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RESEARCH ARTICLE

Comparative Analysis of the Gut Microbiota between Two Horse Species

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ABSTRACT

The composition and structure of gut microbiota are easily influenced by external factors, especially host genetics. While horses are closely related to human life, there is a lack of research comparing the gut microbiota compositions and differences between native Mongolian horse (MH) and imported Dutch Warmblood horse (DH). Here, we collected feces from MH and DH and compared the differences in gut microbiota between the two breeds of horses using amplicon sequencing. Results showed that there was no significant difference in the diversity of gut microbiota between the two breeds of horses. At the phylum level, both *Firmicutes* and *Bacteroidota* were the most dominant phyla in all samples, independent of species. In addition, we also observed significant differences in 78 bacterial genera between the MH and DH, of which 36 genera (*Bifidobacterium*, *Chujaibacter*, *Lactobacillus*, *Rothia*, etc.) were significantly increased in DH and 42 genera (*Aequorivita*, *Aeromicrobium*, *Psychrobacillus*, *Brevibacterium*, etc.) were significantly decreased compared with MH. Altogether, this investigation dissected the compositions and differences of the gut microbiota between DH and MH and observed distinct differences in the gut microbiota between the two breeds of horses. Furthermore, these findings enhance our comprehension of the gut microbiome features of distinct horse breeds, which helps to analyze the differences in traits among different horses.

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INTRODUCTION

Horses are monogastric herbivorous mammals that can easily digest cellulose-containing materials such as grasses and other plant derivatives. Horses efficiently obtain energy through fermentation by the gut microbiota in the hindgut (Venable *et al*., 2017). Horses have evolved over 50 million years and are associated with human civilization and life (Park *et al*., 2021). Throughout history, horses have been used by humans for various purposes such as domestic use, transportation, and battlefields (Fages *et al*., 2019). Nowadays, horses have more uses such as entertainment and competition. MH is an important breed in northern China and have many excellent traits (Wen *et al*., 2022). DH has become the most successful, popular and riding horse in the world. DH is a new product of the 20th century. It is different from the warm-blooded horses that existed before the 20th century, which is a specially bred for equestrian competitions.

The gut microbiota, comprising 100 trillion microbes, is a complex microecosystem proven to play a role in nutrient absorption, metabolism, immune system maturation, injury recovery and intestinal mucosal barrier in different host species (Liao *et al*., 2022; Ren *et al*., 2023). In addition, gut microbiota can also synthesize various beneficial metabolites such as amino acids, vitamins, and short-chain fatty acids, which play an important role in ensuring nutrient intake and maintaining intestinal homeostasis (Li *et al*., 2023). At present, gut microbiota has gradually become the focus of animal breeding and animal health because of the two-way interaction between gut microbiota and the host. Research has shown that diet, various environmental factors, and animal genetics may alter microbial composition and structure (Wu *et al*., 2022; Wu *et al*., 2024). Meanwhile, gut microbiota can also affect animal growth performance, physiological function, meat quality and

motor function (Rashid *et al*., 2023). This intricate network of associations makes the gut microbiota a key factor in understanding the relationship between genotype, phenotype and environment. Thus, different genetic backgrounds can cause different microbial populations.

The development of high-throughput technologies has made it possible to study the composition and structure of complex gut microbiota (Ding *et al*., 2023). It is helpful for disease prevention and control and formulating effective strategies to mitigate the development of diseases (Shen *et al*., 2022). Meanwhile, this is crucial for understanding phenotypic differences between species and production traits by studying gut microbiota. Currently, there has been successful analysis of the composition and structure of gut microbiota in multiple species, leading to the discovery of differences in gut microbiota among them (Wang *et al*., 2022). For example, Park *et al*. (2021) indicated that Thoroughbred horses have more species and diverse bacterial populations as well as beneficial bacteria than Jeju horses in Korea. Moreover, studies by Wen *et al*. (2022) demonstrated that Thoroughbred horses have a higher gut fiber-dissolving bacteria and carbohydrate metabolism capacity as compared to MH. Therefore, there may be differences in the gut microbiota between DH and MH. However, studies regarding the gut microbiota in DH and MH remain scarce. Here, we investigated the gut microbial composition and difference between DH and MH.

MATERIALS AND METHODS

Sample acquisition: Eight DH and eight MH were used in present research. DH were imported from Holland and aged between 2-5 years. All the horses in this study were kept under identical conditions and had the same immune procedures. Moreover, professional veterinarians observed and evaluated these horses to determine their health status and without injecting any antibiotics before sample acquisition. Prior to sample acquisition, each horse was placed in an individual pen and provided with adequate food and water. The following morning, sufficient faeces (approximately 200 g) were collected from each horse using the stool sampler. To minimize pollution from bedding and flooring, the fresh fecal samples were then re-sampled from the intermediate portion. Subsequently, sixteen rectal feces from DH and MH were immediately stored at −80°C for further analysis.

Amplicon sequencing of gut microbiota: We performed DNA extraction and amplicon sequencing based on previous studies (Liao *et al*., 2022). SPSS statistical program (v20.0) was used to conduct data analysis. P-values (means±SD) <0.05 were considered statistically significant.

