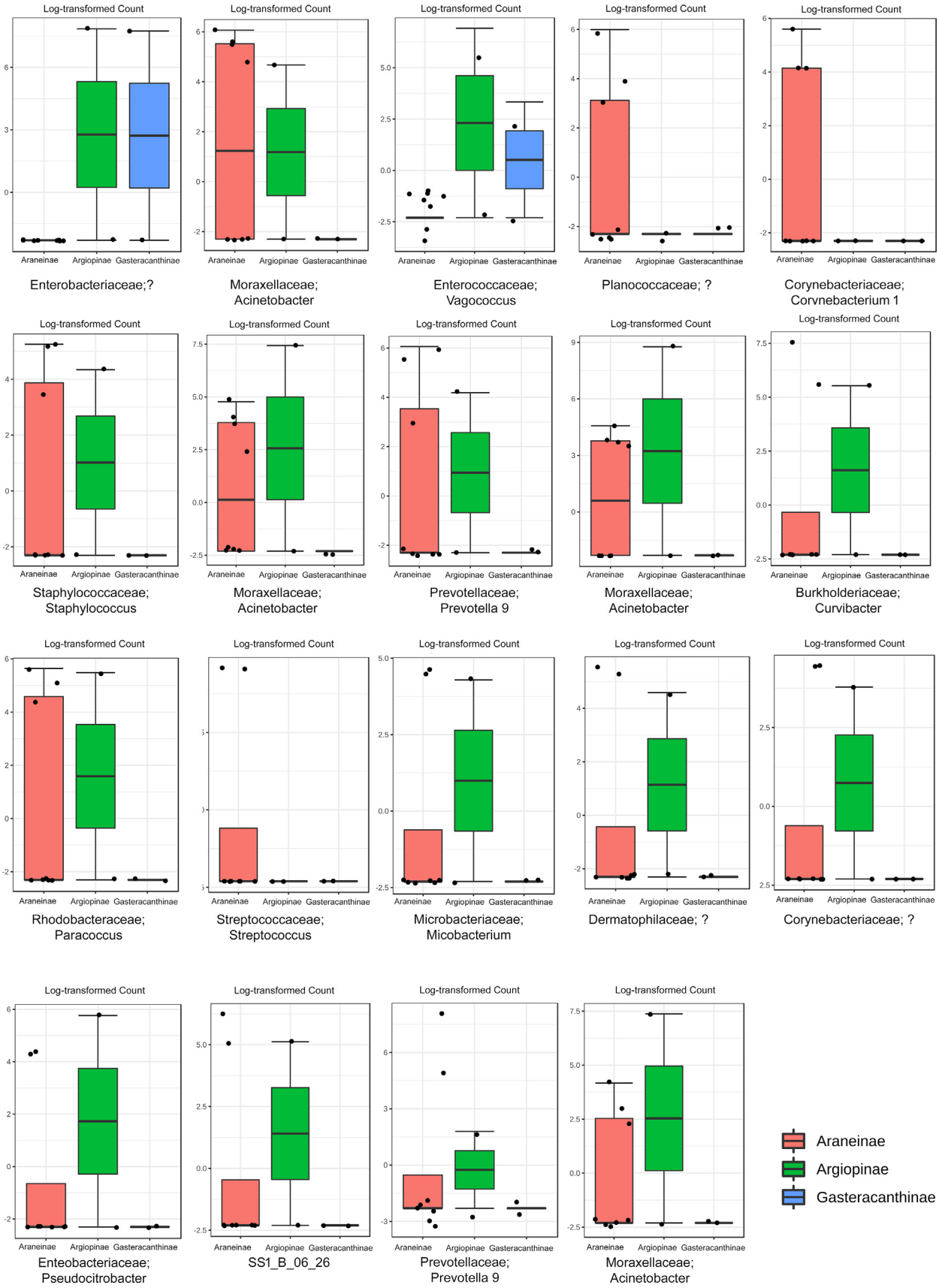

Fig. 5. The representation of the differential abundance analysis DESeq2.

