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RESEARCH ARTICLE

Potential Negative Impact of *Actinobacteria* Phylum on Middle-Aged Equines Based on 16s rDNA Analysis

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ABSTRACT

The gut microbiota plays a crucial role in maintaining the host's health by enhancing immune function and resisting foreign pathogens. However, there remains a lack of comprehensive understanding regarding age-related alterations in the gut microbiota of equines. DNA was extracted from fecal samples collected from juvenile and middle-aged equines, followed by comprehensive data analysis, integration, and refinement. Subsequently, diversity analysis, taxonomic classification, and differential analysis were conducted to investigate the differences in gut microbiota between middle-aged and juvenile equines. The composition and diversity of the intestinal microbiome in middle-aged equines differs significantly from that of juvenile equines. There is an increase in species diversity within the *Firmicutes*, *Verrucomicrobia*, *Anaerovorax*, and *Akkermansia* phyla, while a decrease is observed in the *Bacteroidota*, *Proteobacteria*, *Empedobacter*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* phyla. The population of *Actinobacteria* exhibits a significant increase in middle-aged equine compared to that observed in juvenile equines. Additionally, functional prediction analysis reveals enrichment of outer membrane receptor proteins (mostly Fe transport), AcrA subunit of multidrug resistance transporter (membrane fusion protein), and dipeptidyl aminopeptidase/carboxypeptidase in differential microbiomes. The aging process may induce modifications in the composition of intestinal microbiota, thereby influencing iron transporters, multidrug-resistant transporters, and transcriptional regulatory factors. The enhanced presence of *Actinobacteria* has the potential to modulate the susceptibility of middle-aged equines to diseases through regulation of iron transport proteins and multidrug resistance efflux pumps. These findings provide a theoretical foundation for further exploration into the impact of age on the compositional characteristics of the equine intestinal microbiome.

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INTRODUCTION

The gut microbiota is believed to comprise thousands of species of bacteria, archaea, protozoa, viruses, and fungi that make significant contributions to the host's health (Guzzo *et al*., 2022). These contributions include enhancing local mucosal immune function, producing short-chain fatty acids (SCFAs), promoting antigen tolerance development (Goulet, 2015; Frati *et al*., 2018; Martin-Gallausiaux *et al*., 2021), as well as providing protective effects against colonization by other microorganisms (Caballero-Flores *et al*., 2023). Although over 1000 bacterial species have been identified in the digestive cells of horses with a capacity for accommodating 150 liters of organic matter (Julliand and Grimm, 2016), there remains limited understanding regarding the gut microbiota in horses. Furthermore, the gut microbiota plays a crucial role in providing daily energy for horses (Argenzio *et al*., 1974; Daly *et al*., 2001; Ericsson *et al*., 2016) while also enhancing local mucosal immune function and regulating B cell and T cell responses to modulate immune reactions in horses (Boucher *et al*., 2024).

Age-related changes in the composition of specific bacterial genera and species have been observed, with significant inter-individual variations. Studies have

reported that the overall count of anaerobic bacteria remains relatively stable in elderly fecal samples (Woodmansey *et al*., 2004; Salazar *et al*., 2019), while notable alterations occur in the relative abundance of different genera. Furthermore, a decrease in *Bacteroides* species diversity has been documented among elderly individuals compared to healthy young volunteers (Chung *et al*., 2020). The *Bacteroides* genus is renowned for its nutritional versatility and crucial role in polysaccharide digestion within the colon (MacFarlane and Gibson, 1991; Wang *et al*., 2021), making it an important consideration. These nutritionally valuable subgroups may exert significant effects on both elderly hosts and other bacteria through complex cross-feeding networks at a species level within the gut microbiota (Tamayo *et al*., 2024). Importantly, diminished amylase activity has been observed in healthy aging populations as well as older patients undergoing minor antibiotic treatment (Woodmansey *et al*., 2004), further emphasizing the significance of the *Bacteroides* genus for nutrition. However, it remains unclear whether age influences equine gut microbiota similar to humans, and research on this topic is limited.

By employing high-throughput sequencing technology targeting the 16S rDNA, it becomes feasible to comprehensively sequence the entire bacterial population within specific environmental or habitat samples (Wensel *et al*., 2022). This capability enables the investigation of microbial community composition in environmental samples and facilitates the interpretation of their diversity, abundance, and population structure. Consequently, 16S rDNA can further promote exploration into the interdependent relationship between microorganisms and their environment or host. The conventional approach to microbial research relies on laboratory cultivation, while the advent of high-throughput sequencing technologies such as 16S amplicon sequencing has bridged the research gap about uncultivable microorganisms in traditional laboratories and expanded the potential for harnessing microbial resources (Woo *et al*., 2008). These advanced tools offer effective means for investigating microbial interactions. The 16S rDNA is situated on the small subunit of prokaryotic ribosomes and encodes the DNA sequence that corresponds to the 16S rRNA, which serves as the coding gene for 16S rRNA. This gene has a total length of approximately 1542bp and comprises nine variable regions and ten conserved regions (referred to as V1-V9). These conserved regions elucidate phylogenetic relationships among distinct biological species, while the variable regions exhibit variations across different species, with their diversity closely linked to bacterial system evolution. Therefore, this fragment is widely recognized as an ideal indicator for bacterial system evolution and classification identification.