RESULTS

Data acquisition and analysis: In this research, we collected 1,279,676 (MH = 640,082, DH = 639,594) raw sequences from MH and DH in the range of 79,713 to 80,234 sequences per sample (Table 1). Subsequently, these raw data were quality filtered and 871,932 (MH = 447,789, DH = 424,143) valid sequences were obtained, resulting in an effective rate of approximately 68.14%. We observed that each sample had over 40,000 effective sequences, suggesting sufficient sequencing depth (Fig. 1A, B). In addition, the rank abundance curves tended to be flat when the operational taxonomic unit (OTU) ranks reached 800, indicating that the microbial composition was relatively uniform (Fig. 1C). The valid sequences obtained from MH and DH were clustered into 9,618 (MH = 5,904, DH = 5,246) OTUs, ranging from 872 to 1,170 OTUs per sample (Fig. 1D, E). Among identified OTUs, 1,532 OTUs co-occurred in the MH and DH, accounting for approximately 15.93% of the total OTUs. In addition, the quantity of unique OTUs in the MH and DH was 4,372 and 3,714, respectively.

Table 1: Sequence analysis of each sample from DH and MH

Sample	Raw Reads	Clean Reads	Denoised Reads	Merged Reads	Effective Reads	Effective (%)
MH1	79926	79579	76635	67096	58195	72.81
MH2	80013	79669	76835	65905	55477	69.33
MH3	79899	79542	76934	67280	54527	68.24
MH4	80234	79895	77497	69846	58936	73.45
MH5	80059	79717	76608	67628	58174	72.66
MH6	80072	79751	76458	63212	54588	68.17
MH7	79936	79610	76990	65935	55013	68.82
MH8	79943	79603	76568	65095	52879	66.14
DH1	80155	79826	76903	64329	55161	68.81
DH2	80007	79621	76882	65913	56753	70.93
DH3	79966	79642	76724	65271	54172	67.74
DH4	79890	79507	76241	64614	53292	66.70
DH5	79713	79354	75626	60054	50494	63.34
DH6	80085	79765	76687	62455	51108	63.81
DH7	80016	79701	75768	58300	47117	58.88
DH8	79762	79433	76710	66037	56046	70.266

Comparative analysis of microbial diversity index of different horse species: To further explore the differences in gut microbiota among the MH and DH, we also calculated four indices such as Chao1, ACE, Simpson and Shannon, which reflect microbial abundance and diversity (Fig. 2). The MH had a Chao1 index of 992.91 and an ACE index of 995.37, while the Chao1 and ACE indices of DH were 964.34 and 967.09, respectively. Additionally, the Simpson and Shannon indices of the MH were 0.97 and 8.32, respectively, whereas those of the DH were 0.99 and 8.72. Statistical analysis of alpha diversity indices intuitively demonstrated that the difference of gut microbial abundance and diversity between the MH and DH was non-significant. PCoA scatter, which reflects the similarity and variation of gut microbiota between individuals, was applied to dissect beta diversity. Results showed that the scatter points clustered together, demonstrating that the major components of gut microbiota in MH and DH did not differ dramatically.

Composition and variation of gut microbiota at different taxonomic levels: To further explore the differences of taxonomic compositions in MH and DH, the gut microbiota of these horses were analyzed using Metastats analysis. Specifically, the *Firmicutes* (51.03%), *Bacteroidota* (29.59%) and *Proteobacteria* (9.73%) were the dominant bacterial phyla in the gut microbiota of MH, accounting for more than 90% of all bacterial taxa

