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Analysis on gut microbiota diversity of wild Asian elephants (*Elephas maximus*) from three regions of Yunnan Province

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Studying the gut microbiome diversity of Asian elephants (*Elephas maximus*) is crucial for understanding their environmental adaptability, health status, and conservation needs. In this study, high-throughput sequencing of the 16S rRNA gene was utilized to analyze and compare the microbial community composition and diversity of 50 wild Asian elephants from three regions in Yunnan Province. The results indicated significant differences in gut microbiome richness among the regions, and the lowest diversity observed in the Lincang region. Principal coordinate analysis (PCoA) revealed that the microbial community structure of the Lincang population was markedly different from that of the other two regions. At the phylum level, Firmicutes, Proteobacteria, and Bacteroidetes were the dominant bacterial groups across all three regions. However, in the Lincang region, the abundance of Proteobacteria was the highest and significantly greater than in the other regions. Additionally, the levels of potential pathogenic bacteria, such as *Acinetobacter* and *Stenotrophomonas*, were significantly elevated in the Lincang population compared to the other two regions. Therefore, future conservation efforts need to integrate ecological restoration with microbiome monitoring to mitigate the microbial dysbiosis caused by human disturbances.

Keywords Asian elephant (*Elephas maximus*), Gut microbiome diversity and richness, Gut microbiome function, Wild Asian elephant management

The Asian elephant (*Elephas maximus*) is the largest terrestrial mammal on the Asian continent. Their feeding and migration activities reshape vegetation distribution and soil fertility¹. They also spread seeds and create habitats and food resources, playing a key role in maintaining forest ecosystem balance and protecting biodiversity^{1,2}. Historically, Asian elephants were widely distributed across the Asian continent, covering an area of up to 9 million square kilometers³. Currently, they are found only in 13 countries and regions, including China, with their range reduced by nearly 95%. As a result, the International Union for Conservation of Nature (IUCN) has classified them as an endangered species^{3,4}. In China, Asian elephants are a first-class protected wild animal, mainly found in the regions of Xishuangbanna, Pu'er, and Lincang in Yunnan Province. According to surveys, the population of wild Asian elephants in China reached 290 in 2018, and it is estimated that the current population has exceeded 300⁵.

However, the Asian elephant population still faces threats such as habitat loss, degradation and fragmentation, poaching, and retaliatory killings due to human-elephant conflicts^{6,7}. Therefore, China places great emphasis on the conservation and research of wild Asian elephants, which has now expanded to include studies on their behavior and health conditions^{8,9}. The composition and function of the gut microbiome in mammals are influenced by various factors, and can serve as an important biological indicator for assessing the health status of mammals^{10,11}. Studies have shown that the gut microbiome of Asian elephants (*Elephas maximus*) plays a crucial role in degrading indigestible cellulose and maintaining the host's internal homeostasis¹². The dominant phyla in their gut microbiome are Firmicutes and Bacteroidetes^{13,14}. In Yunnan Province, the core microbiome

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of captive wild Asian elephants are Bacteroidetes, Firmicutes, Planctomycetota, and Verrucomicrobiota¹⁵. Additionally, there are differences in the diversity and structure of the microbiome based on ages³, sex¹⁴, different captive conditions¹⁵, and between captive and wild Asian elephants^{16,17}. Furthermore, the gut microbiome can also influence the adaptability of Asian elephants to a high-fiber diet¹⁸, e.g. Wild Asian elephants have a gut microbiome with rich diversity and a high abundance of hemicellulose-degrading enzymes¹⁴. Moreover, surveys have found that the low abundance of lignocellulose-degrading bacteria and CAZyme content in the gut of the northward-migrating elephant herd in China drove them to migrate in search of low-fiber food sources with suitable nutrition⁶.

At present, most studies on the gut microbiome of Asian elephants in China focus on the characteristics of captive Asian elephants and the impact of human interference on their changes. However, the composition and characteristics of the gut microbiome of wild Asian elephants living in natural ecosystems are still unclear, especially in the Pu'er and Lincang areas. This study used 16S rRNA high-throughput sequencing technology to analyze and compare the gut microbiome structure of wild Asian elephants in three regions of Yunnan, aiming to reveal the characteristics of the gut microbiome of Asian elephants in the wild environment of Yunnan Province, explore the unique features of the gut microbiome of wild Asian elephants in the three regions, and further analyze the changes of gut microbiome under different habitats and their relationship with host adaptability. It is expected to provide valuable insights for the conservation of wild Asian elephants, habitat assessment, gut microbiota-based health management, and habitat restoration and construction.

Results

Sequencing data analysis

After conducting Illumina MiSeq sequencing, a total of 5,956,574 raw reads were generated. After undergoing splicing, filtering, and quality control processes on these raw reads, obtaining a refined dataset of 5,947,652 clean reads. This resulted in an average of 118,953 reads per sample (ranging from 64,603 to 131,355) (Supplementary Table S1). To elucidate the diversity of species composition, operational taxonomic unit (OTU) clustering was performed based on a 97% similarity criterion, yielding a total of 11,157 OTUs, with an average of 1,715 OTUs per sample (ranging from 820 to 2436) (Supplementary Table S1). The rarefaction curves (Fig. 1A) indicated that the sequencing depth was adequate, as additional data were unlikely to yield a significant number of new species. Concurrently, the Shannon diversity curve (Fig. 1B) gradually plateaus with increasing sequencing depth, signifying that the current sequencing depth sufficiently captures the sample diversity, making the data suitable for further analysis.

Alpha diversity and beta diversity analyses

The Kruskal–Wallis rank-sum test was used to evaluate the alpha diversity differences of the Asian elephant gut microbiome across three geographical regions. The results showed significant differences in both the Chao1 index (a species richness indicator; Fig. 2A) ($\text{Chao1: } p = 4.55 \times 10^{-7}$). Dunn's pairwise comparisons revealed that the richness of the Lincang population were significantly Lower than those of the Xishuangbanna ($p < 0.01$) and Pu'er populations ($p < 0.01$). Although there was no significant difference in the Shannon index (a community diversity indicator; Fig. 2B), the alpha diversity of the Lincang population was the lowest overall. Phylogeny-based principal coordinate analysis (PCoA) showed that the weighted UniFrac distance (Fig. 3A) ($R^2 = 0.133, p = 0.001$) indicated greater within-group Beta diversity differences in the Xishuangbanna and Pu'er populations, while the Lincang population exhibited higher within-group homogeneity; the unweighted UniFrac distance (Fig. 3B) ($R^2 = 0.139, p = 0.003$) revealed separation of the Lincang population from the other populations. PERMANOVA analysis further confirmed significant differences in community structure among the three regions ($R^2 = 0.133, p < 0.05$), with the Lincang population showing significant beta diversity differences from the other regions.

Composition of gut microbiome communities of Asian elephants

In this study, sequencing algorithms clustered a total of 11,157 bacterial operational taxonomic units (OTUs), which we annotated into 30 phyla, 70 classes, 167 orders, 302 families, 601 genera, and 737 species. The Venn analysis (Fig. 4A) revealed that among the Asian elephants from Pu'er, Lincang, and Xishuangbanna, there were 3241, 144, and 2896 unique OTUs respectively. Only 1508 OTUs were shared across the three regions, indicating strong regional differentiation (see Supplementary Table S2 for classification details). Our analysis revealed that the shared bacteria among the three regions were mainly from the phyla Proteobacteria, Bacteroidetes, and Firmicutes (Fig. 4B; specific OTUs are listed in Supplementary Table S3). We identified these shared bacteria as including probiotics such as *Allorhizobium*-*Neorhizobium*-*Pararhizobium*-*Rhizobium*, *Lachnoclostridium*, *Devosia*, *Agathobacter*, and *Chryseobacterium*. Additionally, potential pathogens such as *Stenotrophomonas*, *Acinetobacter*, *Enterococcus*, and *Escherichia*-*Shigella* were also identified (see Supplementary Table S3). The potential pathogens may affect host health.