Given the pivotal role of microbiota in maintaining health, alterations in its composition may potentially contribute to disease mechanisms or susceptibility and impact host immunity during aging. Therefore, employing 16S rDNA technology can enhance our comprehension of gut microbiota variations among horses across different age groups, facilitating the exploration of the correlation between age and gut microbiota disparities while shedding light on their underlying interactions. In this study, significant differences were observed in the composition of the *Bacteroidales*, *Bacteroidota*, *Bacteroidia*, *Baclllales*, and *Planococcaceae* between middle-aged and young horses. Furthermore, distinct microbial communities exhibited enrichment in signaling pathways such as outer membrane receptor proteins and multidrug efflux pump subunit AcrA (membrane fusion protein), as well as dipeptidyl aminopeptidase/acylaminoacyl peptidase. These findings provide a theoretical foundation for further investigation into the effects and roles of age on the intestinal microbiota in horses.

MATERIALS AND METHODS

Sample acquisition: Fecal samples were collected from nine Middle-aged equine (older than 10 years) and eight Juvenile equines (younger than 10 years) of comparable body weight and in optimal health conditions, sourced from a commercial farm (Shenzhen, China). Stool samples were rapidly frozen using liquid nitrogen for subsequent analysis of the 16S rDNA sequencing.

DNA extraction and sequencing: Fecal total microbial DNA was extracted using the fecal Genome DNA Extraction Kit (BioTeke, China). Quantitative analysis of the DNA was performed using Qubit from Invitrogen in the United States. PCR amplification was carried out with universal primers F/R (Logue *et al*., 2016). Purification of the PCR products was done using AMPure XT Beads (Beckman Coulter Genomics, Danvers, MA, USA), and quantification was carried out using Qubit from Invitrogen (USA). The quality assessment of qualified PCR products involved the use of an Agilent Bioanalyzer system (Agilent Technologies Inc., USA) and Illumina library Quantification kit provided by Kapa Biosciences (Woburn, MA, USA). These kits were subsequently pooled together and sequenced on an Illumina NovaSeq™6000 platform (PE250) provided by LC-Bio Technology Co., Ltd., Hangzhou China.

Data partitioning, concatenation, and refinement: For paired-end sequencing data, the samples should be divided based on barcode information and adapter sequences must be eliminated. Initially, RawData was processed using cutadapt software (v1.9) with the parameters "-g R1 -G R2 -n 1 -O 17 -m 100", which eliminates primer sequences and balances base sequences from RawData. Subsequently, paired-end reads were merged into longer tags using FLASH software (v1.2.8) with the parameters "-m 10 -M 100 -x 0.25 -t 1 -z", assembling each pair of paired-end reads based on the overlapping region. Following this, quality trimming of reads was performed using fqtrim software with the parameters "-P 33 -w 100 -q 20 –l –m5 –p1 –V –o trim.fastq.gz". A default window size of 100bp was used for window-based quality scanning; if the average quality value within a window was less than or equal to 20, trimming occurs from the start to end of that window in a read sequence. Subsequently, Vsearch software (v2.3.4) with default parameters removes sequences shorter than <100bp after trimming and chimeric sequences containing >5% N (ambiguous bases). Finally, length filtering and denoising were carried out using qiime dada2 denoise-

paired function in DADA2 to obtain Amplicon Sequence Variant (ASV) characteristic sequences and ASV abundance tables while removing singleton ASVs from all samples.

Diversity analysis: The diversity of alpha and beta diversity analysis was assessed by examining the characteristics of ASV sequence and ASV abundance form. Alpha diversity analysis primarily evaluates the within-territory diversity using seven indices: observed_species, shannon, Simpson, chao1, goods_coverage, Pielou_E, and ACE. Beta diversity was evaluated through the calculation of four distances (weighted_unifrac, unweighted_unifrac, jaccard, bray_curtis) and six analyses to assess inter-habitat diversity (between samples/groups).

Taxonomic classification: The species annotation was performed using the SILVA database (Release 138, https://www.arb-silva.de/documentation/release138/) and NT-16S database (Release 20230718) with annotation thresholds based on the ASV sequence files. Subsequently, the abundance of each taxonomic level in each sample was calculated using the ASV abundance table.