Inter- subfamilies comparison reveals that the bacterial community richness of *Neoscona bengalensis* was highest, while that of *Araneus mitificus* was lowest. Further, intra-subfamilies comparison in Araneinae, Gasteracanthinae and Argiopinae revealed that the richness profile was highest for *Neoscona bengalensis, Gasteracantha kuhli* and *Cyrtophora cicatrosa* while lowest for *Araneus mitificus, Gasteracantha hasselti* and *Argiope pulchella* respectively. Based on the values of richness estimators, it can be concluded that Araneinae was richer (in terms of bacterial community) than Argiopinae and Gasteracanthinae (Fig. 6).

Moreover, the α-diversity analysis based on Shannon and Simpson were in the range of 2.33–4.72 and 0.859–0.975 respectively. However, the changes observed in these diversity estimates for three subfamilies were non-significant ($p > 0.05$). Inter-subfamilies comparison based on these measures was different than that of community richness measures and it was observed that *Eriovixia excelsa* possess higher diversity while lowest in *Araneus mitificus*. On the other hand, in terms of intra-subfamilies comparison the vice-versa of community richness measures stands true for Gasteracanthinae and Argiopinae while for Araneinae different results (in terms of highest diversity) for both the diversity measures were observed (Table S3). Hence, based on diversity estimators, it can be concluded that subfamily Araneinae possess higher diversity than Argiopinae and Gasteracanthinae (Fig. 6).

The Bray-Curtis based NMDS ordination plot also suggested a similar type of results as indicated by unweighted unifrac diversity measures. Based on distance matrix (NMDS Stress = 0.055), it was observed that two species i.e. *Araneus mitificus* and *Eriovixia laglzei* were in close resemblance with each other. The gut samples obtained from the two species (*Gasteracantha kuhli* and *G. hassleti*) of subfamily Gasteracanthinae were in close resemblance with one species of Argiopinae (*Argiope pulchella*) and two species of Araneinae (*Neoscona nautica* and *N. bengalensis*). The rest of the members of subfamily Araneinae were in close resemblance with one species of family Argiopinae (*Cytrophora cicatrosa*) (Fig. 7).

3.5. Functional predictions analysis

The PICRUSt2 computational approach was used to decipher the resemblances and differences in metabolic profiles of the