Comparison of gut microbiome of Asian elephants in three locations

At the phylum level (Fig. 5A), the gut microbiome of wild Asian elephants in the three regions (Lincang, Xishuangbanna, and Pu'er) were dominated by Firmicutes, Proteobacteria, and Bacteroidetes, with Verrucomicrobiota being the second most abundant. Specifically, in the Lincang region, the relative abundance of Proteobacteria was the highest (58.05%), followed by Bacteroidetes (22.92%), Firmicutes (10.22%), and Verrucomicrobiota (5.05%), with the abundance of Verrucomicrobiota being higher than in the other two regions. In the Xishuangbanna region, Firmicutes (35.34%) and Proteobacteria (34.76%) were more abundant, while Bacteroidetes had a relatively lower abundance (17.69%). The Pu'er region was similar to Xishuangbanna, with the highest abundance of Firmicutes (56.23%), followed by Proteobacteria (20.19%) and Bacteroidetes (16.58%).

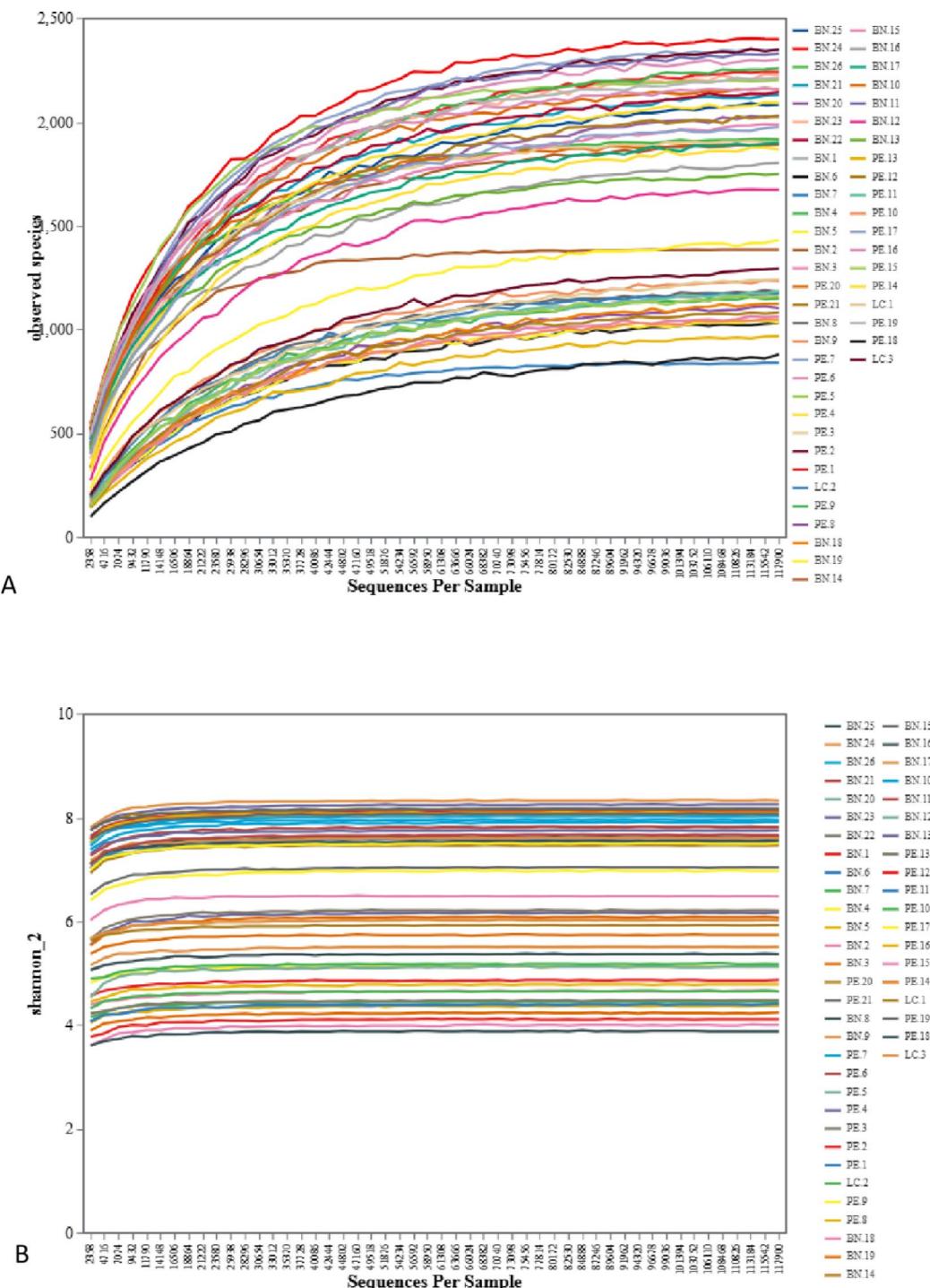


Fig. 1. The observed species rarefaction curves (A) and Shannon diversity index curves (B) for 50 Asian elephant samples collected across the three regions.

At the genus level (Fig. 5B), in the gut microbiome of Asian elephants from Pu'er and Xishuangbanna, the abundance of unclassified bacteria from the family Lachnospiraceae was relatively high (> 14%). In the Lincang region, *Brevundimonas* was the most prominent (9.81%), with higher abundances of *Acinetobacter* (7.67%) and *Stenotrophomonas* (8.01%) compared to Pu'er and Xishuangbanna. Additionally, the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* group was present in all regions at relatively high proportions (> 4%), with the highest abundance in Xishuangbanna (7.43%), followed by Lincang (4.39%) and Pu'er (4.19%).