Differential analysis: Based on the species abundance statistics, appropriate statistical methods were employed to analyze the differences between the comparison group based on sample characteristics: Fisher's exact test was utilized when there were no biological repeated samples for comparison; Mann-Whitney U test (also known as Wilcoxon rank sum test) was used for comparing two sets of biologically repeated samples; Kruskal-Wallis test was employed for comparing multiple groups with biologically repeated samples. The significance threshold for screening was set at $P<0.05$.

RESULTS

Alpha diversity: The Shannon, Simpson, Chao1, Goods coverage, and Pielou-e indices were employed to evaluate the overall diversity of equine gut microbiota in terms of richness, evenness, biological coverage, and diversity (Fig. 1A-E). Our findings revealed no significant variation in species richness, evenness, and sequencing depth across different age groups of equines. Moreover, the validity of these five indices was further confirmed by the results obtained from rarefaction curve (Fig. 1F-J).

Beta diversity: The concept of beta diversity pertains to the dissimilarity in species composition among distinct environmental communities. Together with alpha diversity, beta diversity contributes to the overall ecological heterogeneity or biodiversity of specific environmental communities. To analyze beta diversity, it is customary to initiate by calculating a distance matrix between environmental samples, encompassing the distances between any two samples. Techniques such as principal component analysis (PCA), principal coordinate analysis (PCoA), cluster analysis (UPGMA), non-metric multidimensional scaling (NMDS), similarity analysis (ANOSIM), and multivariate variance analysis (PermANOVA or Adonis) were employed for discerning dissimilarities among samples. Based on PCA, PCoA1, and MDS1 algorithms (Fig. 2A-C), we observed that the distances between biological replicates within the Middle-aged equine and Juvenile equine groups were similar, indicating sample reliability. Moreover, there was a resemblance in microbial composition structure with minor variations between the two groups. Similarly, hierarchical clustering analysis using 1UPGMA also yielded consistent results, demonstrating minimal differences in microbial evolution between Middle-aged equine and Juvenile equines (Fig. 2D). Additionally, Anoism analysis revealed no significant disparities between Middle-aged equine and Juvenile equines (Fig. 2E).

Species and differential analysis: In order to investigate the species differences between Middle-aged equines and Juvenile equines, we employed stacked bar chart, clustered stacked bar chart, and heatmap techniques to identify disparities in species at the phylum and genus levels. Utilizing the abundance table and annotation table of species, we selected the top 30 abundant species classifications and visually represented their relative abundances across samples using different graphical formats (Fig. 3A and 3B). To explore deeper into the dissimilarities and similarities among various samples, cluster analysis was conducted based on Bray-Curtis distance calculated from the bar charts (Fig. 3C and 3D). Upon comparison with Juvenile equines, it was observed that *Firmicutes*, *Verrucomicrobiota*, *Anacrovorax*, and

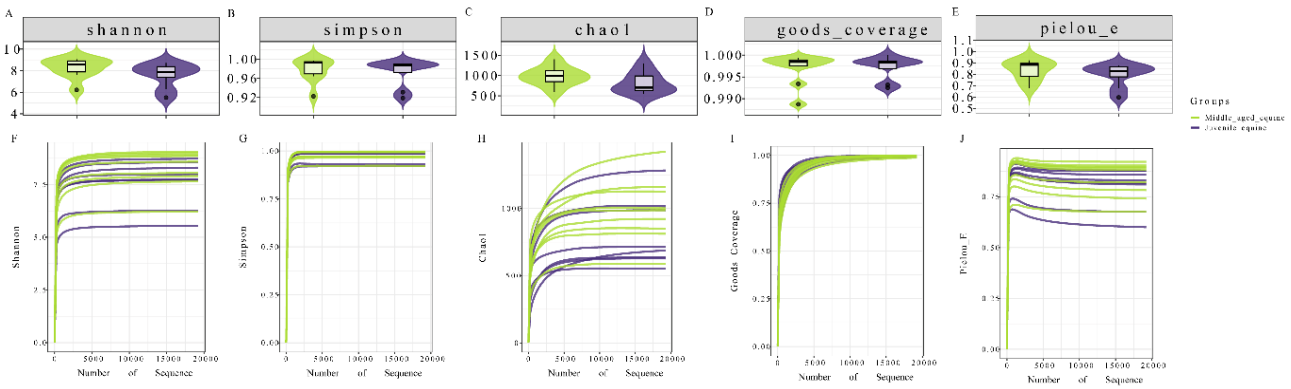

Fig. 1: Alpha diversity analysis. (A) Shannon index. (B) Simpson index. (C) Chao 1 index. (D) Goods coverage index. (E) Pielou e index. Dilution curve of (F) Shannon index. (G) Simpson index. (H) Chao 1 index. (I) Goods coverage index. (J) Pielou e index.

