Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.007

RESEARCH ARTICLE

Effect of High-Density Rearing of Pregnant Ewes on the Intestinal Microbiota of their Offsprings

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ARTICLE HISTORY (24-789)

Received: December 4, 2024
Revised: January 21, 2025
Accepted: January 23, 2025
Published online: February 7, 2025

Key words:
Han sheep
Intestinal microbiota
Offspring
Pregnant ewes
Rearing density

ABSTRACT

This study explored the effects of high-density rearing of pregnant ewes on the intestinal microbiota of their offspring. A total of 40 small-tailed Han sheep were distributed into two groups randomly including the high-density group (1 sheep/m^2) and the control/ low-density group (1 sheep/2m^2). Fecal samples from ewes and offspring were collected for high-throughput sequencing and multiple-significance analysis. We uncovered the response of gut microbiota in ewes and offspring to different rearing densities. The number of potentially harmful bacteria (*Ralstonia pickettii*, *Ruegeria*, Rhodobacteraceae, etc.) was increased in high-density groups, while the abundance of several probiotics (*Oscillibacter*, *Akkermansia*, Ruminococcaceae-UCG-010, etc.) was found significantly lesser than that of the control group ($P<0.05$). In addition, gut microbiota in the high-density groups exhibited more variability with age, indicating that an increase in the housing density has a significant correlation. Taken together, improper increase in the rearing density of pregnant sheep can harm themselves and their offspring, which not only fails to improve economic benefits but also produces harmful effects. This study may provide new ideas for healthy and sustainable sheep reproduction and farming.

To Cite This Article: Wei M, Han Z, Lu S, Gao Q, Liu J, Yang Y, Yang J, Almutairi MH, Almutairi BO, Lv S and Yang S, 2025. Effect of high-density rearing of pregnant ewes on the intestinal microbiota of their offsprings. Pak Vet J, 45(1): 236-245. http://dx.doi.org/10.29261/pakvetj/2025.007

INTRODUCTION

Small-tailed Han sheep breed is an important local small ruminant species in China, originating from low-altitude areas later it accommodated to the pasturage lifestyle in the plateau of China and became an indispensable breed for herdsmen and the local economy (Chang *et al.,* 2022). Small-tailed Han sheep have multiple advantages including strong battening ability, low-fat contents, high meat yield, strong resistance against diseases, and stable genetic properties. In addition, this breed of sheep shows an estrus cycle throughout the year. It has excellent reproductive performance and the average lambing rate is above 250% (Han *et al.,* 2021; Chang *et al.,* 2022). Therefore, the small-tailed Han sheep occupies a dominant position in animal husbandry and is widely raised in China.

Reproductive efficiency is one of the critical factors affecting the economic benefits of small ruminants and promoting the quantity and quality of lambs is a direct method to increase the economic returns of livestock farming (Han *et al.,* 2021). Reducing prenatal stocking density has a profound economic impact on dairy operations as the fixed cost of construction facilities increases. Multiple pieces have shown that appropriately reducing feeding density can effectively improve the immune and antioxidant capacity of sheep. When housed in the same pen, especially at a higher density, pregnant ewes may collide and grab food due to insufficient activity space and lack of food (Tüfekci and Sejian, 2023). The

enclosure environment may also become harsh, as a result applying adverse effects on both pregnant ewes and offspring (Xiaoqing *et al.,* 2020). Consequently, finding a suitable breeding density to reduce costs without negatively affecting sheep health seems to be a good approach. Silva *et al.* (2014) explored the effects of prenatal stocking density on the metabolism, health, and reproduction performance of dairy cows. Bonnie et al. studied the effects of stocking density and restricted trough space on sheep behavior and biological functioning (Mayes *et al.,* 2022). However, few studies have focused on the impact of changing the rearing density of pregnant sheep on their offspring, and hence the purpose of the current study was to reveal the answer to this question.

The excellent characteristics of small-tailed Han sheep are not only related to their genetics but may also be related to gut microbiota. The gut of mammals has a large microbial community that plays an indispensable role in maintaining intestinal barriers, immune function, and metabolic changes. For decades, it has been believed that the activity of gut microbiota and its metabolites can directly or indirectly affect various aspects of animal physiology (Lee and Hase *et al.,* 2014). Intestinal bacteria can promote nutrient absorption and maintain physical health by secreting enzymes to break down many hard-to-digest substances and participate in inflammation and immune responses.