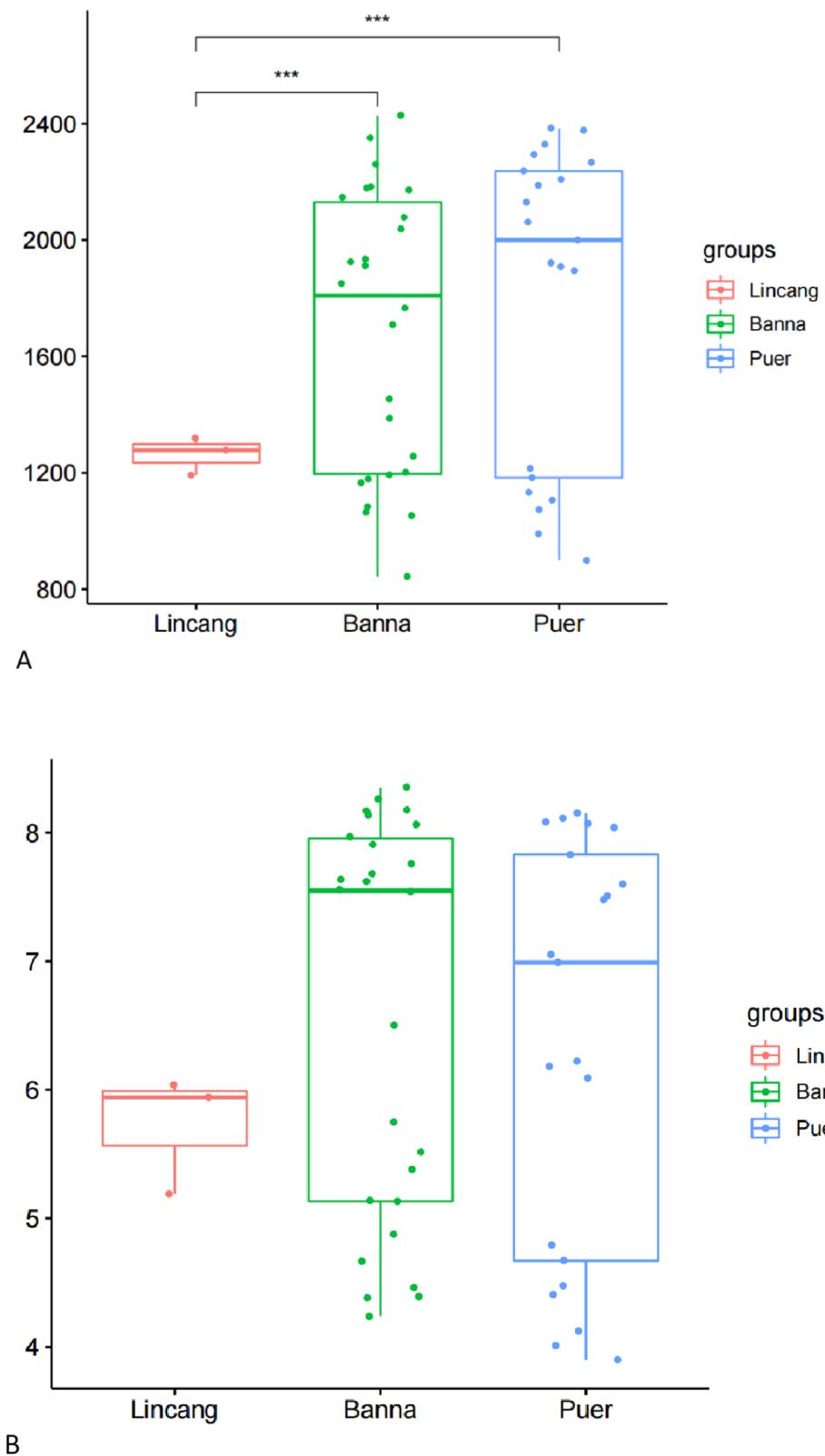


Fig. 2. Boxplots of differences in gut microbiome Chao1 index (A) and Shannon index (B) alpha diversity of wild Asian elephants in three places. Lincang (Red), Xishuangbanna (Green), Pu'er (Blue); Chao1 index ($p < 0.05$): a method to estimate the total number of unknown species in a community based on the number of rare species in the sample. Shannon index ($p > 0.05$), which considers both species richness and evenness, was used to measure the community diversity. The higher the value of the Shannon index, the higher the diversity of the community. The absence of asterisks in the figures indicates no significant differences, $***P < 0.001$.

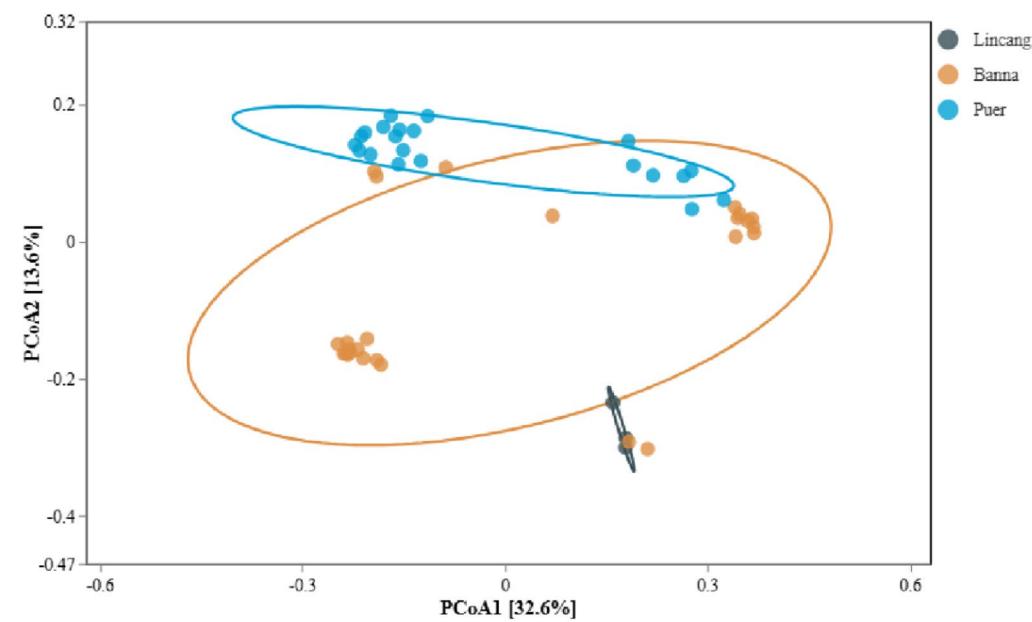
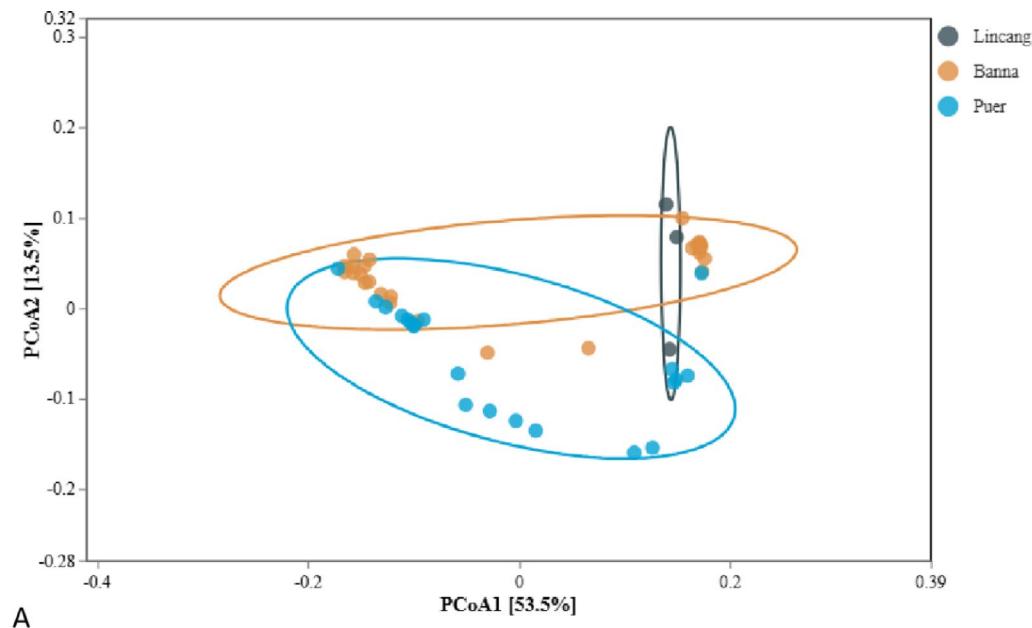


Fig. 3. PCoA diagram of beta diversity differences between the gut microbiomes of Asian elephants in three regions based on weighted_unifrac (A) and unweighted_unifrac (B). Lincang group (gray), Xishuangbanna (orange), Pu'er (blue); (A) The PCoA plot based on the weighted_unifrac algorithm shows the percentage changes in PCoA1 (53.5%) and PCoA2 (13.5%) in different samples; (B) The percentage change of PCoA1 (32.8%) and PCoA2 (13.5%) based on the unweighted_unifrac algorithm.