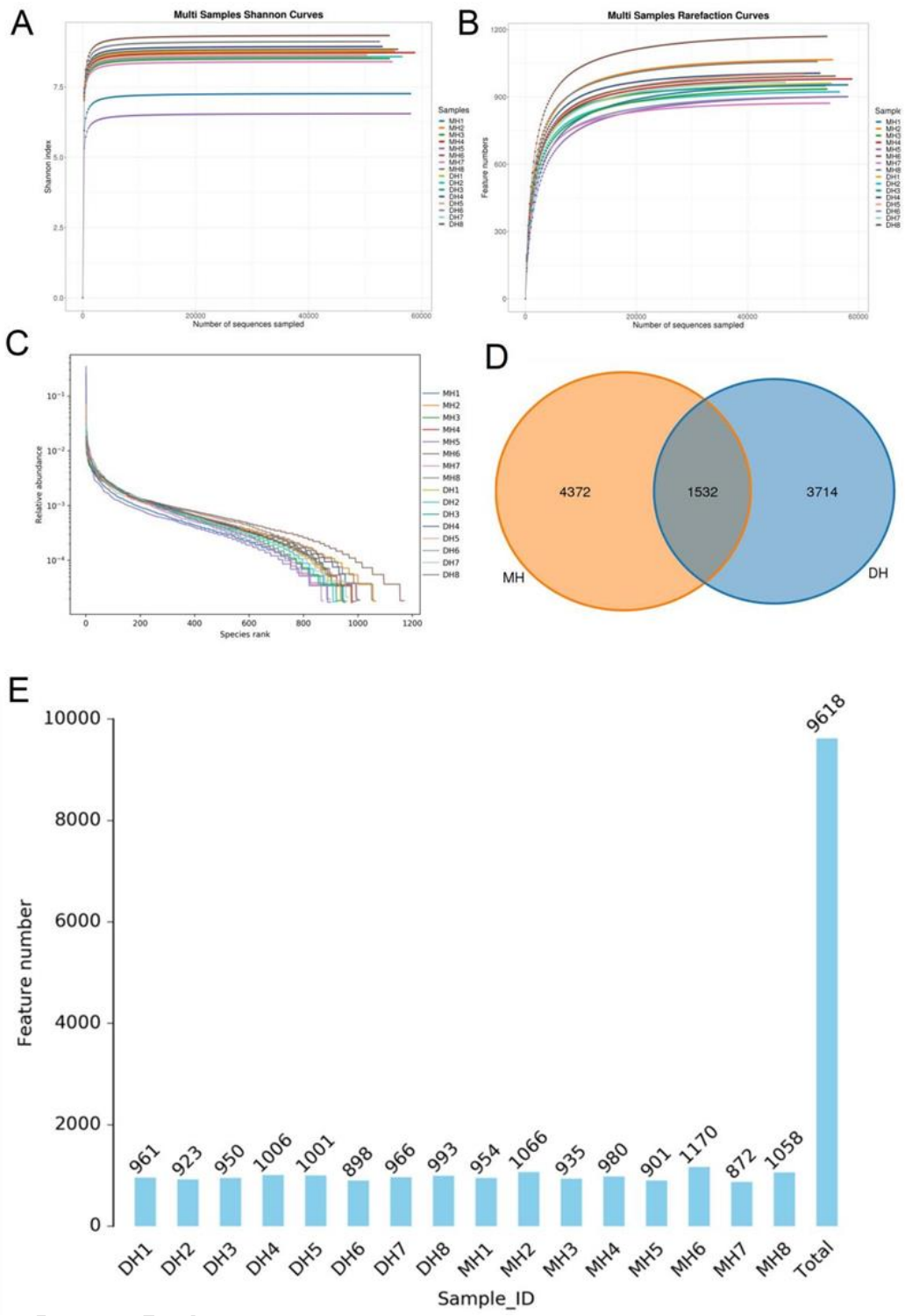

Fig. 1: Sequencing depth assessment and OTUs quantity statistics.

(Fig. 3A). Moreover, the gut microbiota in the DH were predominated by *Firmicutes* (57.07%), *Bacteroidota* (30.32%), *Fibrobacterota* (5.98%) and *Verrucomicrobiota* (2.14%) in descending order. The abundances of other bacterial phyla such as *Patescibacteria* (0.62%, 0.88%), *Desulfobacterota* (0.66%, 0.45%), *Actinobacteriota* (0.51%, 0.56%) and *Synergistota* (0.25%, 0.16%) in MH and DH were shown to be less than 1% of the total bacterial composition. In addition, a total of 316 genera were recognized in the gut microbiota of MH and DH, ranging from 128 to 160 genera per sample. Among them, *Acinetobacter* (9.33%), *unclassified_p_251_o5* (8.09%), *unclassified_Lachnospiraceae* (7.68%), *Rikenellaceae_RC9_gut_group* (5.85%) and *unclassified_F082* (4.11%), which accounted for more than 4% of total sequences on average, were abundantly present in the gut microbiota of MH (Fig. 3B). However, *unclassified_Lachnospiraceae* (10.16%), *unclassified_p_251_o5* (9.32%), *Fibrobacter* (5.98%) and *Lachnospiraceae_AC2044_group* (5.20%) were the most abundant bacteria in the gut microbiota of DH. The clustering heatmap analysis of genus-level showed that the samples within the same group were more similar to each other than to those in other groups. Additionally, it also indicated a change in the bacterial genus-level compositions between the MH and DH (Fig. 3C).

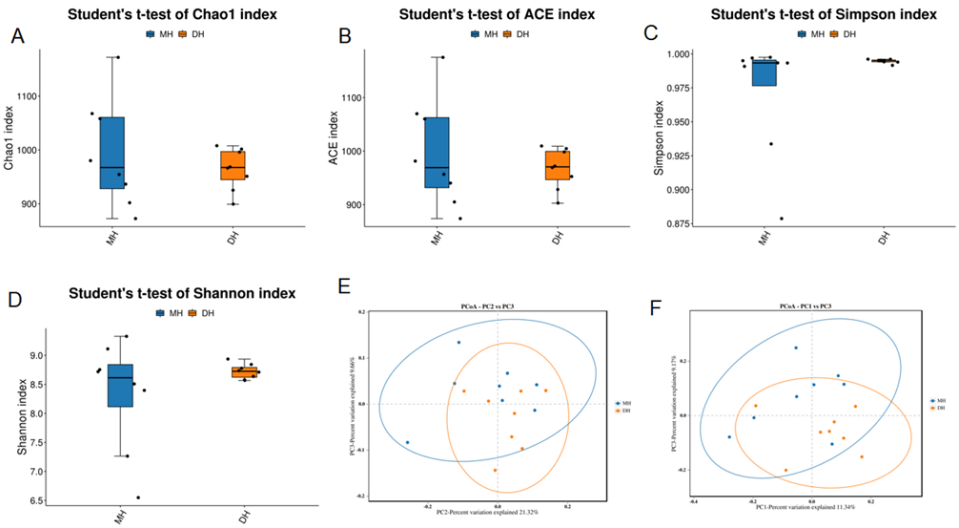

Fig. 2: Changes in diversity indices related to gut microbiota between DH and MH.