Evolutions in the structure of microbial communities sometimes suggest a link to diseases, including metabolic diseases (obesity and malnutrition), autoimmune diseases, intestinal parasitic diseases, and inflammatory bowel diseases (Xu *et al.,* 2023). Hence exploring the composition and alternations of gut microbiota can find out the physiological effects of experimental variables on ruminants, especially on small-tailed Han sheep with significant economic benefits.

In the current study, the effect of different rearing densities during ewe's pregnancy on the microbiome of the offspring and the connection between variations in the gut microbiota of ewes and their offspring were explored. The reproduction of livestock and the health of young animals have always been top priorities in animal husbandry. Therefore, this research may be a new insight into improving the efficiency and benefits of sheep farming.

MATERIALS AND METHODS

Experimental design and animal handling: A total of forty pregnant small-tailed Han sheep were employed and set into two groups randomly, including the high-density group (1 sheep/m^2, n=20) and the control/ low-density group (1 sheep/2m^2, n=20). The subjects were kept in the same sheep shed, fed the same diet, and were provided unified management. After the end of the pregnancy, all sheep were fed in the shed like the control group, and the newborn animals were kept with ewes before weaning. At 7 days of age, the lambs were able to take a starter diet and arbitrary access to feeds and drinking water. Furthermore, the ewes in the high-density group (1 sheep/m^2) were group P1 and their offspring F1, while ewes in the low-density/ control group (1 sheep/2m^2) were group P2 and their offspring F2.

Sample collection: Rectal fecal samples from each ewe and their offspring were collected utilizing sterile fecal collection tubes when the offspring was at the age of 4 months (H/T, samples were collected twice), 6 months (R), and 8 months (C). Each stool sample was collected in a sterile tube and was immediately frozen with liquid nitrogen before storing at -80°C for further analysis. The grouping of samples is shown in Table 1.

Table 1: Sample collection information

Age	Groups	Source	Samples
4 months	high-density group	Ewes	HP1(H.P.1-H.P.6), TP1(T.P.1-T.P.6)
		Offspring	HF1(H.F.1-H.F.6), TF1(T.F.1-T.F.6)
	Control group	Ewes	HP2(H.P.7-H.P.13), TP2(T.P.7-T.P.13)
		Offspring	HF2(H.F.7-H.F.12), TF2(T.F.7-T.F.12)
6 months	high-density group	Ewes	RP1(R.P.1-R.P.6)
		Offspring	RF1(R.F.1-R.F.6)
	Control group	Ewes	RP2(R.P.7-R.P.12)
		Offspring	RF2(R.F.7-R.F.12)
8 months	high-density group	Ewes	CP1(C.P.1-C.P.6)
		offspring	CF1(C.F.1-C.F.6)
	Control group	fecal samples from ewes	CP2(C.P.7-C.P.13)
		fecal samples from offspring	CF2(C.F.7-C.F.12)

DNA extraction and PCR amplification: Six to seven samples from each group were selected randomly for further analysis. Fecal samples were unfrozen, mixed with a small amount of sterile double distilled water, and homogenized before DNA extraction. Genomic DNA from fecal samples was extracted using the commercial Omega E.Z.N.ATM Stool DNA Kit (Omega Bio-Tek, Inc., United States) according to their specifications. To assess the integrity and purity of extracted DNA, UV–Vis spectrophotometer (NanoDrop 2000, United States) and 0.8% agarose gel electrophoresis were performed (Wang *et al.,* 2022; Ren *et al.,* 2023).

According to the previously reported PCR amplification procedure, we used conventional PCR to amplify the bacterial 16S rRNA genes variable regions (V3-V4) piloting the universal primers 338F/806R (Li *et al.,* 2023). Then, all PCR products went through gel extraction and purification by utilizing PureLink™ PCR Purification kit (Invitrogen ™, USA) and were quantified using 1.5% agarose gels (w/v). Finally, the concentrations of the products were detected using QuantiFluor™-ST (Promega, USA) (Li *et al.,* 2022; Li *et al.,* 2023)

High-throughput sequencing and library construction: Sequencing libraries were prepared using a commercially available NEXTFLEX Rapid DNA-Seq kit (Bio Scientific, USA). Then, sequencing libraries were sequenced using a paired-end configuration by using the NovaSeq 2000 platform (Illumina, United States) (Wang *et al.,* 2022).