Analysis of different species of gut microbiome of wild Asian elephants in three places

To clarify the differences in species abundance of gut microbial communities among wild Asian elephants in the three regions (Xishuangbanna, Lincang, and Pu'er), we first used the Linear Discriminant Analysis Effect Size (LEfSe) method to identify significantly different species (LDA score ≥ 4.0 , $p < 0.05$). The results showed that the Xishuangbanna group had 3 enriched taxa, the Lincang group had 26, and the Pu'er group had 16 (Fig. 6). Specifically: Xishuangbanna group: Enterobacteriaceae in Proteobacteria was significantly enriched

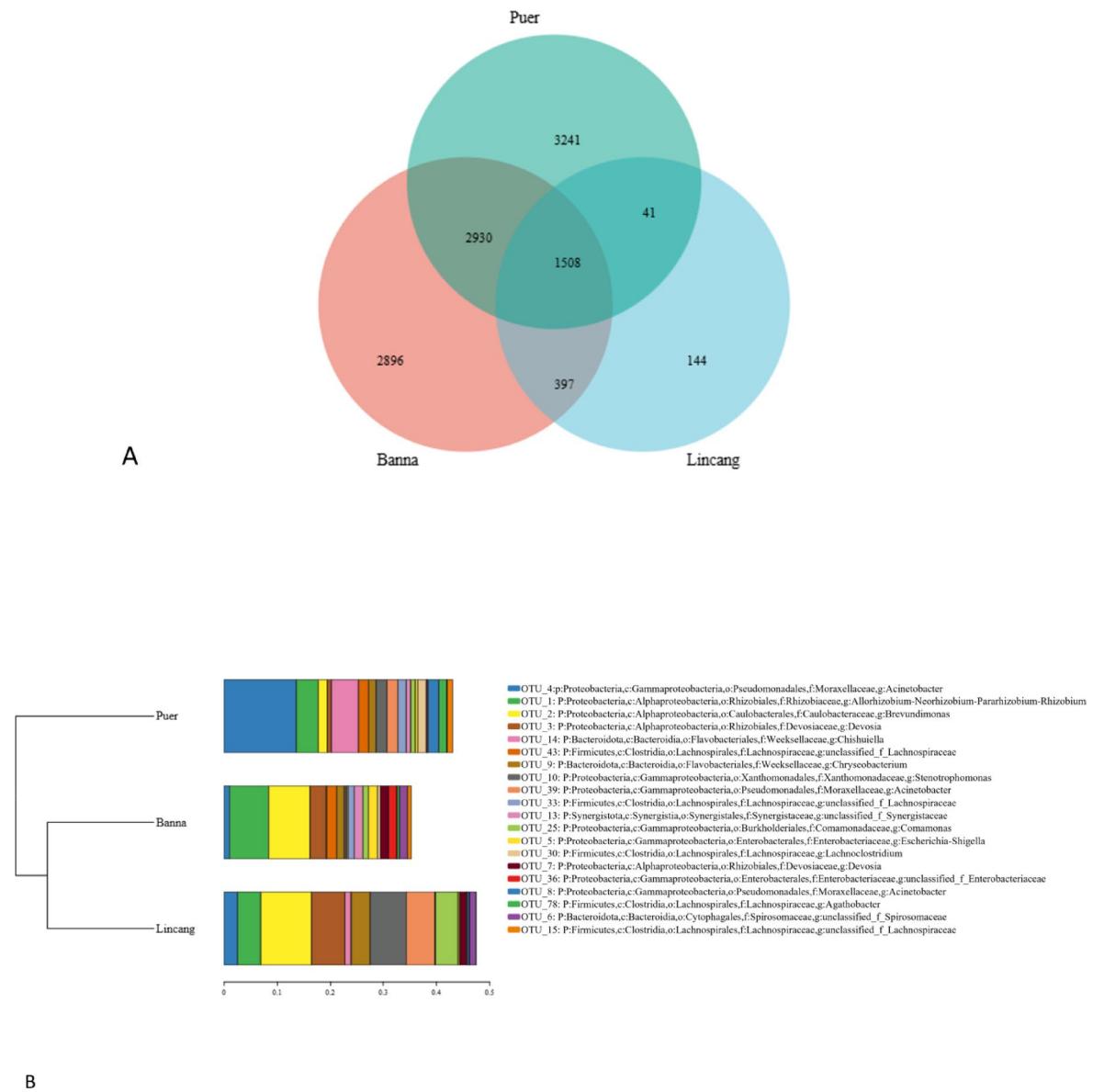


Fig. 4. Venn diagram of shared and unique OTUs of Asian elephant gut microbes at three locations (A) and the top 20 OTUs shared by the three regions (B). In Fig. B, the legend p represents bacterial phylum, c represents bacterial class, o represents bacterial order, f represents bacterial family, and g represents bacterial genus.

($p < 0.05$). Lincang group: Chitinophagaceae ($p < 0.01$), Spirosomaceae ($p < 0.01$), *Sphingobacterium* ($p < 0.01$) in Bacteroidota, and Sphingomonadaceae ($p < 0.01$), Comamonadaceae ($p < 0.05$), *Stenotrophomonas* ($p < 0.05$) in Proteobacteria were significantly enriched. Pu'er group: *Chishuiella* ($p < 0.05$) in Bacteroidota, and Lanococcaceae ($p < 0.01$), Lachnospiraceae ($p < 0.01$), *Oscillospirales* ($p < 0.05$) in Firmicutes were significantly enriched (see Supplementary Table S4).

Further verification of species abundance differences among the three groups was conducted using the Kruskal-Wallis rank sum test (Supplementary Table S5, $p < 0.05$). At the phylum level, the abundance of Proteobacteria in the Lincang group was significantly higher than that in the Pu'er and Xishuangbanna groups ($p = 0.01$); the abundance of Firmicutes in the Pu'er group was significantly higher than that in the other two groups ($p = 0.02$); the abundance of Verrucomicrobiota ($p = 0.03$), Patescibacteria ($p = 0.02$), Bdellovibrionota ($p = 0.01$), Campylobacterota ($p = 0.001$), Fusobacteriota ($p = 0.001$), and Planctomycetota ($p = 0.02$) in the Xishuangbanna and Lincang groups was significantly higher than that in the Pu'er group. At the genus level, the abundance of *Sphingobacterium* in the Pu'er group was significantly higher than that in the other two groups ($p = 0.01$); the abundance of *Enterococcus* ($p = 0.02$), and *Stenotrophomonas* ($p = 0.02$) in the Lincang group was significantly higher than that in the Pu'er and Xishuangbanna groups.