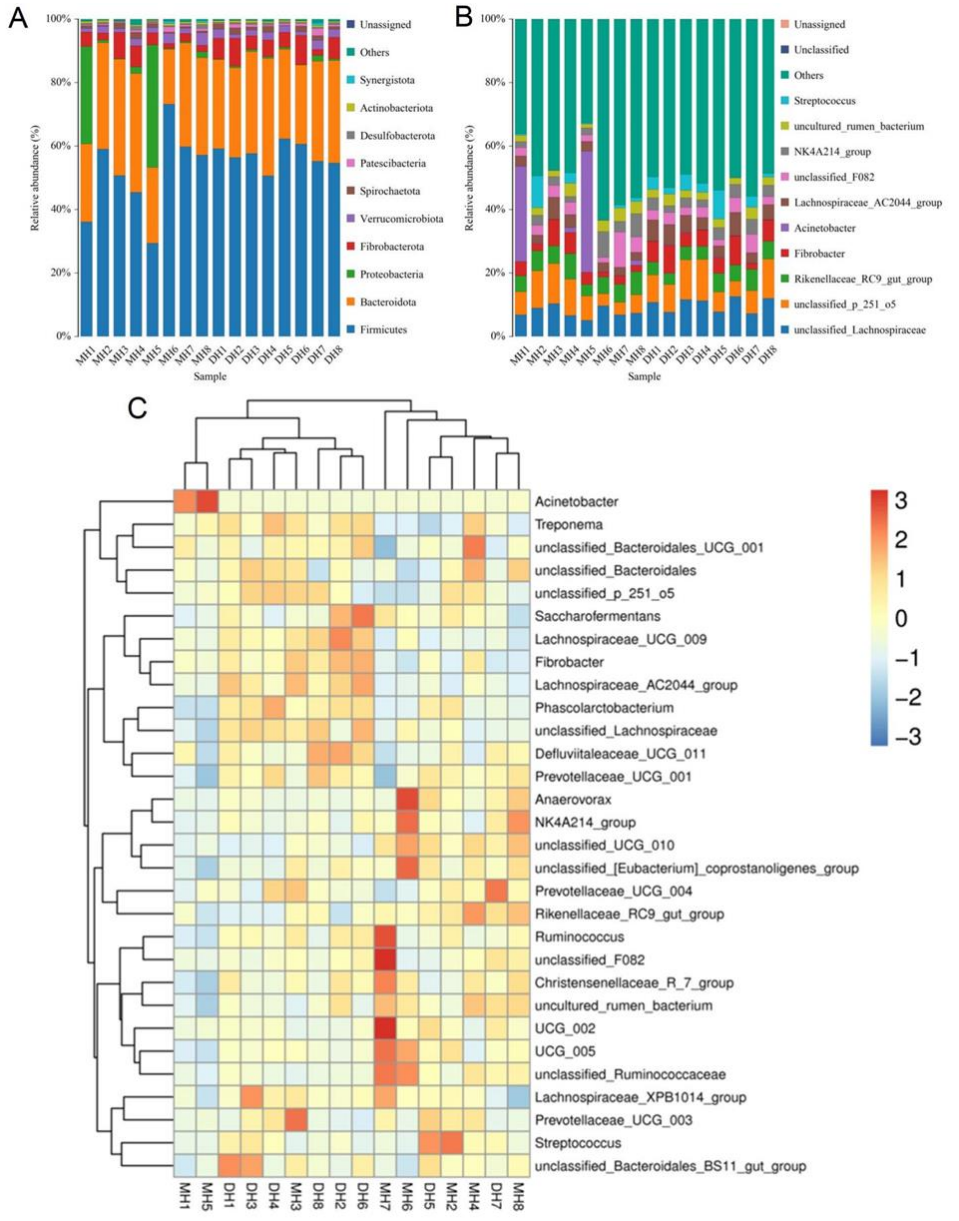

Fig. 3: Comparative analysis of relative abundances of bacterial phylum and genus between DH and MH. The heatmap analysis presents the distribution and relative abundance of bacteria in the DH and MH.

We also observed that 78 genera exhibited significant differences between the MH and DH by Metastats analysis (Fig. 4). Compared with the MH, the relative abundances of 36 genera (*Bifidobacterium*, *Chujaibacter*, *DNF00809*, *Inquilinus*, *Kurthia*, *Fretibacterium*, *Lachnospiraceae_UCG_010*, *Lactobacillus*, *Rothia*, *unclassified_Actinobacteria*, *Succinivibrio*, *unclassified_Clostridiaceae*, *unclassified_Coriobacteriia*,