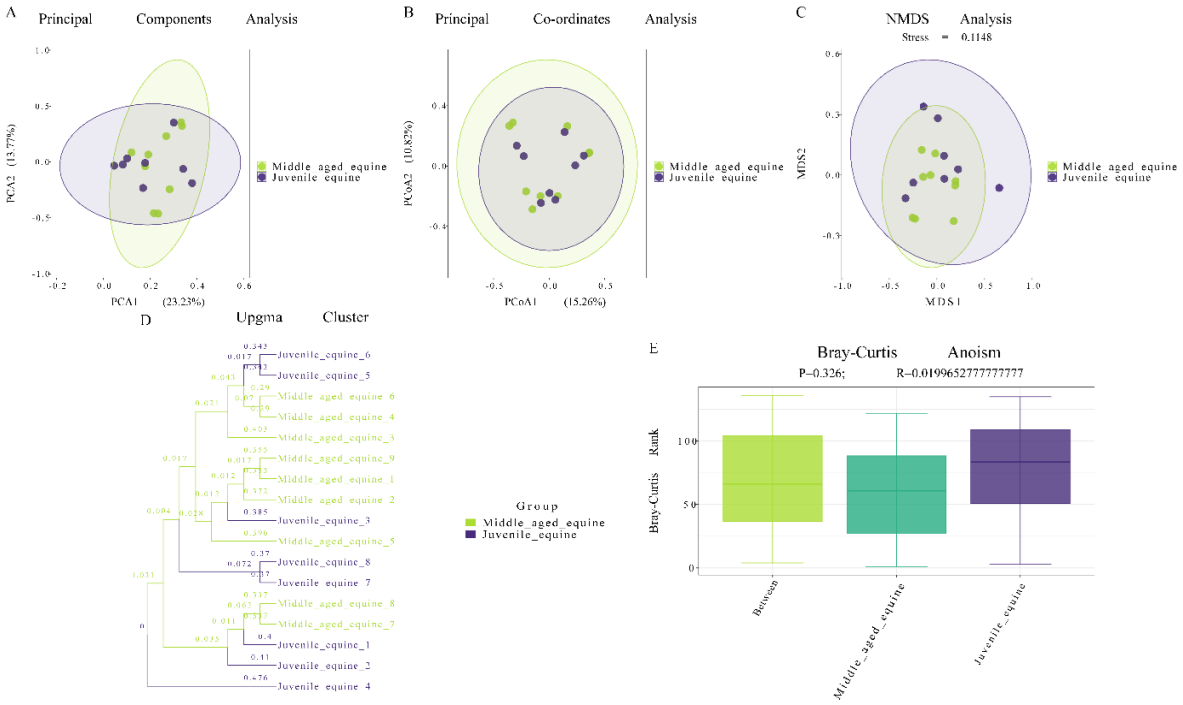

Fig. 2: Beta diversity analysis. (A) PCA analysis. (B) PCoA analysis. (C) NMDS analysis. (D) Sample cluster. (E) Anosim analysis.