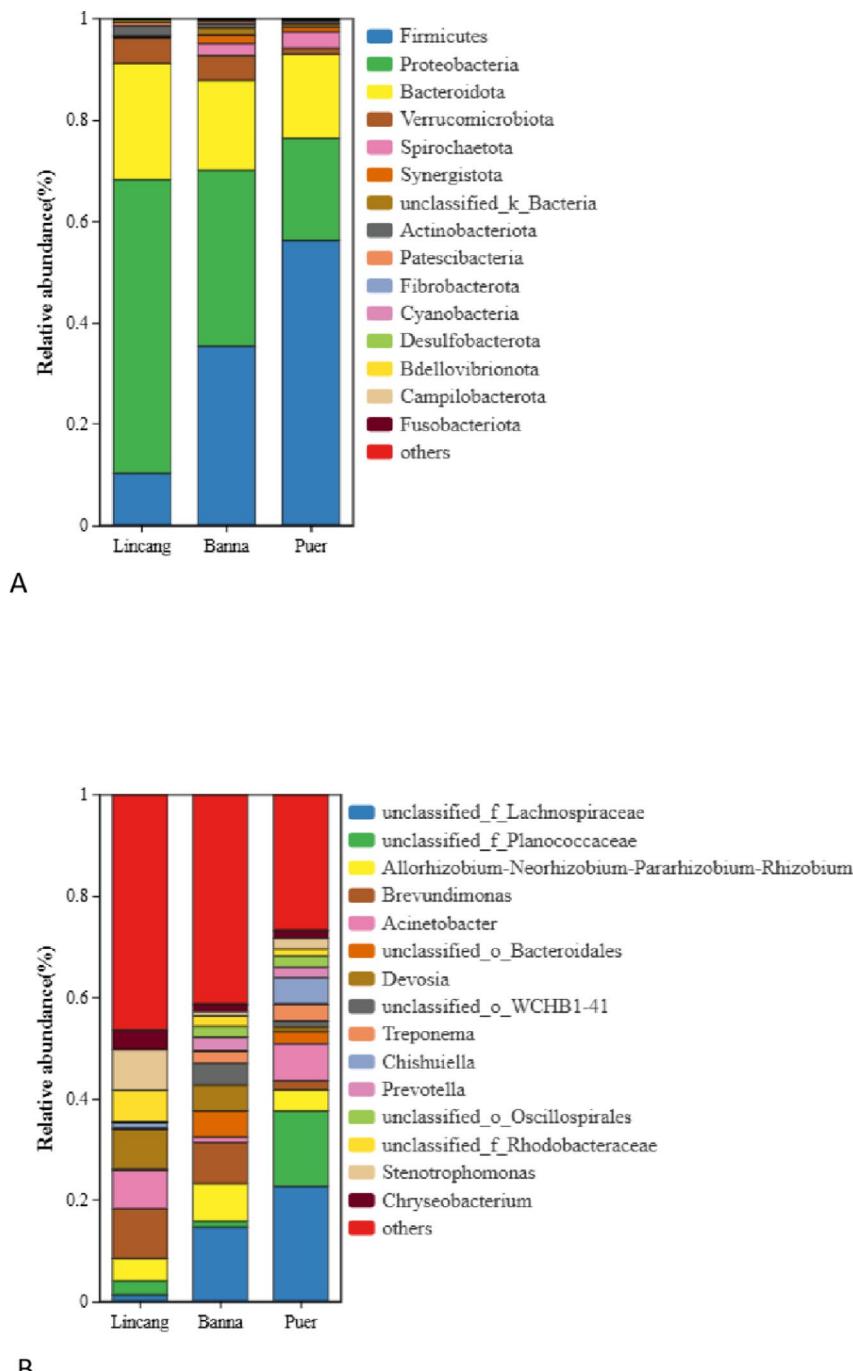
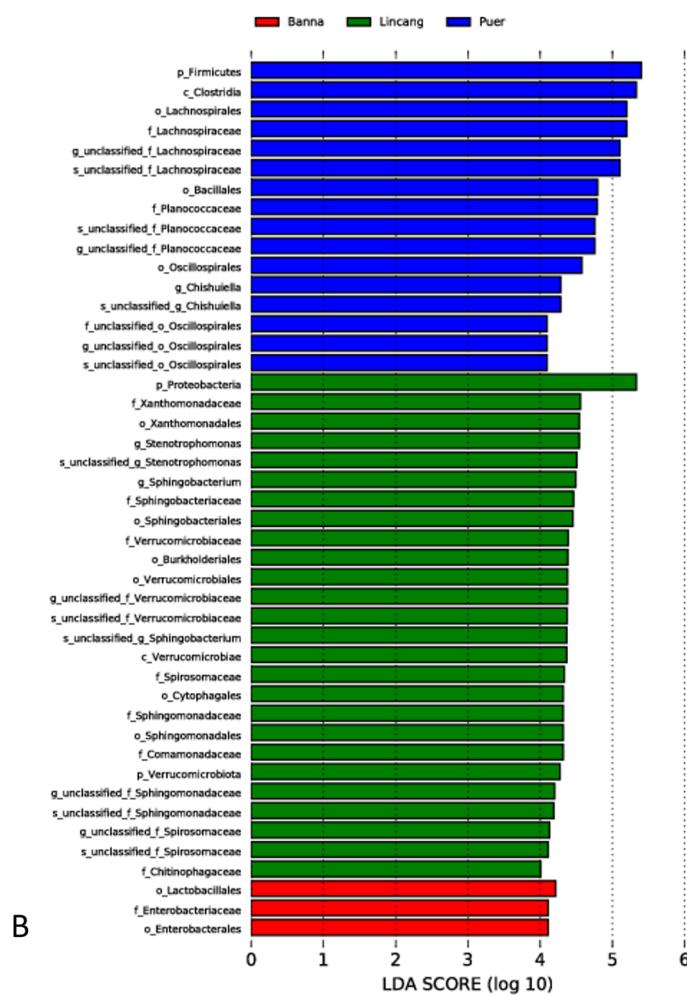
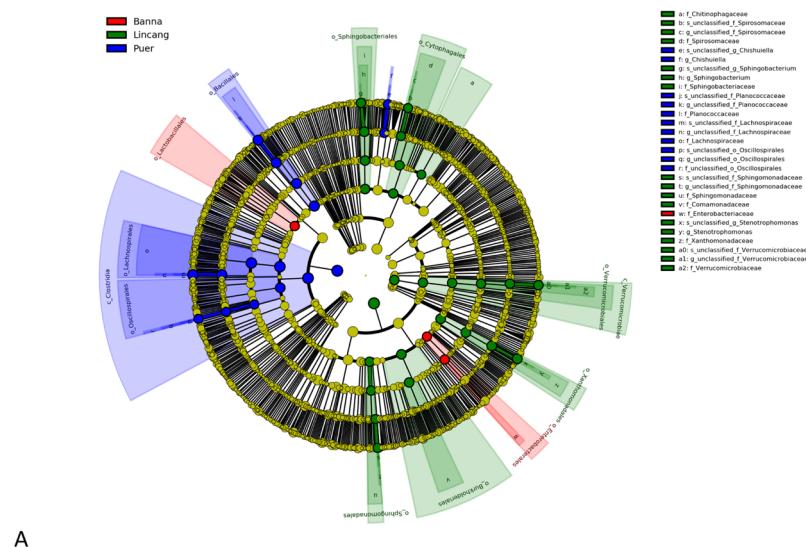


Fig. 5. Histogram of the top 15 bacteria in relative abundance at the level of the Asian elephant phylum (A), and genus (B) in the three locations.

Prediction of gut microbiome function

The functions of the gut microbiome of Asian elephants were analyzed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) tool, which predicts metabolic pathways based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A total of 7,535 KEGG Orthologs were identified, classified into 28 KEGG level 2 pathways and 175 KEGG level 3 pathways. The KEGG level 2 pathways (Fig. 7A) mainly included various metabolic processes, such as, Carbohydrate metabolism, Amino acid metabolism, Metabolism of cofactors and vitamins, Metabolism of terpenoids and polyketides, and energy metabolism. The KEGG level 3 pathways (Fig. 7B) provided detailed descriptions of the specific functions of the microbiome in biosynthesis and metabolism, e.g. biosynthesis of ansamycins, valine, leucine and isoleucine, fatty acids, and the metabolism of C5-branched dibasic acids, D-glutamine and D-glutamate. Cluster analysis in Fig. 7B showed that the gut microbiome of wild Asian elephants in Lincang had higher abundance in ketone body

Cladogram



synthesis and degradation and fatty acid biosynthesis pathways, the gut microbiome of those in Xishuangbanna had higher abundance in vancomycin biosynthesis pathways, and those in Pu'er had higher abundance in the pentose phosphate pathway. However, the Kruskal–Wallis rank sum test indicated that there were no significant

◀ **Fig. 6.** (A) Linear discriminant analysis effect size (LEfSe) analysis of species evolution and (B) LEfSe (LDA Effect Size) analysis plot. (A) Three microbial species with significant grouping differences. The evolutionary map corresponds to different taxonomic levels of phyla, class, order, family and genus from inside to outside, and the lines between the levels indicate the affiliation. Each circle node represents a species, the Yellow node indicates that the difference between groups is not significant, and the non yellow node indicates that the species is the characteristic microorganism of the corresponding group (the abundance is significantly higher in this group). The color sector indicates the classification range of characteristic microorganisms, including Lincang (Green), Xishuangbanna (Red), Pu'er (Blue). (B) The bar represents the LDA score. This visual representation clearly delineates the distinct microbial communities associated with the Lincang group (depicted in green), Xishuangbanna (in red), and Pu'er (in blue), highlighting their significant differences (LDA score ≥ 4.0 ; $p < 0.05$).

differences in the functions of the gut microbiome among the three regions ($p > 0.05$), suggesting that microbial functions did not show statistical heterogeneity among regions.