Bioinformatics and statistical analysis: The method for obtaining reliable clean reads was the same as previously reported (Wang *et al.,* 2022). Operational taxonomic units (OTUs) were generated by clustering and obtained high-quality reads at over 97% sequence similarity by utilizing the QIIME Uclust algorithm (http://qiime.org/scripts/assign_taxonomy.html) according

to the previous database (Ding *et al.*, 2023; Ren *et al.*, 2023). Rarefaction for quality and depth of current sequencing evaluation was illustrated by R (Version 2.15.3) software. The software QIIME (Version 1.9.1) was employed for calculating microbial alpha diversity indices including Chao1and ACE (showing species richness), Shannon, Simpson, and PD_whole_tree indexes (showing species diversity and evenness) and observed species to find the diversity of bacteria in different samples. Beta diversity was performed through principal component analysis (PCA) and principal coordinates analysis (PCoA), indicating the variation of bacterial community between high-density and control groups. In addition, methods of metastatic analysis, T-test analysis, and LEfSe were analyzed to assess the bacterial community variety between groups (Mandal *et al.*, 2015).

Statistical analysis: Data was analyzed by utilizing GraphPad Prism (v7.0) and SPSS software (v22.0). The values were depicted as the mean ± SD, with statistical significance set at $P<0.05$.

RESULTS

Alpha diversity assessment: The rarefaction curves terminally tending to be horizontally implied that the sequencing depth and scope were adequate for further analysis (Fig. 1a). The rank abundance plot showed the richness and uniformity of each sample (Fig. 1b). A total of 35188 OTUs were obtained from all of the samples based on 97% sequence similarity. Among them, 11573 OUTs were obtained from group HFs and CFs. A total of 2740 and 2091 OTUs were found in group HF1 and group HF2 respectively, with 1759 OTUs shared, while 2983 and 3759 OTUs were examined in group CF1 and group CF2 respectively, with 2257 OTUs shared. Besides this, shared OTUs (n=1484) were revealed in the four sheep groups (HF1, HF2, CF1, and CF2) (Fig. 1c).

According to Table 2, the abundance and evenness of sheep gut microflora in lambs of both the high-density group and the control group were higher at the age of 8 months. The small-tailed Han sheep in the high-density group showed a significantly higher species richness while the sheep in the control group showed a high index in species diversity and evenness when growing up. However, no obvious difference in these indices was observed between the control group and the high-density group in these two periods. In addition, Table 2 imply that high density harms intestinal microbial diversity in ewes. The alpha diversity indices in lambs were lower than those in ewes at the age of 4 months while the indices turned higher in lambs than those in ewes at the age of 8 months.

The similarity between the individuals or groups: The PCA plot showed that points belonging to groups HF1 and CF2 were relatively scattered in Fig. 2a, while the points of group HF2 and CF1 were observed aggregated. This result indicated high similarities in community structure observed within the group HF1 and CF2. Similar results can be summarized from the PCoA plot and NMDS as well (Fig. 2b and 2c). In addition, there is a certain overlap between the points in group HF1 and HF2, while the distance between the groups CF1 and CF2 was relatively longer, showing the difference in bacterial community structure between group CFs was relatively more significant. Besides this, the UPGMA clustering tree (Fig. 2d) revealed a strong correlation between groups HF1 and HF2 compared to groups CF1 and CF2.

Table 2: Alpha diversity shows that high-density rearing changed the abundance and evenness of intestinal microflora in various groups (HF1 vs CF1 and HF2 vs CF2), and Ewes' vs offspring at the age of 4 months (M4 vs L4) and 8 months (M8 vs L8)

Indices	Groups			
	HF1	CF1	HF2	CF2
ACE	1648.58±61.60^{a}	1886.08±69.97^{b}	1451.69±14.09^{a}	1869.71±173.80^{b}
Chao1	1738.32±151.47^{a}	1866.80±74.93^{a}	1441.38±13.15^{a}	1975.86±193.92^{b}
Shannon	8.17±0.04^{a}	8.49±0.15^{a}	8.09±0.04^{a}	8.52±0.09^{a}
	Groups (Ewes' vs offspring)			
	M4	L4	M8	L8
ACE	1827.87±71.07^{a}	1530.55±39.33^{b}	1555.16±29.80^{a}	1873.78±101.85^{b}
Chao1	1842.45±74.82^{a}	1520.53±43.08^{b}	1529.07±21.99^{a}	1841.98±95.04^{b}
Shannon	7.61±0.17^{a}	8.13±0.03^{b}	8.25±0.05^{a}	8.50±0.08^{a}

Data is represented as Mean±SD. Different superscript letters in the rows indicate significance difference ($P<0.05$) between groups.