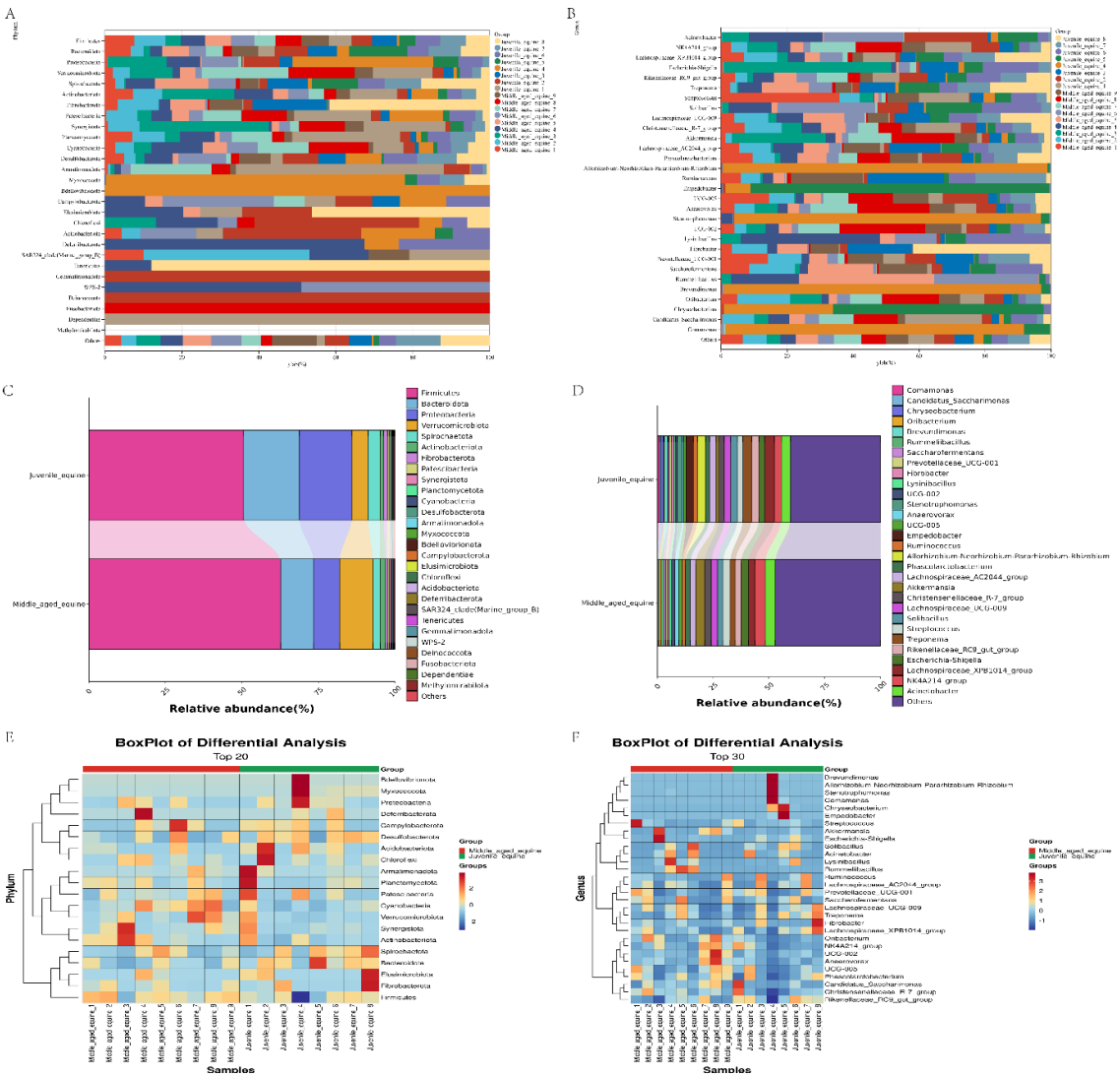

Fig. 3: Species diversity analysis. (A) Stacked bar chart of Phylum. (B) Stacked bar chart of Genus. (C) Relative abundance of Phylum. (D) Relative abundance of Genus. (E) Bosplot of differential analysis of Phylum. (F) Bosplot of differential analysis of Genus.

Akkermansia exhibited an increase in Middle-aged equine while *Bacteroidota*, *Proteobacteria*, *Spirochaetota*, *Fmpedobacter*, and *Allorhizobium-Ncorhizobium-Pararhizobium-Rhizobium* showed a decrease. Furthermore, the classification units were sorted according to clustering results and presented through a heatmap. The heatmap distinguished high-abundance from low-abundance classification units, and reflected similarity and difference among multiple samples at various taxonomic levels through color gradients (Fig. 3E and 3F).

Furthermore, significant differences were observed at the phylum level between Middle-aged and Juvenile equines in terms of *Bacteroidota*, *Actinobacteriota*, *Fibrobacterota*, and *Myxococcota* ($P<0.05$). At the genus level, a notable distinction was found between *Ruminococcus* and *Escherichia-Shigella* ($P<0.01$) (Fig. 4A and 4B). Similarly, LEfSe analysis comparing the gut microbiome composition of Middle-aged and Juvenile equines revealed species with significant intergroup variations. The results demonstrated that *Bacteroidales* and *Bacteroidota* were highly abundant species in Juvenile equines, while *Bacillales* and *Planococcaceae* were more prevalent in Middle-aged equines (Fig. 4C and 4D).

Correlation network analysis and function prediction: By utilizing bubble plots, phylogenetic trees, and correlation networks, we investigated the correlations between phylum and genus of Middle-aged equines and Juvenile equines (Fig. 5A-C). The findings revealed that *Bacteroidota*, *Fibrobacterota*, *Firmicutes*, *Patescibacteria*, *Proteobacteria*, *Spirochaetota*, and *Verrucomicrobiota* were the predominant microbial communities enriched in these two groups (Fig. 5A). Furthermore, the phylogenetic tree depicted the evolutionary distances among these microbial communities (Fig. 5B). Moreover, we elucidated the interrelationships among these microbial communities (Fig. 5C). To investigate the functional differences of these microbial communities, we employed PICRUSt2 for COG functional classification. Our findings revealed that the differential microbial communities were predominantly enriched in DNA-binding transcriptional regulator LysR family, outer membrane receptor proteins (primarily involved in iron transport), and multidrug efflux pump subunit AcrA (membrane-fusion protein) signaling pathways (Fig. 5D).