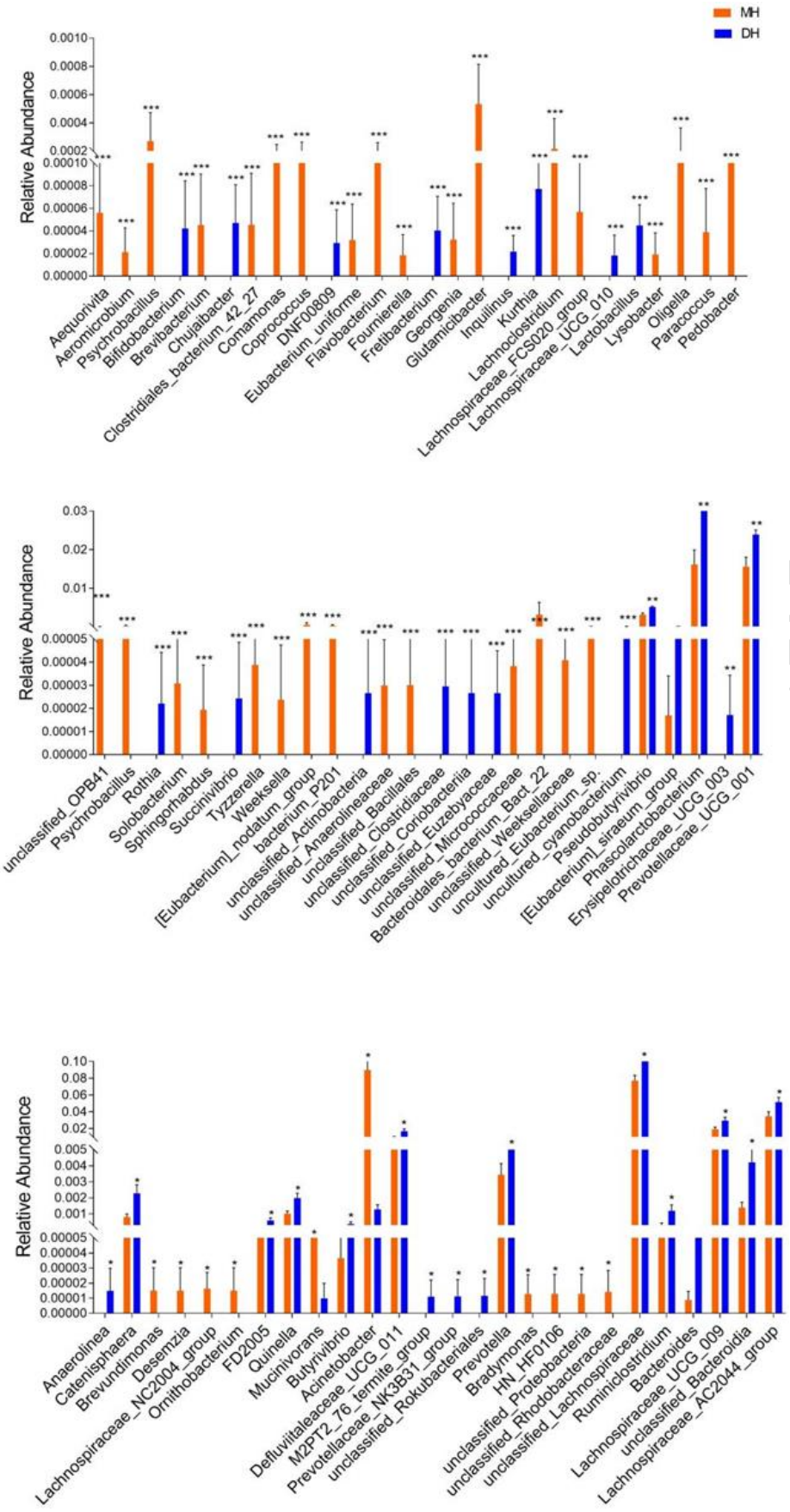

Fig. 4: The bacterial phyla and genera differed significantly between the DH and MH. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

unclassified_Euzebyaceae, *uncultured_cyanobacterium*, *Pseudobutyrivibrio [Eubacterium]_siraeum_group*, *Phascolarctobacterium*, *Erysipelotrichaceae_UCG_003*, *Prevotellaceae_UCG_001*, *Anaerolinea*, *Catenisphaera*, *FD2005*, *Quinella*, *Butyrivibrio*, *Defluviitaleaceae_UCG_011*, *M2PT2_76_termite_group*, *Prevotellaceae_NK3B31_group*, *unclassified_Rokubacteriales*, *Prevotella*, *unclassified_Lachnospiraceae*, *Ruminiclostridium*, *Bacteroides*, *Lachnospiraceae _ UCG _ 009*,