Taxonomic composition of bacterial populations: The top ten taxa with the highest abundance are shown in Fig. 3a. At the phylum level, the primary phyla in all of the groups were Firmicutes (37.74%, average), followed by Bacteroidota (31.61%, average). However, Proteobacteria has a higher abundance in the high-density group and was relatively higher when the lamb was eight months old. In addition, the abundance of Actinobacteria was found to increase explosively when the lamb was eight months old, which was rarely detected in the group HFs. Campylobacterota had a higher proportion in the control/ low-density group while Verrucomicrobiota was more in the high-density group.

At the genus level, *Treponema*, *Rikenellaceae* RC9 gut group, UCG-005, and *Bacteroides* were the main genera among the groups. It can be seen from Fig. 3a that *treponema* has a higher abundance in the high-density group. On the contrary, the Rikenellaceae RC9 gut group, UCG-005, and *Bacteroides* were more in the control group. The abundance of *Akkermansia* was observed higher when the sheep were four months old. In addition, interestingly, the abundance of *Ralstonia* was significantly higher in group HF1 (1.77%) than in other groups (0.0018% on average) while the abundance of GWE2-31-10 was observably higher in ruminants in group HF2 (2.96%) than in other groups (0.59% average).

The abundance of bacteria in different taxa in the feces of ewes is displayed in Fig. 3b. Phylum Firmicutes and Bacteroidota were the top two among all Phylum detected and became more in numbers when the offspring was at the age of 8 months. On the contrary, *Proteobacteria* became less at the same time. At the genus level, the abundance of Succinivibrio and Ralstonia was relatively higher when the offspring was at the age of 4 months while the Rikenellaceae_RC9_gut_group was observed lower. Besides this, it can also be found that Bacteroides were always more in the control group. Moreover, the clustering heatmap showing the top 35 genera could display the gut microbial distribution and variability among groups in more detail (Fig. 3c and 3d).

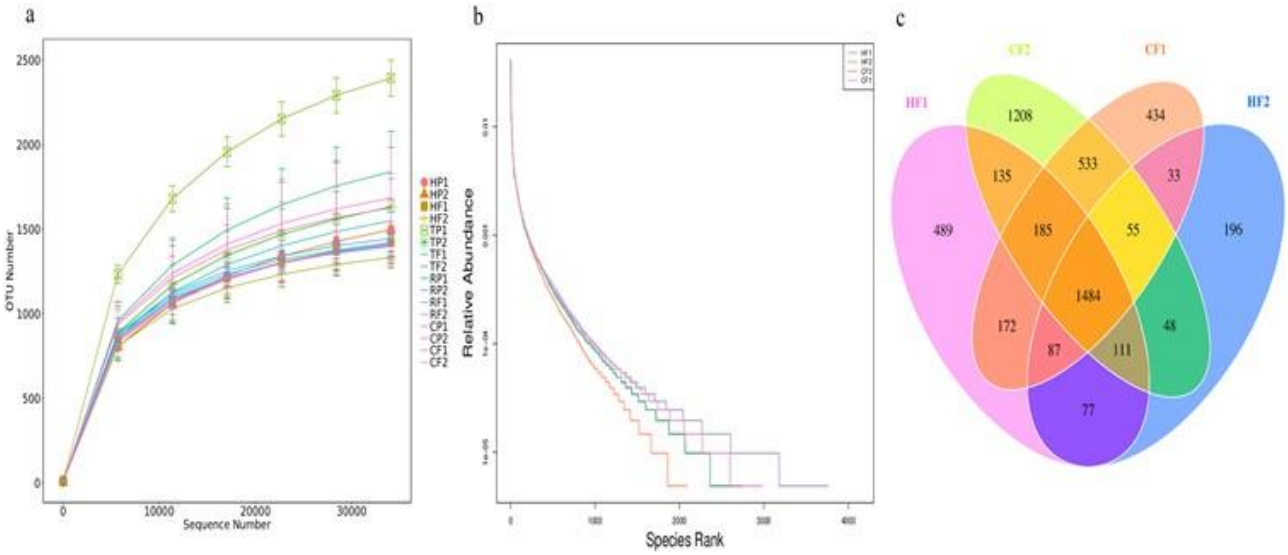

Fig. 1: Samples using 16S rRNA high-throughput sequencing. (a) Rarefaction curves of each sample (HP1, HP2, HF1, HF2, TP1, TP2, TF1, TF2, RP1, RP2, RF1, RF2, CP1, CP2, CF1, CF2). (b) Rank abundance plot of each sample. (c) Venn diagram shows the number of shared and unique OTUs in groups HF1, HF2, CF1, and CF2.