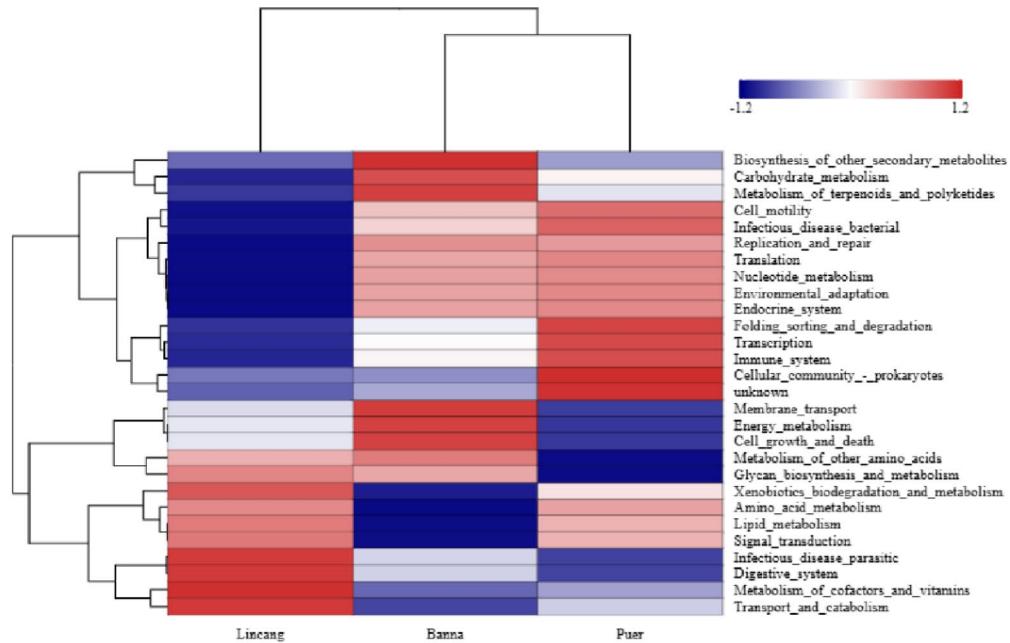
Discussion

The southern region of Yunnan Province in China, which includes Xishuangbanna, Pu'er, and Lincang, serves as the primary habitats for wild Asian elephants. This area is characterized by a unique monsoon climate and a diverse array of vegetation types³. The habitat selection of Asian elephants is highly oriented towards nutritional needs, favoring habitat types with high vegetation cover and abundant food resources (such as bamboo forests, shrublands, and grasslands) to meet their energy intake and survival needs^{19,20}. This study analyzed the gut microbiome characteristics of wild Asian elephants across three regions of Yunnan Province to gain insights into their natural gut microbiome. It is expected to provide valuable references for assessing the suitability of Asian elephant habitats, formulating conservation strategies, and implementing effective habitat restoration measures.

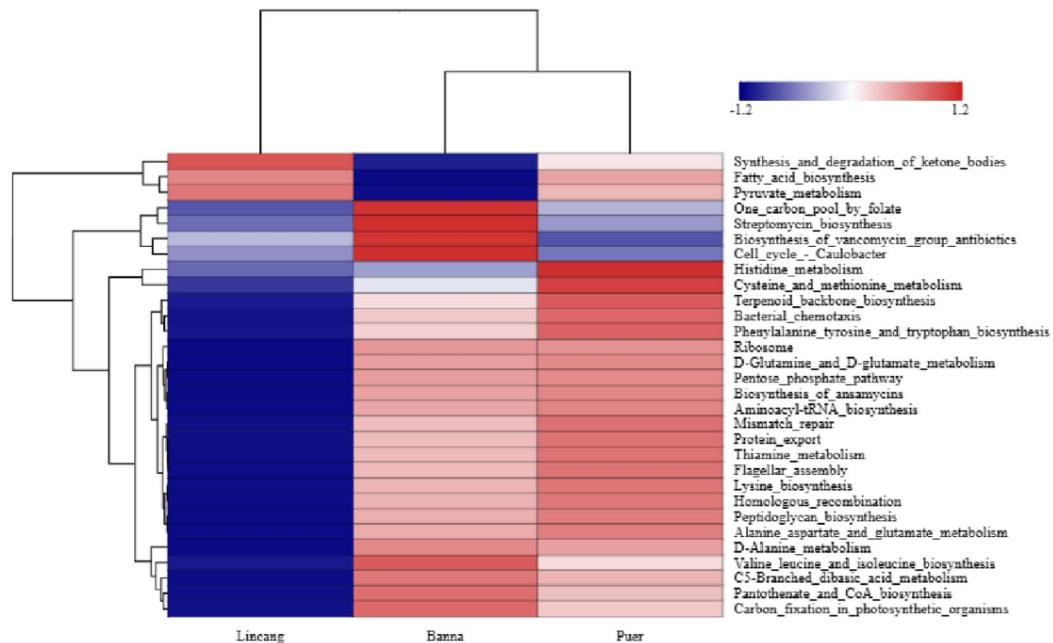
This study utilized high-throughput sequencing of the 16S rRNA gene to analyze the gut microbiome of wild Asian elephants across three regions in Yunnan. The findings indicate that the microbiome is predominantly composed of the phyla Firmicutes, Proteobacteria, Bacteroidetes, and Verrucomicrobiota. This composition aligns with the results of a previous study on healthy captive wild Asian elephants in Yunnan, which identified a core microbiome consisting of Firmicutes, Bacteroidetes, and Verrucomicrobiota¹⁵. This suggests that the core gut microbiome of wild Asian elephants in Yunnan Province is largely similar, although distinct differences exist between captive and wild populations. Among the various bacterial phyla present, Firmicutes and Proteobacteria are the most abundant. These bacteria can produce short-chain fatty acids (SCFAs) through the fermentation of dietary fiber, which is consistent with the feeding habits of wild Asian elephants in Yunnan, which mainly consume high-fiber plants such as Poaceae, Moraceae, Musaceae, and Fabaceae (Supplementary Table S6)^{21,22}. At the same time, Firmicutes can enhance energy acquisition by improving the efficiency of cellulose degradation¹², supporting the high energy needs of wild Asian elephants.