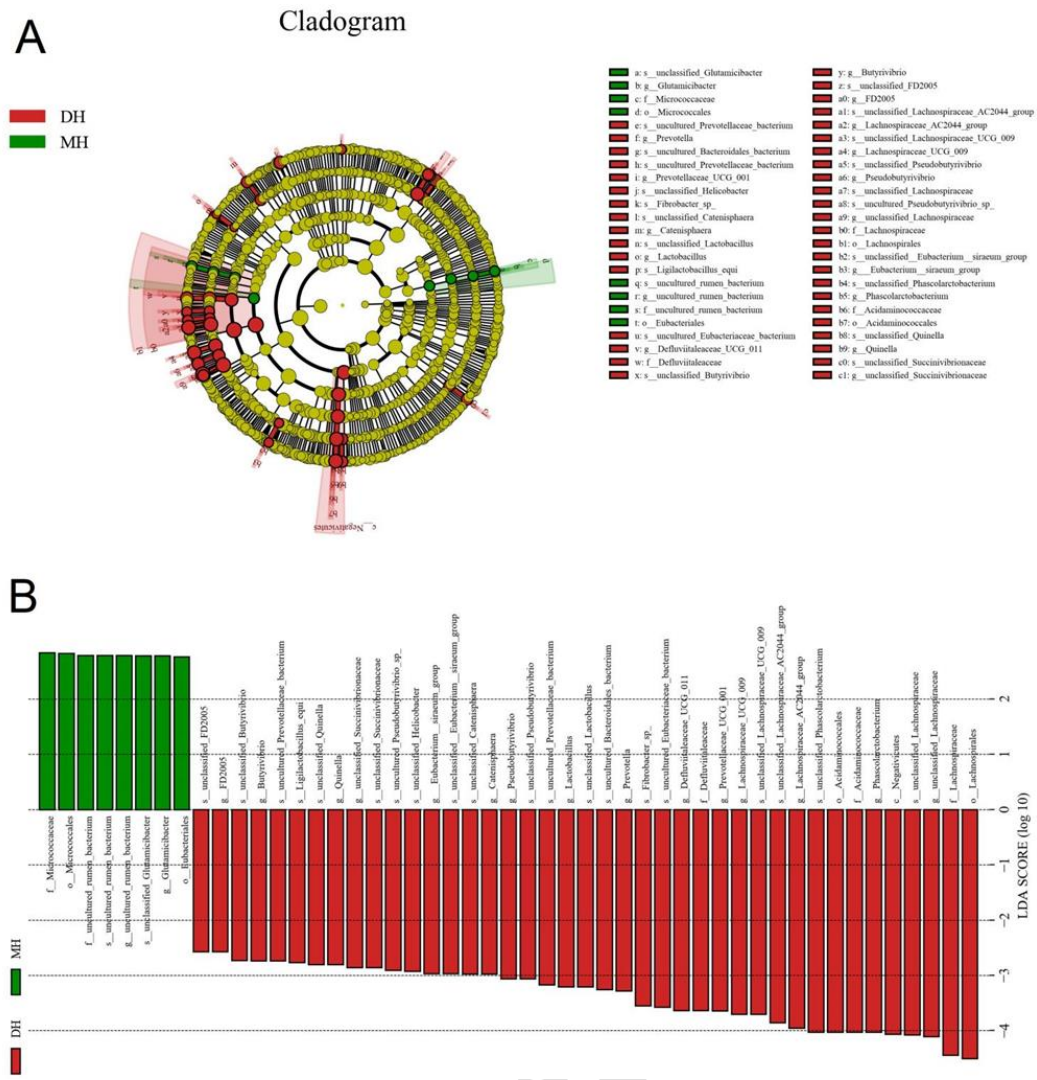

Fig. 5: LEfSe analysis was used to identify differential taxa in the gut microbiota between DH and MH.

unclassified_Bacteroidia and *Lachnospiraceae_AC2044_ group*) dramatically increased, while the relative abundances of 42 genera (*Aequorivita*, *Aeromicrobium*, *Psychrobacillus*, *Brevibacterium*, *Clostridiales_bacterium _42_27*, *Comamonas*, *Coprococcus*, *Eubacterium_ uniforme*, *Flavobacterium*, *Fournierella*, *Georgenia*, *Glutamicibacter*, *Lachnoclostridium*, *Lachnospiraceae_ FCS020_group*, *Lysobacter*, *Oligella*, *Paracoccus*, *Pedobacter*, *unclassified_OPB41*, *Psychrobacillus*, *Solobacterium*, *Sphingorhabdus*, *Tyzzerella*, *Weeksella*, *[Eubacterium]_nodatum_group*, *bacterium_P201*, *unclassified_Anaerolineaceae*, *unclassified_Bacillales*, *unclassified_Micrococcaceae*, *Bacteroidales_bacterium_ Bact_22*, *unclassified_ Weeksellaceae*, *uncultured_ Eubacterium_sp*, *Brevundimonas*, *Desemzia*, *Lachnospiraceae_NC2004_ group*, *Ornithobacterium*, *Mucinivorans*, *Acinetobacter*, *Bradymonas*, *HN_HF0106*, *unclassified_Proteobacteria* and *unclassified_ Rhodobacteraceae*) dramatically decreased in DH. To further explore the differences in the gut microbiota between the MH and DH, we also used LEfse analysis to further identify the differential taxa (Fig. 5). Results showed that the MH was dramatically enriched for *uncultured_rumen_bacterium*, while the DH showed a dramatically higher abundances of *unclassified_ Succinivibrionaceae*, *Eubacterium__siraeum_group*, *Lachnospiraceae_AC2044_group*.

Correlation network analysis: *Pseudobutyrivibrio* was positively related to *Defluviitaleaceae_UCG_011* (0.73), *Prevotellaceae_UCG_001* (0.70), *unclassified_Lachnospiraceae* (0.65), *Phascolarctobacterium* (0.65). *Prevotella* was positively related to *Phascolarctobacterium* (0.8), *unclassified_p_251_o5* (0.76), *Alloprevotella* (0.61) but negatively related to *unclassified_Clostridia* (0.70). *Lachnospiraceae_UCG_009* was positively related to *Lachnospiraceae_AC2044_group* (0.77) and *Fibrobacter* (0.60) but negatively related to *Acinetobacter* (0.63) (Fig. 6).

DISCUSSION

Interactions between gut microbiota and host occur throughout life, involving nutrient absorption, metabolism, immunity, and growth performance. In addition, the composition of the gut microbiota can also affect the overall physiology of the host, including feed conversion, exercise capacity, etc. (Yang *et al*., 2021). Therefore, exploring the composition of gut microbiota among different species is helpful to analyze the differences in their traits. Currently, host genetics is considered an endogenous factor influencing gut microbial diversity, with genetically related individuals tending to have a more similar gut microbial composition than unrelated individuals (Li *et al*., 2021).