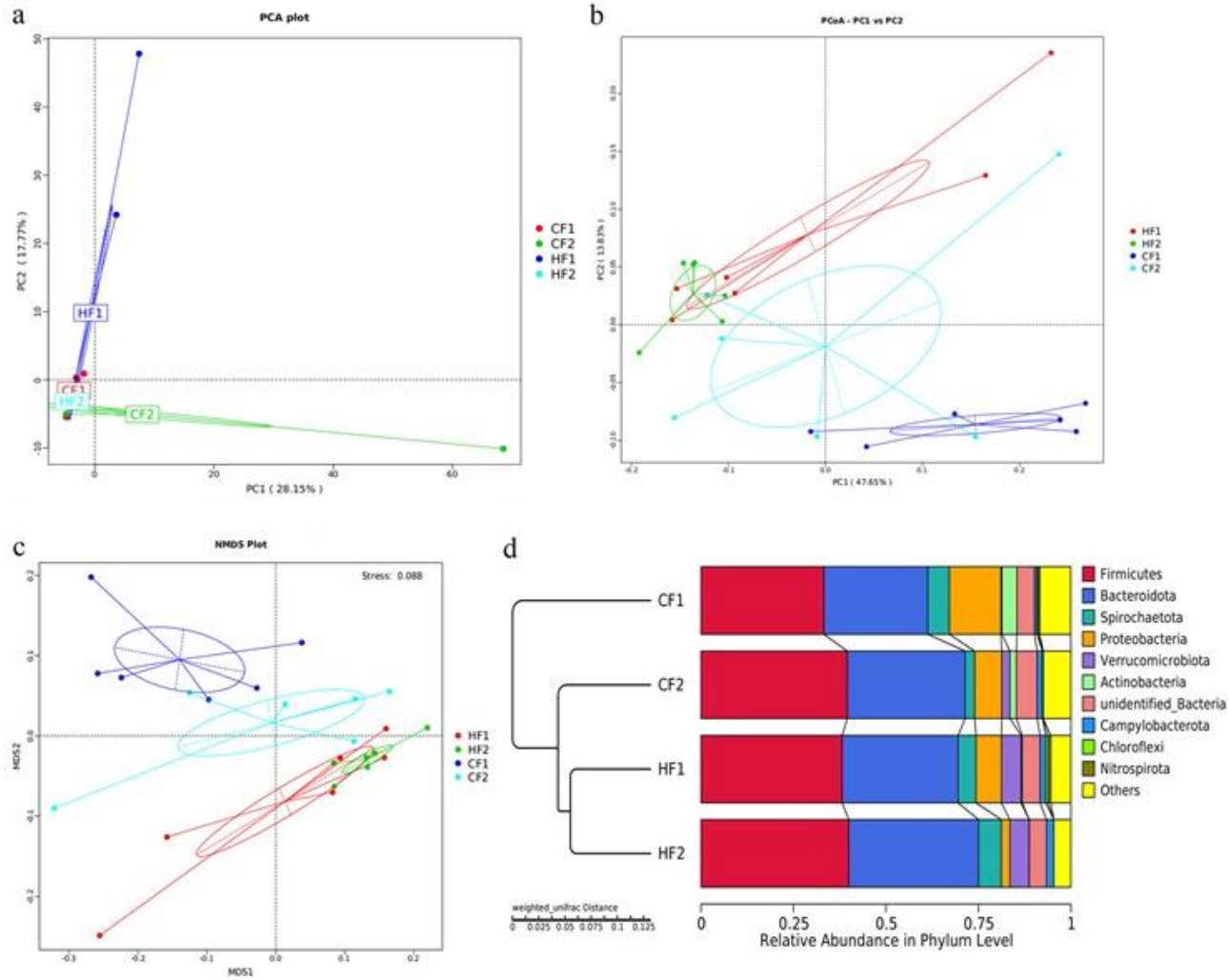

Fig. 2: Beta diversity showing that high-density rearing changed the intestine microbiota structure of small-tailed Han sheep (groups HF1, HF2, CF1, CF2). (a) Principal component (PCA) analysis. (b) Principal coordinate (PCoA) analysis. (c) Non-metric multidimensional scaling (NMDS) analysis. (d) UPGMA clustering tree.