However, in the gut microbiome of wild Asian elephants in Pu'er City and Xishuangbanna Prefecture, Firmicutes are significantly dominant (with higher relative abundance). In contrast, in the wild Asian elephant population in Lincang City, the abundance of Proteobacteria is significantly higher than in the other two places. Studies have shown that plant composition can significantly influence microbial structure (e.g., the regulatory effect of leaf nitrogen content on the ratio of Firmicutes to Proteobacteria, high-fiber plants promote the enrichment of Firmicutes, while low-fiber or high-protein diets may lead to the proliferation of Proteobacteria^{23,24}). The differences in gut microbiome among Asian elephants in the three regions of Yunnan Province may stem from the feeding differences of elephants in different habitats (Supplementary Table S6), but the exact correlation between diet and microbiome still needs to be verified by multi-omics approaches in combination with plant physiological characteristics and host metabolic pathways. It is worth noting that the gut microbiome of healthy elephants is predominantly composed of the phylum Firmicutes, while an increased abundance of Proteobacteria is significantly associated with gastrointestinal diseases²⁵. The high abundance of Proteobacteria in the Lincang population suggests potential health risks. Therefore, there is an urgent need to establish a dynamic database of gut microbiome for Chinese Asian elephants. Long-term monitoring of microbial changes at different times (seasons, ages), states (healthy/sick), and habitats will help elucidate the interaction mechanisms between diet, microbiome, and the health of wild Asian elephants in Yunnan Province.

By comparing with the gut microbiome composition of captive Asian elephants in Yunnan Province in the previous period, it was found that the abundance of Bacteroidetes was the highest in the gut microbiome of captive Asian elephants²⁴, which may be related to their long-term intake of high-sugar, high-fat artificial diets (such as bananas, sugarcane, and commercial feed)^{15,22,26}. In contrast, the wild populations are mainly dominated by Firmicutes or Proteobacteria, further emphasizing the impact of diet structure (such as high-fiber natural food) on microbial community composition. In the three regions of this study, the abundance of Bacteroidetes generally ranks second, while the abundance of Verrucomicrobia in specific regions (such as Lincang) is significantly higher than that in other groups, indicating that geographical and breeding environmental factors may lead to spatial heterogeneity of the microbiome. It is worth noting that an increase in the Firmicutes/Bacteroidetes ratio and a decrease in the abundance of Verrucomicrobia have been associated with metabolic disease risks (such as obesity and inflammatory bowel disease) in humans and mouse models^{16,27,28}. Although this ratio cannot be directly used as an assessment marker for obesity in Asian elephants, it does suggest that in order to optimize the health management of Asian elephants, it is urgent to establish an association model between gut microbiome characteristics and disease phenotypes (such as obesity) and implement obesity monitoring strategies for wild populations to mitigate potential health risks in the wild.



A



B

Fig. 7. Clustering of KEGG functional relative abundance of gut microbiota in wild Asian elephants from three locations in Yunnan Province: (A) KEGG Level 2 pathways and (B) KEGG Level 3 pathways. The functional information shown in the figure is a predictive analysis based on PICRUSt sequencing data, which can be regarded as an inference based on evolutionary relationships, but not an exact functional performance.

At the genus level, the *Rhizobium-Neorhizobium-Pararhizobium-Rhizobium* group was detected in the guts of wild Asian elephants in all three regions. As a key indicator of plant rhizosphere, it may enter their gut through the feeding behavior of these elephants^{29,30}. Given the high abundance of Lachnospiraceae in the gut microbiome of wild Asian elephants, this family is commonly found in mammalian guts, and its members produce short-chain fatty acids (SCFAs) through the fermentation of polysaccharides, which have anti-inflammatory properties that

maintain intestinal immune homeostasis³¹ and inhibit the colonization of opportunistic pathogens (such as *Pseudomonas*)³². Therefore, their feces can be considered a potential reservoir of beneficial bacteria for the development of probiotic resources.

This study found that the gut microbiome of the wild Asian elephant population in Xishuangbanna contains unique bacterial communities such as *Gemmamimonadota*, MBNT15, and *Dependentiae* (Supplementary Table 4). These bacterial communities are commonly found in soil and water environments and are involved in the dissimilatory reduction of iron^{33,34}. The unique climatic characteristics of Xishuangbanna, such as high temperatures, heavy rainfall, and distinct dry and wet seasons, lead to an abundance of iron and aluminum oxides in its soil environment, which mainly results from the ferruginous and aluminous weathering processes under strong weathering^{35,36}. In this study, we also identified potential pathogens such as *Stenotrophomonas*, *Acinetobacter*, *Enterococcus*, and *Escherichia-Shigella* which are known to cause diseases in a variety of animals, including humans^{37,38}. *Enterococcus* in captive Asian elephants in China has been detected with multidrug resistance (such as tet(M) and erm(B) genes) and virulence factors (such as gelE and biofilm formation), and the genus *Escherichia* is known to cause diarrhea in Asian elephants^{39,40}. The abundance of *Enterococcus* and *Stenotrophomonas* in the gut of the Asian elephant population in Lincang was significantly higher than in the other two areas. Therefore, it is essential to establish a long-term monitoring network for pathogens and antibiotic resistance genes in the Lincang area to reduce the risk of zoonotic diseases.

Alpha diversity analysis indicates that there are significant differences in the richness of gut microbiome among wild Asian elephants in Lincang, Pu'er, and Xishuangbanna in Yunnan Province. And the Lincang population having the lowest Alpha diversity and the Lincang population the lowest. This suggests that habitat environments may indirectly affect gut microbiome. Studies have shown that Asian elephants with gastrointestinal symptoms generally experience a decline in microbial diversity and functional disorders^{25,41}. In light of the findings of this study, it can be inferred that the Lincang population may have a higher potential health risk and needs enhanced health monitoring. Beta diversity analysis shows that the Lincang population is significantly separated from the other two regions, which is consistent with the conclusion in African elephant studies that “habitat type and diet composition shape the microbiome”⁴². The long-term geographical isolation of the Lincang population from the other two regional populations and their different genetic adaptations may also affect microbial colonization^{43,44}.

Based on PICRUSt2 functional prediction, the core metabolic functions of the gut microbiome in wild Asian elephants are concentrated in carbohydrate and amino acid metabolic pathways, which is consistent with previous studies^{12,45,46}. This is functionally coupled with the high abundance of the phylum Firmicutes, which is characterized by high activity of hemicellulose-degrading enzymes encoded by this phylum, confirming its evolutionary adaptation to the energy conversion of high-fiber plants in the habitat¹⁴. Although there is no significant difference in the gut microbial functions of wild Asian elephants from different geographical regions, the community composition reflects adaptive adjustments to the habitat environment¹⁷. Studies have confirmed that human interventions such as migration, captivity, and deworming^{17,47}, age⁴⁸, diet⁴⁹, and health status²⁵ both may significantly reduce the gut microbial diversity of Asian elephants. Based on the above findings, it is recommended to prioritize the construction of ecological corridors in fragmented habitat areas and optimize food resource supply to maintain gut microbiome homeostasis, thereby supporting the long-term health of Asian elephant populations.

However, the limitations of 16S rRNA gene sequencing and PICRUSt2 prediction technology, coupled with the limited sample size and individual information, make it difficult to achieve precision at the species level and to accurately infer the relationship between metabolic pathways and health phenotypes. Therefore, future studies should focus on expanding the sample size and combining metagenomic sequencing to validate the predicted metabolic pathways. Meanwhile, it is essential to establish individual tracking databases and integrate multi-omics approaches to identify the drivers of long-term microbiome changes, thereby creating a dynamic database of gut microbiome for wild Asian elephants in China. This will provide a solid scientific basis for the conservation of Asian elephants.

Conclusion

This study employed high-throughput sequencing technology to systematically analyze the gut microbiome of wild Asian elephants in the Lincang, Pu'er, and Xishuangbanna regions of the Yunnan Province. The study found that although these populations share common bacterial groups, such as Firmicutes, Proteobacteria, and Bacteroidetes, there are significant differences in the composition and richness of their gut microbiome across different regions, which may be related to habitat and dietary differences. Notably, the gut microbiome of the three populations exhibited high functional conservation, indicating the adaptive capacity of the Asian elephants to the environment through their gut microbiome. Future studies need to expand the sample size and integrate multi-omics analysis methods, such as metagenomics, to obtain a comprehensive dynamic feature library of gut microbiome in wild Asian elephants in Yunnan Province.