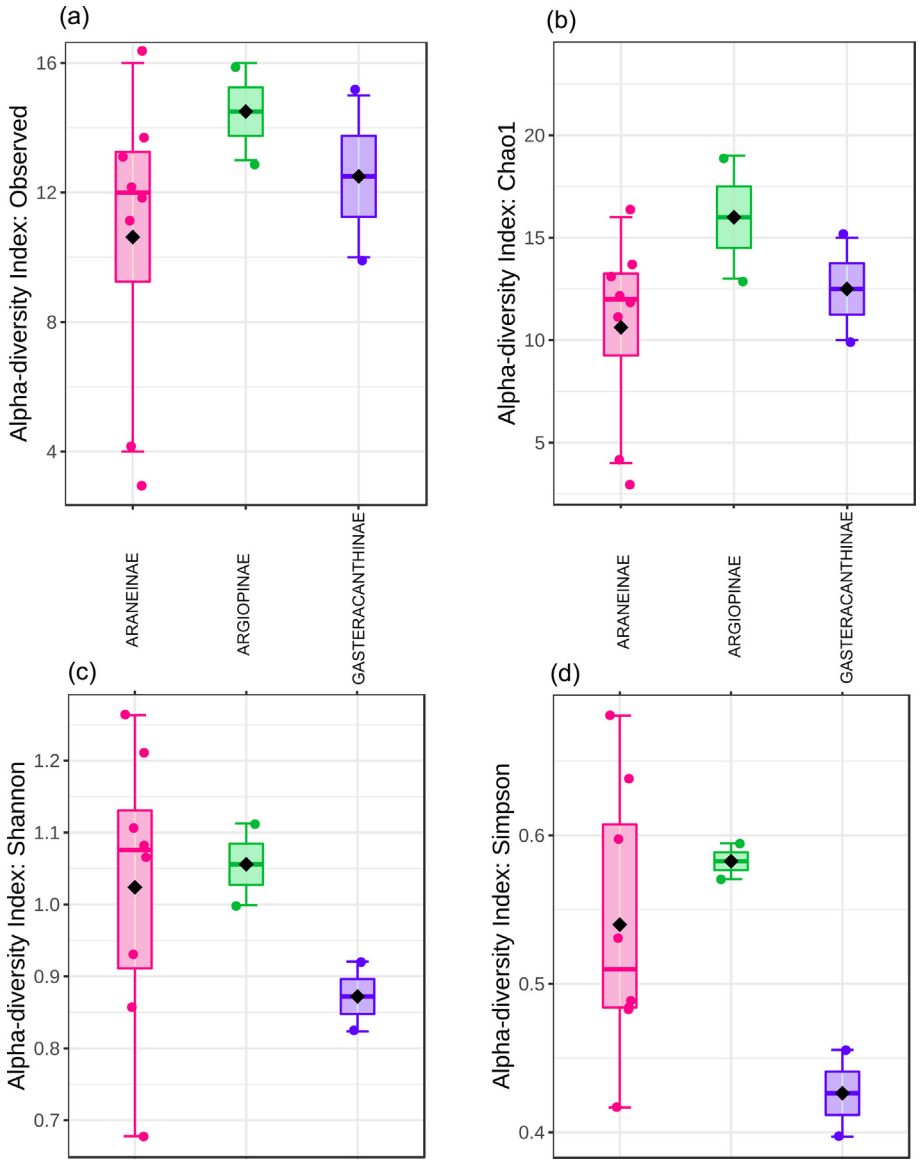

Fig. 6. Box plot for the alpha-diversity index (a) observed, (b) Chao1, (c) Shannon and (d) Simpson of the gut bacterial diversity in three subfamilies of family Araneidae. The ends of the whiskers represent the minimum and maximum while the line inside the box represents the median.