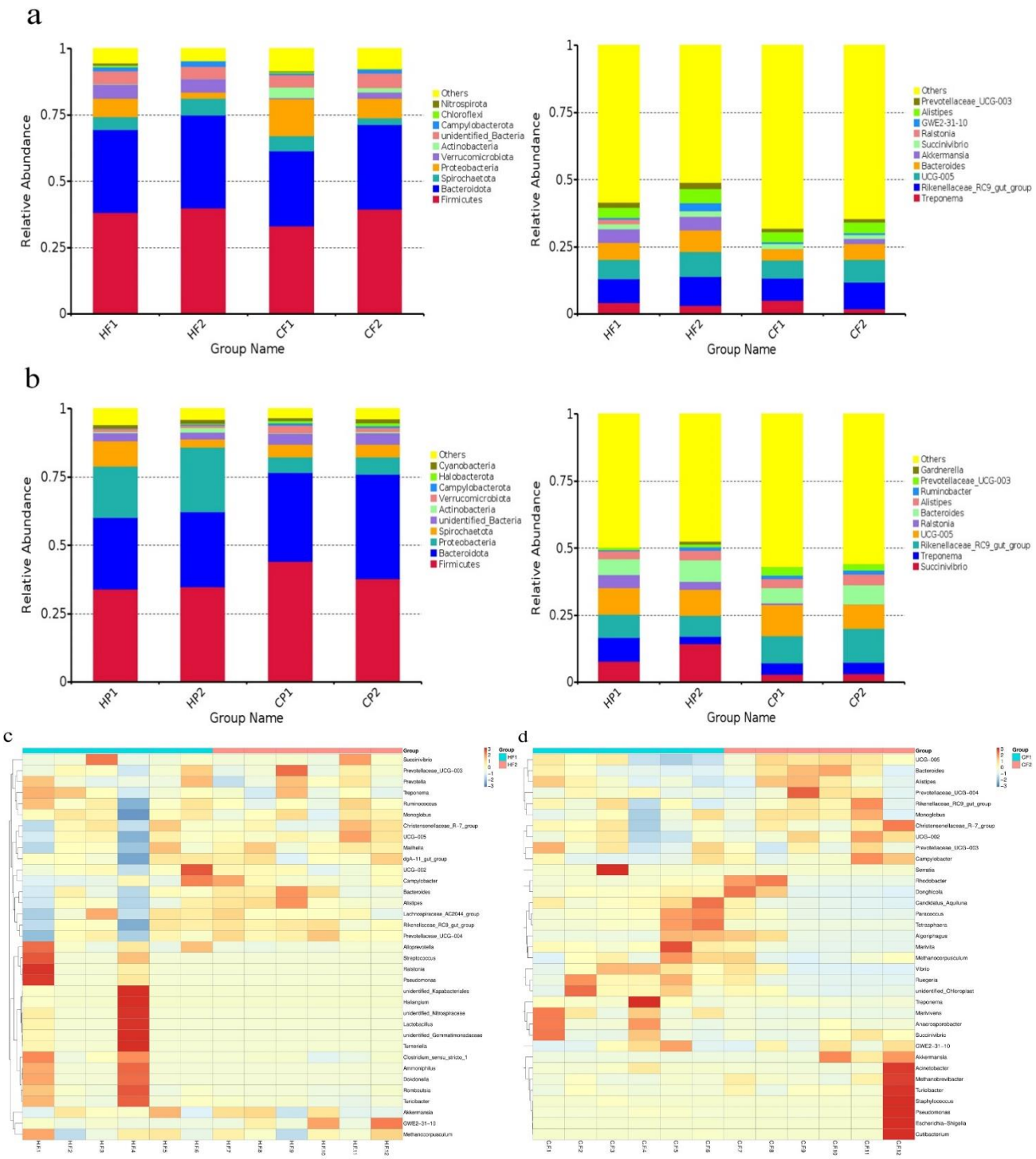

Fig. 3: High-density rearing changed gut microbiota in small-tailed Han sheep in different taxa. Top ten taxa with the highest abundance among groups (a) At the phylum level and the genus level of groups HF1, HF2, CF1, CF2. (b) At the phylum level and the genus level of groups HP1, HP2, CP1, CP2. Heatmap of the top 35 genera (c) Groups HF1 and HF2 (d) Groups CF1 and CF2.