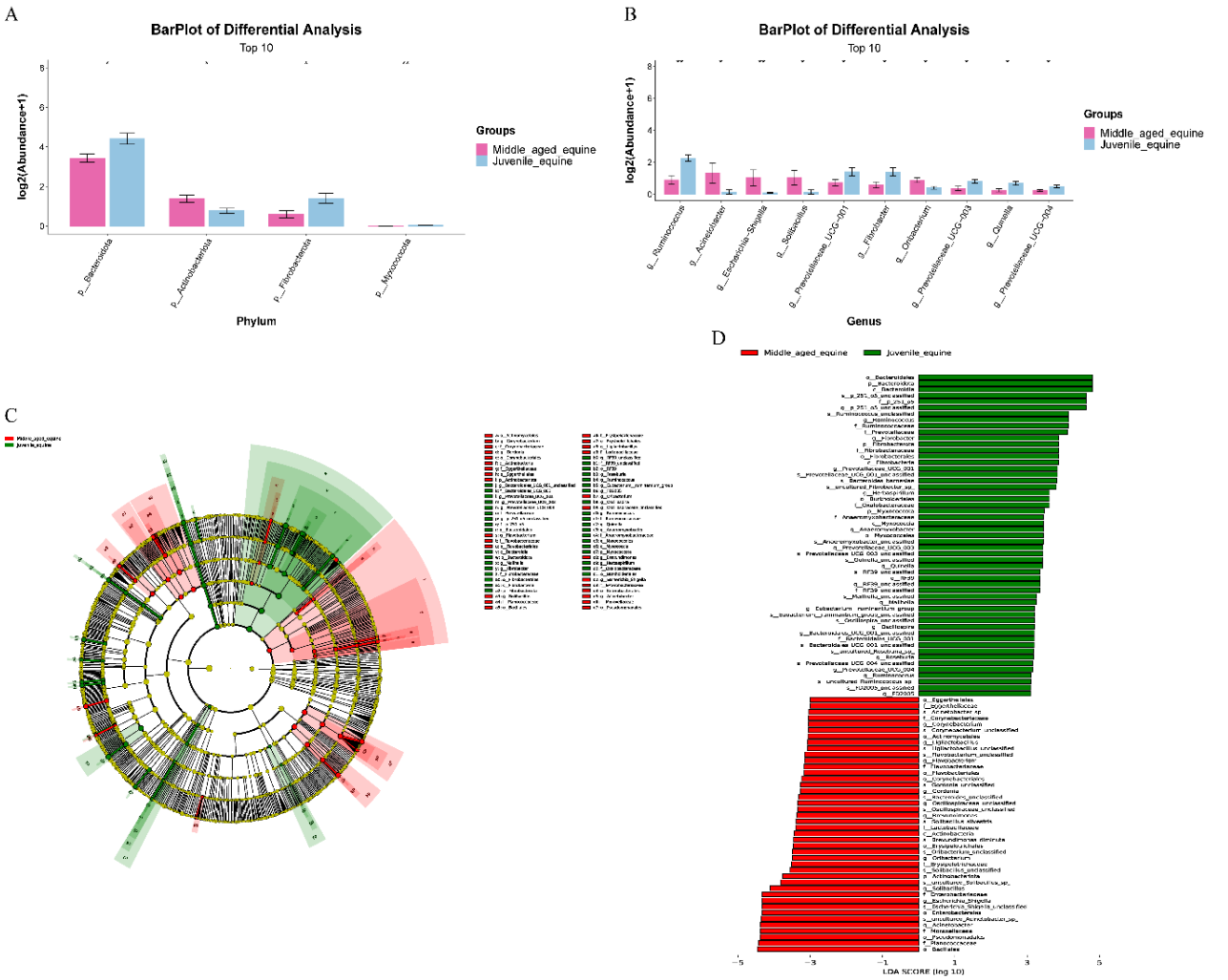

Fig. 4: Flora diversity analysis. (A) Barplot of differential analysis of Phylum. (B) Barplot of differential analysis of Genus. (C) Evolutionary branch. The distinct taxonomic levels are represented by different circles, arranged in the sequence of kingdom, phylum, class, order, family, genus and species from inner to outer. Each node corresponds to a specific species, with larger nodes indicating higher abundance of that particular species. (D) Split pillar diagram. The distribution bar chart illustrates the species that exhibit a statistically significant difference in LDA score, with the default threshold set at 3.0, i.e., the Biomakers displaying a marked distinction.