Methods

Sample collection

From 2022 to 2023, our research team collected 50 fecal samples from wild Asian elephants across three regions in southern Yunnan Province: Xishuangbanna Dai Autonomous Prefecture (100°03'–101°50'E, 21°08'–22°36'N), Pu'er City (100°57'E, 22°49'N), and Lincang City (98°57'–99°26'E, 23°9'–23°40'N). From 2022 to 2023, our research team collected 50 fecal samples from wild Asian elephants in Xishuangbanna, Pu'er City, and Lincang City, Yunnan Province. With the assistance of the Yunnan Provincial Forestry and Grassland Bureau and local departments, we utilized the “Asian Elephant Monitoring and Early Warning App” and drone technology to collect fresh fecal samples after the elephants had vacated the area. We stored the samples in 50-ml sterile

Parameter	Xishuangbanna	Pu'er	Lincang city
Latitude/longitude	100°03' ~ 101°50'E, 21°08' ~ 22°36'N	100°57'54.1"E, 22°49'38.1"N	98°57' ~ 99°26'E, 23°9' ~ 23°40'N
Vegetation characteristics	Tropical rainforest, monsoon evergreen broad-leaved forest, and seasonal rainforest	The vegetation is dominated by monsoon evergreen broad-leaved forest, thermophilic coniferous forest, and hot-shrub and shrub-grassland	The vegetation is primarily characterized by monsoon evergreen broad-leaved forest, thermophilic coniferous forest, hot shrub and shrub-grassland, tropical rainforest and seasonal rainforest, as well as xerothermic shrub and shrub-grassland
Average elevation (m)	1450	1050	1401
Climate type	Tropical Monsoon Climate	Subtropical monsoon climate	Low-latitude plateau and mountain monsoon climate
Annual mean temperature (°C)	>22	15–23	18.8
Annual precipitation (mm)	1193.7–2491.5	1514	1259
Topographical features	Generally gentle relief	High in the northwest and low in the southeast	Uplift in the center and subsidence around
Soil characteristics	The soil is predominantly laterite with relatively high fertility	The soil consists of red soil and yellow soil, both of which have good fertility	Red soil is predominant, with yellow soil in some areas, and the fertility is moderate
Soil iron and aluminum content	Highest	Lowest	Low
Soil pH	4.5–6.2	5–6	Average 5.27
Soil texture	Heavy in forested areas	Loose to moderate	Loose to moderate
Soil water retention	High moisture but strong drainage	Best	Strongest drainage

Table 1. Characteristics of the three research areas.

Location	Place of collection (No.)	Number of samples
Xishuangbanna	Mengla County–Huimanhe (BN1–9)	9
	Menghai County–Naka Village (BN10–19)	10
	Jinghong City–Xiamancha (BN20–26)	7
Pu'er	Simao District–Simaogang Town (PE1–4)	4
	Simao District–Liushun Town (PE5–15)	11
	Simao District–Yunxian Yi Ethnic Township (PE16–17)	2
	Simao District–Liushun Town Elephant Canteen (PE18–21)	4
Lincang city	Nangun River nature reserve (LC1–3)	3

Table 2. Information on the collection of fecal samples from Asian elephants.

centrifuge tubes, transported them using dry ice, and preserved them in a –80 °C laboratory freezer. When we collected the samples, none of the wild Asian elephants displayed any obvious signs of disease. Details of the three regions are shown in Table 1, and more information about the samples can be found in Table 2.

DNA extraction and Illumina sequencing

All the collected fecal samples from each Asian elephant were subjected to microbial DNA extraction utilizing the ALFA-SEQ Magnetic Soil DNA Kit (Qiagen, USA) and a nucleic acid extractor. The concentration, purity, and integrity of the DNA were then assessed using Nanodrop One (Thermo Fisher Scientific, MA, USA) and 1.5% Agarose gel electrophoresis. After detecting qualified DNA nucleic acids as templates, we used the sample utilizing universal primers for the 16S rRNA V3–V4 region, specifically 338F(5'-ACTCCTACGGAGGC AGCA-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') to carry out Polymerase chain reaction (PCR) amplification. The PCR reaction system was composed of a total volume of 50 μL: premix taq (2 ×) 25 μL; Primer: 338F (10 μM) 1 μL; Primer: 806R (10 μM) 1 μL; DNA 50 ng; nuclease free water added to 50 μL. Reaction procedure: 94 °C initial denaturation for 5 min; 30 cycles including (94 °C, 30 s; 53 °C, 30 s; 72 °C, 30 s); 72 °C, 8 min. After the reaction was completed, we used GeneTools Analysis Software (v.4.03) for concentration comparison and pooled the PCR products. We recovered the PCR mixed products using the E.Z.N.A.® Gel Extraction Kit, and eluted and recovered DNA fragments with a main length of 400–450 bp using TE buffer. The library construction was performed according to the standard protocol of NEBNext® Ultra III DNA Library Prep Kit for Illumina®. Finally, we sequenced the constructed amplicon library using the Illumina platform with paired-end sequencing sequencing (PE250).

DNA sequence and statistical analysis

The raw sequence data obtained from sequencing were processed using fastp (an ultra-fast all-in-one FASTQ preprocessor, version 0.14.1, <https://github.com/OpenGene/fastp>) and usearch-fastq_mergepairs (V10, <http://www.drive5.com/usearch/>) for splitting, sequence correction (polishing), format conversion, removal of host sequences, and elimination of sequences that did not match the primers. This processing resulted in valid data known as clean reads⁵⁰. Using the UPARSE(Uclust-based Parser for ASV and OTU clustering) method, clean sequences with 97% identity were clustered into OTUs (Operational Taxonomic Units)⁵¹. Use usearch -sintax/

blast to compare the OTU representative sequences with databases such as SILVA(16S)⁵² to obtain species annotation information. R software (version 5.1.3) was utilized to calculate the relative abundance of species at each taxonomic level, and the results of species annotation were visually analyzed. Based on the operational taxonomic unit (OTU) abundance table, the Chao1 and Shannon indices were calculated using the usearch-alpha_div command (version 10, <http://www.drive5.com/usearch/>) and the R package to evaluate the alpha diversity of the intestinal microbial community and to test for diversity differences between various groups. Additionally, the alpha_div_rare function from usearch (version 10) was employed to generate the diversity index dilution curve. Apply the vegan package in R software (version 5.1.3) to create Principal Coordinates Analysis (PCoA) for Beta diversity comparison based on the unweighted and weighted UniFrac distance algorithms. Furthermore, we combined the vegan and pegas packages with Lefse software to identify, evaluate, test the significance of, and correct for species with significant abundance differences among groups. Subsequently, we standardized the OTU abundance table using PICRUSt2 and compared it with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to obtain functional annotation information for KEGG Orthology (KO), pathways, and Enzyme Commission (EC)⁵³.

Data availability

The raw 16S rRNA gene sequence data and metadata files in this study are available in the NCBI sequence read archive under the accession number PRJNA1221814.

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Author contributions

Zeya Yang and Yixuan Wang conducted the selection of experimental materials and the experimental operations. and the drafting of the initial manuscript was written by Zeya Yang; Yixuan Wang, Ruimei Wang, Mingfei Shi, Yuhan Wang, and Mingwei Bao conducted the collection of experimental samples; and Jiuxuan Zhou, Ruiling Yuan, and Binghui Wang analyzed the results and revised the article. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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