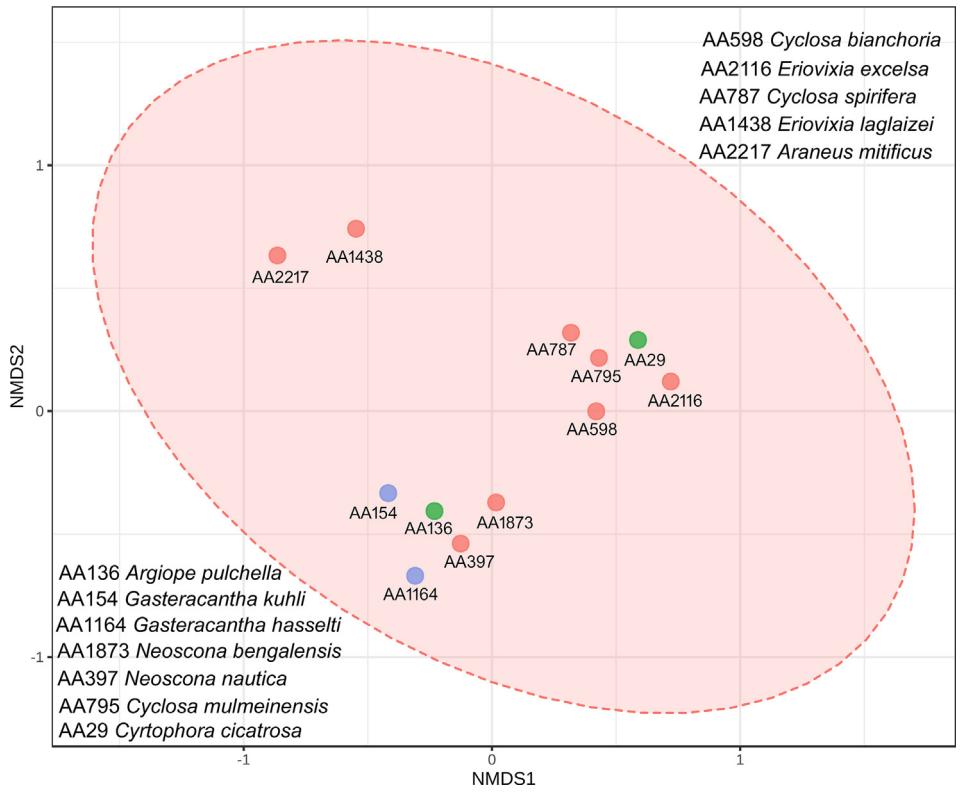

Fig. 7. Bray-Curtis dissimilarity-based Non-metric Multidimensional Scaling (NMDS) ordination plot of twelve spider species.

microbiome of three subfamilies, i.e. Araneinae, Argiopinae, and Gasteracanthinae. Results revealed the presence of 443 Metabolic functional pathways which were further analysed with MicrobiomeAnalyst and obtained significant (p value < 0.05) pathways on the basis of their ASVs abundance/metagenome hits (Table S4). These pathways included profiles related to metabolism (carbon, amino acids, nitrogen, sulphur, biotin, pyruvate Butanoate and fatty acids etc), biosynthesis (amino acids, carbapenem), Glycolysis, TCA cycle, degradation (fatty acids, Valine, Leucine, Isoleucine, Lysine, Geraniol), Drug and enzymes metabolism etc. (Table S4). Furthermore, the bacterial taxa Like *Bacillus*, *Acinetobacter*, *Lactococcus*, *Thermus Staphlococcus*, *Blastomonas*, Enterobacteriaceae ambiguous Taxa, and Burkholderiaceae ambiguous taxa, were mainly responsible for these predicted metabolic pathways (Fig. 8). Moreover, the gut bacteria associated with carbon metabolism and amino acid biosynthesis have higher relative abundance than those involved in other metabolic activities.

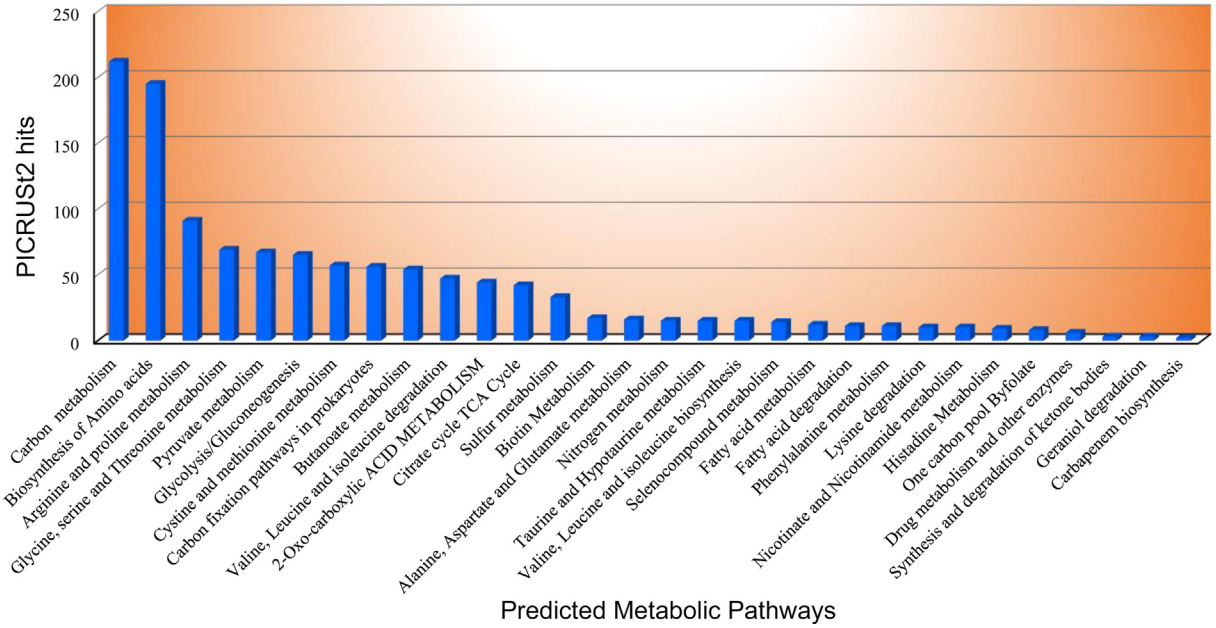

Fig. 8. The predicted functional metabolic pathway of twelve species under three subfamilies of family Araneidae.