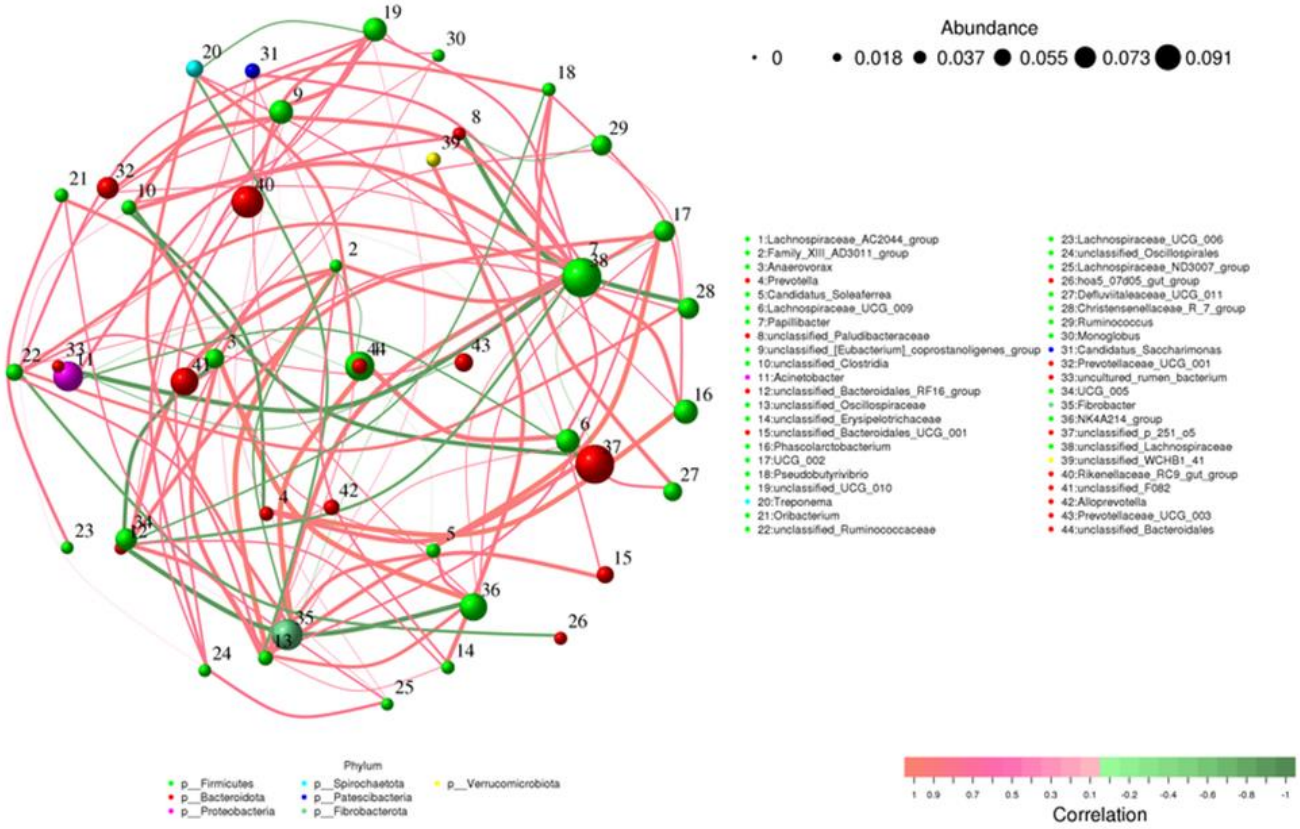

Fig. 6: Correlation analysis of gut microbiota in DH and MH.

To date, the gut microbiota of different breeds of pigs, sheep, cattle, and chickens have been studied and the variability of gut microbiota among different breeds has been demonstrated (Yang *et al*., 2020). However, limited research has been conducted on the variances in gut microbiota between MH and DH. Thus, this study aims to compare the differences in gut microbiota of MH and DH.

Early investigations showed that the intestine harbors trillions of microbes that interact with each other to form a relatively constant state (Yu *et al*., 2021). However, the gut microbiota, as a dynamic system, is easily affected by antibiotics, environmental pollutants, and dietary factors (Ding *et al*., 2019). In addition to the aforementioned factors, species was also found to be an important factor leading to changes in gut microbiota (Liu *et al*., 2021). For instance, Park *et al*. (2021) observed distinct differences in the diversity of gut microbiota between Jeju Horses and Thoroughbred Horses in Korea. Additionally, similar results were observed in Lusitano horses and Hanoverian horses (Massacci *et al*., 2020). Alpha diversity is an effective tool to evaluate the species diversity and abundance of gut microbiota, while beta diversity can reflect the differences in the main components of gut microbiota (Li *et al*., 2021). Generally, higher alpha diversity means more abundant and diverse gut microbiota, which is also considered a sign of mature gut microbiota (Roswall *et al*., 2021). In this case, the gut microbiota has a higher resistance to the environment and is not easily affected by external factors. Research has shown that a greater variety and quantity of gut microbiota is associated with improved intestinal function and the ability to perform complex physiological functions. Additionally, gut microbiota with higher microbial diversity and abundance have been found to promote energy utilization. This study found no significant differences in the alpha and beta diversity of gut microbiota between MH and DH. We speculated that the similarity in microbial diversity may be attributed to their shared diet and habitat.

This study found that both MH and DH had high abundances of *Firmicutes* and *Bacteroidetes*, regardless of species. These results align with previous research that also found these phyla to be abundant in the donkey, steer, sheep, and yak (Liu *et al*., 2022). *Firmicutes* and *Bacteroidetes* are main component of the gut microbiota in many animals, which may contribute significantly to maintaining gut microbial balance and function. *Firmicutes* is a group of Gram-positive bacteria, and some members are generally considered beneficial for maintaining a healthy balance in the gut microbiota and preventing the invasion of harmful pathogens. Studies have demonstrated that *Firmicutes* are crucial in the digestion of fiber and cellulose, while *Bacteroidetes* primarily aid in the digestion of carbohydrates and proteins, as well as promoting the maturation of the intestinal immune system (Gavande *et al*., 2021). The greater abundance of *Firmicutes* and *Bacteroidetes* in the gut microbiota is likely linked to the energy and nutritional requirements of animals.