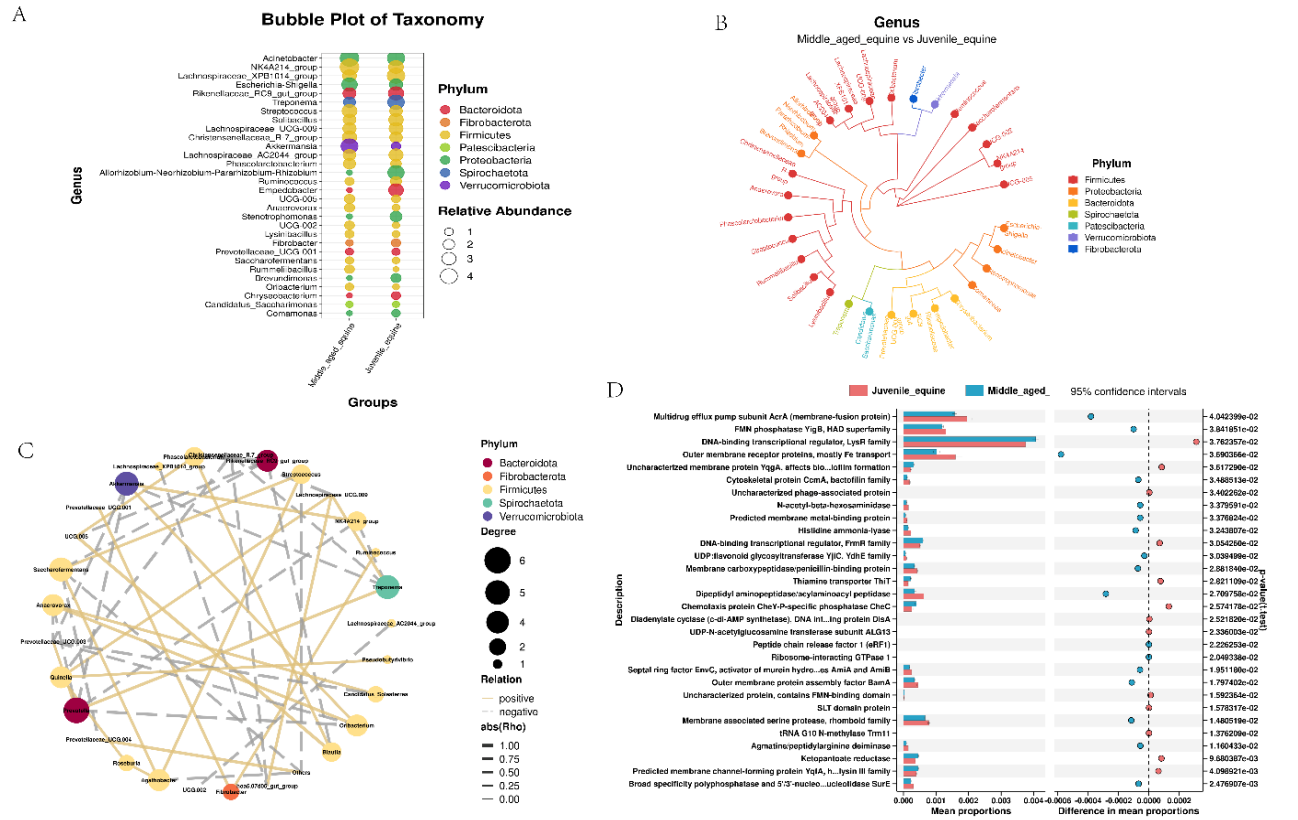

Fig. 5: Analysis of correlation function of network and enrichment. (A) Bubble plot. The plot presents detailed species-level annotation data and relative abundance (indicated by the size of the circles) across various sample groups, along with species-level annotation information for the phylum (denoted by the color of the circles) corresponding to each species. (B) Evolutionary tree of species. The classification of different branches represents distinct hierarchical levels, with each level corresponding to a specific color; however, it is emphasized that all branches belong to the same category or domain. Furthermore, the proximity between two species signifies a closer evolutionary relationship between them. (C) Correlation network diagram. Distinct genera are represented by different nodes, where the color of each node denotes species belonging to a specific gate level. The connections between these nodes indicate correlations between two genera. (D) Function prediction PICRUSt2.