3.6. Comparison of gut bacterial composition based phylogeny with their host phylogeny

To evaluate the phylosymbiotic relationship, the host species phylogeny (BI phylogeny based on COI sequences) and bacterial composition phylogeny (Unweighted Unifrac based dendrogram) were compared (Fig. 9). The dendrogram revealed two clades and can be discriminated by the relative abundance of phylum Deinococus-Thermus. Clade I with negligible abundance of phylum Deinococus-Thermus was observed in one species of subfamily Argiopinae (*Cyrtrophora cicatrosa*) + all the members of subfamily Araneinae in this clade while clade II contain all the members of subfamily Gasteracanthinae + one species of subfamily Argiopinae (*Argiope pulchella*) + two species of subfamily Araneinae (*Neoscona nautica* and *N. bengalensis*).

Clade I was further divided into two subclades A and B: The subclade IA possesses a similar type of bacterial abundance, except in the species *Cyclosa mulmeinensis* which diverged due to increased abundance of the phylum Bacteroidetes. The subclade IB also possesses a similar type of bacterial abundance, except in the species of *Cyclosa bianchoria*, in which the abundance of phylum Proteobacteria was higher than the other three species. Two species of Araneinae (*Eriovixia excelsa* and *Cyclosa spirifera*) and one species of Argiopinae (*Cyrtophora cicatrosa*) in subclade IB have similar type of bacterial diversity, while *Cyclosa spirifera* was branched out from this subclade due to the high abundance of phylum Actinobacteria.

The clade II with a considerable abundance of Deinococcus-Thermus contains five species (*Neoscona nautica*, *N. bengalensis*, *Gasteracantha kuhli*, *Gasteracantha hasselti* and *Argiope pulchella*). The branch of species *Argiope pulchella* (Argiopinae) was the first branch out due to the high abundance of phylum Firmicutes. This clade further sub divided into two subclades A and B and have similar type of bacterial diversity except a few changes in the diversity of Phylum Chalamydiae and Cyanobacteria.

In host species phylogeny, the species of the subfamily Gasteracanthinae (*Gasteracantha hassleti* and *G. kuhli*) showed sister relationship with subfamilies Araneinae and Argiopinae (Fig. 9) while these species are closer to *Neoscona* species and *Argiope pulchella* in bacterial composition phylogeny. However, the subfamily