There is mounting evidence to suggest that certain bacterial variations can show the potential relationship between gut microbiota and phenotype of host. In addition to these common features mentioned above, we also observed obvious shifts in several functional bacterial genera between the MH and DH, which may play important roles in host intestinal function and

homeostasis. For instance, the gut microbiota of DH was significantly enriched by *Bifidobacterium*, *Lachnospiraceae_UCG_010*, *Lactobacillus*, *Succinivibrio*, *Pseudobutyrivibrio*, *Prevotellaceae_UCG_001*, *Prevotella*, *Butyrivibrio*, *Ruminiclostridium*, *Prevotellaceae_NK3B31_group*, *Bacteroides*, *Ligilactobacillus*, and *Lachnospiraceae_UCG_009* ect. in comparison with MH. As a crucial anaerobic bacterium, *Bacteroides* has been shown to decompose polysaccharides and positively affect the intestinal ecosystem (Schwalm and Groisman, 2017). *Ligilactobacillus* has been reported to possess multiple vital biological properties such as enhancing immunity, antibacterial, maintaining intestinal health, and improving growth performance (Iniesta *et al*., 2022). Previous research indicated that *Prevotellaceae* and *Butyrivibrio* have the ability to digest high carbohydrate, pectin, and hemicellulose. Similarly, *Prevotella* has also been shown to have a significant impact on the utilization of carbohydrates and nitrogen in the foregut of yaks. *Ruminiclostridium*, a crucial beneficial bacterium, has been shown to produce beneficial metabolites, thereby playing a pivotal role in improving host growth performance and maintaining a healthy intestinal ecosystem. As acknowledged beneficial bacterium, *Bifidobacterium* and *Lactobacillus* were previously reported to involve in the positive regulation of the gut microbial homeostasis, immune system, gastrointestinal function, intestinal environment and growth performance (Song *et al*., 2022). Recent investigations on *Bifidobacterium* have also revealed its important roles in anti-aging, anti-tumor, disease prevention and nutrient regulation (Kim *et al*., 2022). *Bifidobacteria* and *Lactobacillus* have the ability to produce antimicrobial peptides, which can inhibit the growth of harmful bacteria and resist pathogenic bacterial infections (Iram *et al*., 2022). In addition, they are also capable of synthesizing essential vitamins for the body, promoting mineral absorption, and producing various organic acids (Zhang *et al*., 2022). Research has shown a negative correlation between *Lachnospiraceae* and intestinal inflammation, further highlighting its potential as intestinal beneficial bacteria (Awoniyi *et al*., 2022). Remarkably, some of the bacteria mentioned above, such as *Bifidobacterium*, *Lachnospiraceae_UCG_010*, *Lactobacillus*, *Lachnospiraceae_UCG_009*, *Ruminiclostridium*, *Pseudobutyrivibrio* and *Butyrivibrio* have been shown to be potential producers of short-chain fatty acids (SCFAs) (Berger *et al*., 2021). SCFAs are beneficial metabolites produced by bacteria that have multiple important physiological functions such as weakening inflammation, maintaining the gut microbial balance, regulating energy intake, and reducing oxidative stress (Silva *et al*., 2020). Recent studies have also revealed that SCFAs play important roles in cell proliferation, immune system function, and intestinal barrier function. Moreover, SCFAs have the ability to alter the pH levels in the intestine and enhance the activity of digestive enzymes, thereby playing the role of antibacterial and growth-promoting. In addition, we also observed some SCFAs-producing bacteria such as *Lachnospiraceae_FCS020_group*, *Lachnospiraceae_NC2004_group* and *Coprococcus* in the MH. However, the abundances of some pathogenic bacteria such as *Comamonas*, *Flavobacterium*, *Acinetobacter*, *Brevundimonas* and *Tyzzerella* in the MH were significantly higher than those in the DH. *Comamonas* was potentially pathogenic bacteria associated with bacteremia (Opota *et al*., 2014). *Flavobacterium* is an opportunistic pathogen that could lead to sepsis, meningitis, and pneumonia during immune dysregulation. *Acinetobacter* is an opportunistic pathogen that primarily inhabits the gastrointestinal tract, respiratory tract, skin, and genitourinary tract. It can cause various infections such as bacteremia, endocarditis, pneumonia, as well as urinary and skin infections (Xiao *et al*., 2019). *Brevundimonas* and *Tyzzerella* are both pathogenic bacteria, with the former accelerating bacteremia and the latter causing cardiovascular disease. We noted that despite variations in the enriched bacteria among MH and DH, they all exhibit a high degree of specialization in performing complex intestinal functions. We speculated that both types of horses possess similar dominant bacterial communities that enable them to perform common functions, while the relative abundance of specific bacterial species may contribute to their distinct functions.

Conclusions: In conclusion, we investigated the gut microbial composition of native MH and imported DH and characterized their differences. Results showed that although the diversity of the gut microbiota did not differ between the two horse species, some bacterial genera exhibited high variability. We speculated that the similar dominant microbial communities in MH and imported DH contribute to achieving common functionality, while the differential taxa contribute to achieving own specific functions. In addition, exploring the gut microbial composition and variability will also contribute to understanding the differences in traits between MH and imported DH. However, some limitations of this study need to be noted including individual differences, sample size, etc.

Author contributions: DW and ZS conceived and designed the experiments. DW, JZ, HM and ZS contributed sample collection and reagents preparation. DW analyzed the data. DW wrote the manuscript. DF revised the manuscript. All authors reviewed the manuscript.

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Conflict of interest: The authors declare that they have no competing interests.

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