DISCUSSION

A potential correlation exists between the hindgut health of equines and microbial equilibrium, subsequently impacting their overall well-being. Additionally, horses undergo hindgut fermentation in the colon and cecum to produce volatile fatty acids for metabolic processes, thereby serving as an energy source (Perricone *et al*., 2022). The intricate and complex interactions of the gut microbiota play a pivotal role in disease susceptibility, maintenance of health, immune response, and therapeutic potential. Perturbation in the equine microbiome can disrupt fermentation patterns and ultimately result in metabolic imbalances (Dougal *et al*., 2013; Stewart *et al*., 2021). Extensive human studies have provided substantial evidence supporting the correlation between gut microbiota and clinical conditions including inflammatory bowel disease, colorectal cancer, and diabetes (Ahn *et al*., 2013; Fujimoto *et al*., 2013; Giamarellos-Bourboulis *et al*., 2015; Aydin *et al*., 2018; Zheng *et al*., 2018). The investigation of diverse animal species has revealed that the dynamics of gut microbes can be influenced by a multitude of factors, including environmental conditions, dietary patterns, gestational age, hospitalization history, administration of antibiotics, mode of childbirth delivery, exposure to pressure variations, and feeding methods (Asano *et al*., 2012; Paun and Danska, 2015; Manfredo-Vieira *et al*., 2018). Therefore, the gut microbiota exerts a wide range of effects on the organism. However, it is intriguing to consider whether alterations in the host itself can influence the gut microbiota and subsequently impact its own functioning. Consequently, this study aimed to investigate the age-related impact on gut microbiota composition in horses. Current research suggests that aging may lead to a decline in microbial populations within the gastrointestinal tract, potentially attributed to changes in bacterial diversity (Badal *et al*., 2020). The perception of diversity may be attenuated due to physiological changes associated with aging, such as prolonged chyme transit time and reduced dietary energy requirements (Dougal *et al*., 2014; Mshelia *et al*., 2018). The present study identified *Bacteroidota*, *Fibrobacterota*, *Myxococcota*, *Ruminococcus*, *Prevotellaceae*, *Fibrobacter* and *Quinella* in middle-aged equines. Concurrently, *Actinobacteriota*, *Acinetobacter*, *Escherichia-Shigella*, *Solibacillus* and *Oribacterium* exhibited increased abundance. Therefore, it is hypothesized that aging may exert an influence on the relative abundance of these bacterial taxa potentially impacting immune function and digestive system homeostasis.

The presence of diverse alleles in transferrin genes is hypothesized to potentially influence bacterial iron acquisition, thereby potentially accounting for the substantial variations in susceptibility to infectious respiratory diseases observed among individual equines (Brandon *et al*., 1999; Mousel *et al*., 2003). Meanwhile,

accumulating evidence strongly suggests a substantial genetic component in the susceptibility/resistance of equines to clinical infectious respiratory diseases, with one notable biomarker being the transferrin phenotype. This discovery holds significant clinical implications as it enables effective identification of individuals at the highest risk for infectious respiratory diseases based on their transferrin genotype, thereby facilitating appropriate preventive and therapeutic interventions from birth to mitigate associated infections and respiratory complications (Maynard-Smith *et al*., 2022). Additionally, both humans and animals undergo a rapid decline in plasma or serum iron concentrations as a consequence of inflammatory processes (Feldman and Kaneko, 1980; Chiari *et al*., 1995; Cunietti *et al*., 2004;). The action is believed to function as a crucial defense mechanism for the host, given that iron plays an indispensable role in bacterial virulence and replication (Haschka *et al*., 2021). Low plasma iron concentration is a sensitive indicator for detecting systemic inflammation in horses, with significantly reduced levels observed regardless of the duration of inflammation. In an experimental model of equine streptococcal infection, serum iron levels decreased prior to the onset of clinical signs (within 24 hours) and fever, indicating that serum iron concentration can predict the severity of clinical symptoms (Varma *et al*., 1984). The presence of inflammatory processes, such as infection or endotoxins, can rapidly induce a significant decline in iron concentration (within 24 hours). This reduction is deemed pivotal in augmenting non-specific antibiotic resistance against bacterial infections (Forsberg and Bullen, 1972; Kluger and Rothenburg, 1979). The presence of systemic inflammation consistently results in a reduction in iron concentration in horses, irrespective of its duration. This suggests that the restoration of normal iron levels is contingent upon the resolution of the inflammatory process. Therefore, based on the findings of this study, we postulate that age induces modifications in the host gut microbiota, leading to upregulation of iron transport proteins. Consequently, this may trigger or facilitate an inflammatory response in the host and subsequently result in compromised immune function and a weakened self-defense system in elderly horses, thereby enhancing their susceptibility to health impairments. These matters necessitate further investigation to elucidate the impact of age disparities on the interplay between microbiota and iron transport proteins.

Conclusions: Analysis of 16S rDNA revealed a significant age-related impact on the composition of gut microbiota in horses, which subsequently affects ferroportin and multidrug resistance transporters. Investigating this phenomenon holds potential significance for understanding disease susceptibility, maintaining health, and enhancing host immune response. Furthermore, it can elucidate the underlying mechanisms behind age-induced changes in equine gut microbiota and their subsequent effects on the host.

Consent for publication: Not applicable.

Authors contribution: WZ, RL, RL, JC and RW developed the project. DW and CY performed the statistical analysis on the data. All authors reviewed and approved the structure and writing of the manuscript.

Availability of data and materials: The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest: The authors declare that there are no conflicts of interest.

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