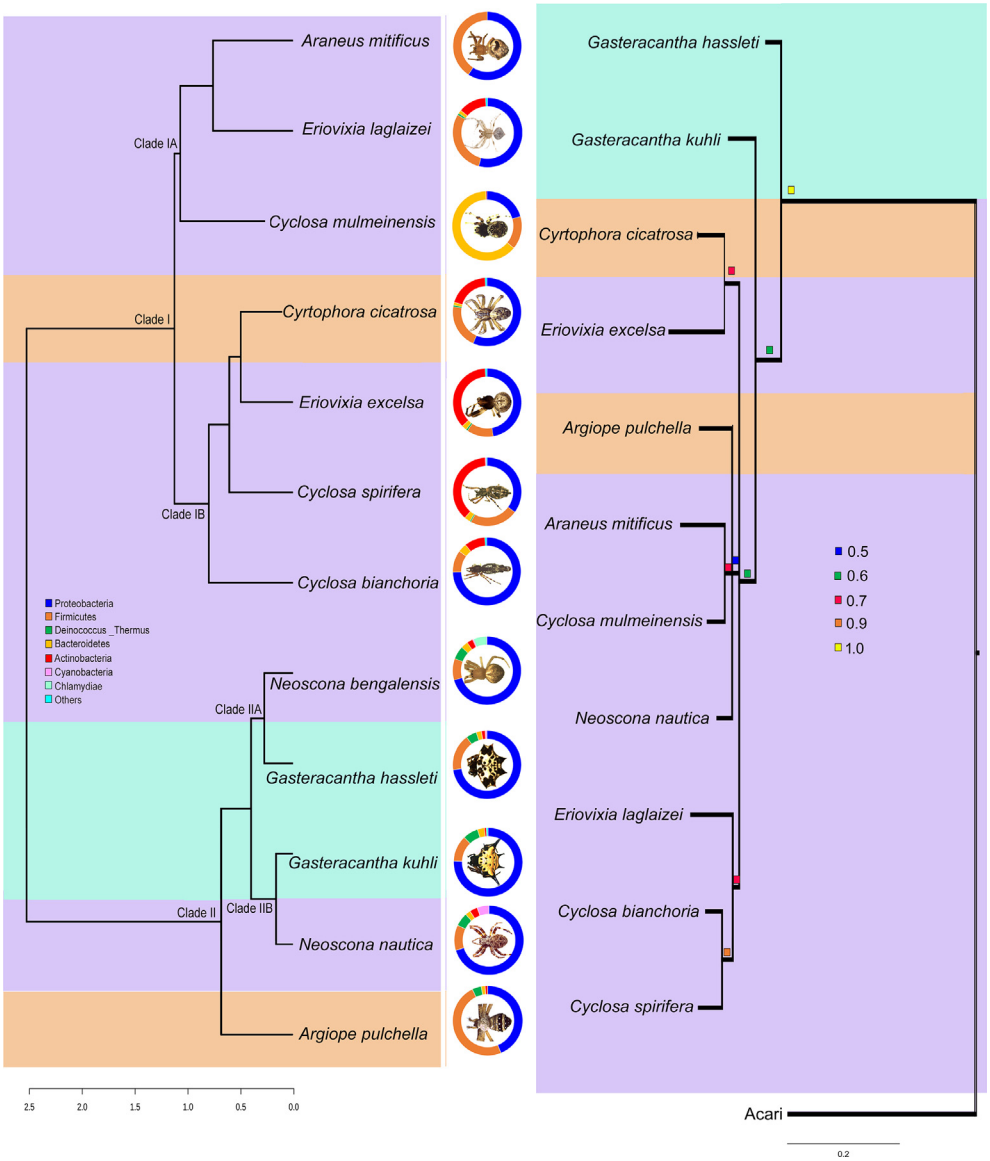

Fig. 9. Dendrogram of β-diversity of twelve species from three subfamilies of the family Araneidae, along with phylum gut bacterial diversity (left side); host species phylogeny (right side).

Argiopinae species *Cyrtophora cicatrosa* is closer to *Eriovixia excelsa* in both phylogenies, while *Argiope pulchella* showed different topologies in both phylogenies. Furthermore, the phylogeny of the species of family Araneinae is not congruent with bacterial composition phylogeny.

Further, the correlation between the host and bacterial composition phylogenies was observed based on statistical test like Mantel test ($r = 0.0674$, $P = 0.31$) with 9999 permutations, and indicating that there is no correlation between the host species and gut bacterial composition phylogeny.

4. Discussion

In the present study, an effort has been made to elucidate the gut bacterial diversity of twelve species of the family Araneidae under three subfamilies collected from 5 states of India along with their predicted metabolic pathways. Further, we have also compared the pattern of gut bacteria with their host species phylogeny.

The family Araneidae comprises 3100 species in 175 genera (World Spider Catalog, 2020). Recently, Scharaff et al. 2019 observed that the family Araneidae is not monoplyletic. This study used multiple molecular markers to determine the classification implications and intrafamilial relationships of family Araneidae and classify it into outgroups (22 genera and 11 families), ingroups (three Zygiellinae and four Nephilinae genera) and ARA Clade genera with 10 informal groups (85 genera). The genera *Argiope* and *Gasteracantha* were included under informal groups of ARA clade Argiopines and Gasteracanthines respectively, whereas the placement of the genera *Araneus*, *Cyclosa*, *Eriovixia* and *Neoscona* is ambiguous. Our BI phylogeny also depicted the similar topology.

The data analysis indicated that the dominant phyla in these twelve Araneidae species were composed of Proteobacteria and Firmicutes, which play an important role in nutrient and energy metabolism as also reported in previous study (Hu et al. 2019). The high abundance of phylum Proteobacteria in the spiders' gut is in line with previous studies on spider and other insects (Hu et al., 2019; Chen et al., 2016; Ruokolainen et al., 2016; Hammer et al., 2017). The other bacterial phyla like Bacteriodetes and Chlamydiae dominated in *Cyclosa mulmeinensis* and *Neoscona bengalensis* respectively.

At the genus level, the dominant genera were *Acinetobacter*, *Bacillus*, *Pseudomonas*, *Cutibacterium*, *Staphylococcus*, and *Coryneobacteria* with considerable abundance throughout the spider species, while an endosymbiont genus *Wolbachia* was observed only in *Eriovixia laglaizei*. The genus *Prevotella* was observed in *Cyclosa mulmeinensis*, it may be due to abnormal r digestion at the time of capturing (Suenami et al 2019). The high relative abundance of the genera *Wolbachia* and *Prevotella*_9 in the gut of these two species have greatly reduced the abundance of other bacteria. Similar results were observed in three spider species (Hu et al. 2019), wherein, the presence of *Wolbachia* and *Rickettsiella* have caused significant differences in the relative abundance of other gut bacteria.

We used the PICRUSt2 for the prediction of the metabolic functional pathways of gut bacteria in spiders. The predicted metabolic function of gut bacteria in spiders were involved in metabolism of amino acids, carbon, fatty acids, glycolysis, TCA cycle, degradation of amino acids and fatty acids, drug and enzyme metabolism etc. Our result is also similar to previous spider gut microbiome study wherein gut bacteria were associated with host nutrient and energy metabolism (Hu et al. 2019). This indicated that the gut bacteria involved in amino acid biosynthesis and carbon metabolism have the highest relative abundance than those involved in other metabolic functions. The alpha diversity based on the richness and diversity estimators concluded that the subfamily Araneinae possess high richness and diversity than Argiopinae and Gasteracanthinae.

Moreover, the host species phylogeny was not congruent with those of bacterial compositions based phylogeny. Our study indicated that the gut bacterial composition may not be influenced by the host species phylogeny. Several other factors like environment conditions, diet and physiology might be shaping the gut bacterial diversity. The structure of gut bacteria in twelve species of spider and their predicted functional metabolic pathways in the current study is the preliminary effort based on 16 s rRNA. It needs future analyses based on metagenomics and metatranscriptomics approaches to explain the relationship of host-microbiome, their structure and functions.

5. Conclusion

This study provides the first baseline data for gut bacterial diversity of twelve spider species of the family Araneidae collected from wild. The result showed that species of three subfamilies shared gut bacterial structure except *Cyclosa mulmeinensis* and *Eriovixia laglaizei* with high abundance of *Prevotella and Wolbachia* respectively. Further, the gut bacterial composition of these twelve species are not in coherence with their species phylogeny. To clarify the relationship between the gut bacteria and their host species phylogeny, extensive sampling along with metagenomics and metatransciptomics analyses with different parameters is needed.

6. Data accessibility

The raw reads were submitted to National Center for Biotechnology Information (NCBI) GenBank under the BioProject ID: PRJNA638522.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

KT involved in specimen collection, identification and DNA isolation; KT, IT and VK involved in Conceptualization, Data Curation, Methodology, Software; KT and IT involved in Bioinformatics analysis, Manuscript writing and Editing. VK supervised the project and Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.06.059.

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