Influence of different rearing densities on the gut bacterial composition of small-tailed Han sheep: LEFSe analysis was utilized to investigate the valid changes in the bacterial structure at phylum, class, order, family, genus, and species levels (Threshold value=3). When the small-tailed Han sheep cubs were four months old, 1 taxon (Pseudomonadales) was remarkably different between the high-density group and the control group at the order level, which was observed at higher abundance in the group HF1. Two families were detected as enriched in the high-density group, which were Pseudomonadaceae and Burkholderiaceae, conversely, the family Planococcaceae was higher in the control group. At the genus level, five genera had different abundances in the combined panel. Genera *Oscillospira*, *Lysinibacillus*, and *Ureaplasma* were observed in higher abundance in the HF2 group while *Pseudomonas* and *Ralstonia* were detected in higher abundance in the HF1 group. At the species level, Lysinibacillus_sp_YS11 was higher in the HF2 group while Anaerotignum_lactatifermentans, Treponema_porcinum, and Ralstonia_pickettii were significantly more plentiful in the HF1 group (Fig. 4a).

When the small-tailed Han sheep cubs were eight months old, three phyla were found to have significant rearing-density-related variations. Desulfobacterota was found enriched in the control group while Nitrospirota and Actinobacteriota were found enriched in the high-density group. At the class level, Saccharimonadia, Nitrospira, Acidimicrobiia, Cyanobacteriia, and Alphaproteobacteria were observed in higher abundance in the high-density group. At the family level, two families (UCG_010 and Akkermansiaceae) were significantly higher in the CF2 group while eight families (Comamonadaceae, Halieaceae, Cryomorphaceae, Ilumatobacteraceae, Nitrospiraceae, Flavobacteriaceae, Microbacteriaceae, and Rhodobacteraceae) were significantly higher in the CF1 group. Several taxa at the order level were detected to have obvious variations in both groups. The high signals of the CF1 group of bacterial were enriched at these genera levels: Chitinophagales, Saccharimonadales, Nitrospirales, Microtrichales, PeM15, Flavobacteriales, Micrococcales, and Rhodobacterales. At the genus level, *Phaeocystidibacter*, *Ilumatobacter*, *Tetrasphaera*, *Marivivens*, *Marivita*, *Ruegeria*, *Candidatus_Aquiluna*, and *Paracoccus* were detected higher in the high-density group while *Akkermansia* was observed higher in the control group. In addition, Treponema_porcinum was found to have a higher richness in group CF1($P<0.05$) (Fig. 4b).

Moreover, differences in bacterial structure at varied ages in the same experimental group were also displayed in Fig. 4c and 4d. In the control group, the variations of flora were less than in the high-density groups. Among the variations, the abundance of a class of Alphaproteobacteria, order of Rhodobacterales, and family of Rhodobacteraceae in the control groups were by those in the high-density groups. In addition, the abundance of genera of *Akkermansia* declined while that of families of Microbacteriaceae and Rhodobacteraceae increased with age in the high-density groups. The family of Spirochaetaceae reduced with age in control groups ($P<0.05$).

Similar results can be summarized from the T-test analysis. No significant difference was found between groups HF1 and HF2 at the phylum level. While three genera (*Oscillibacter*, (*Ruminococcus*)_torques_group, and Family_XIII_AD3011_group) were detected as obviously higher in the HF2 group (Fig. 5a).

At the phylum level, the abundance of Proteobacteria, Nitrospirota, and Bdellovibrionota was higher in group CF2 while the abundance of Desulfobacterota was observed lower (Fig. 5b). At the genus level, a significantly high abundance of 8 genera was observed in the CF1 group, which were *Candidatus_Aquiluna*, *Ruegeria*, and *Marivivens* (Fig. 5c).

From the perspective of the time change (HF1 VS CF1, HF2 VS CF2), variations of bacterial population structure in the control groups were rarer, indicating that the high-density-housing exerted a significant impact on intestinal microflora structure of the subsequent offspring (Fig. 5d).

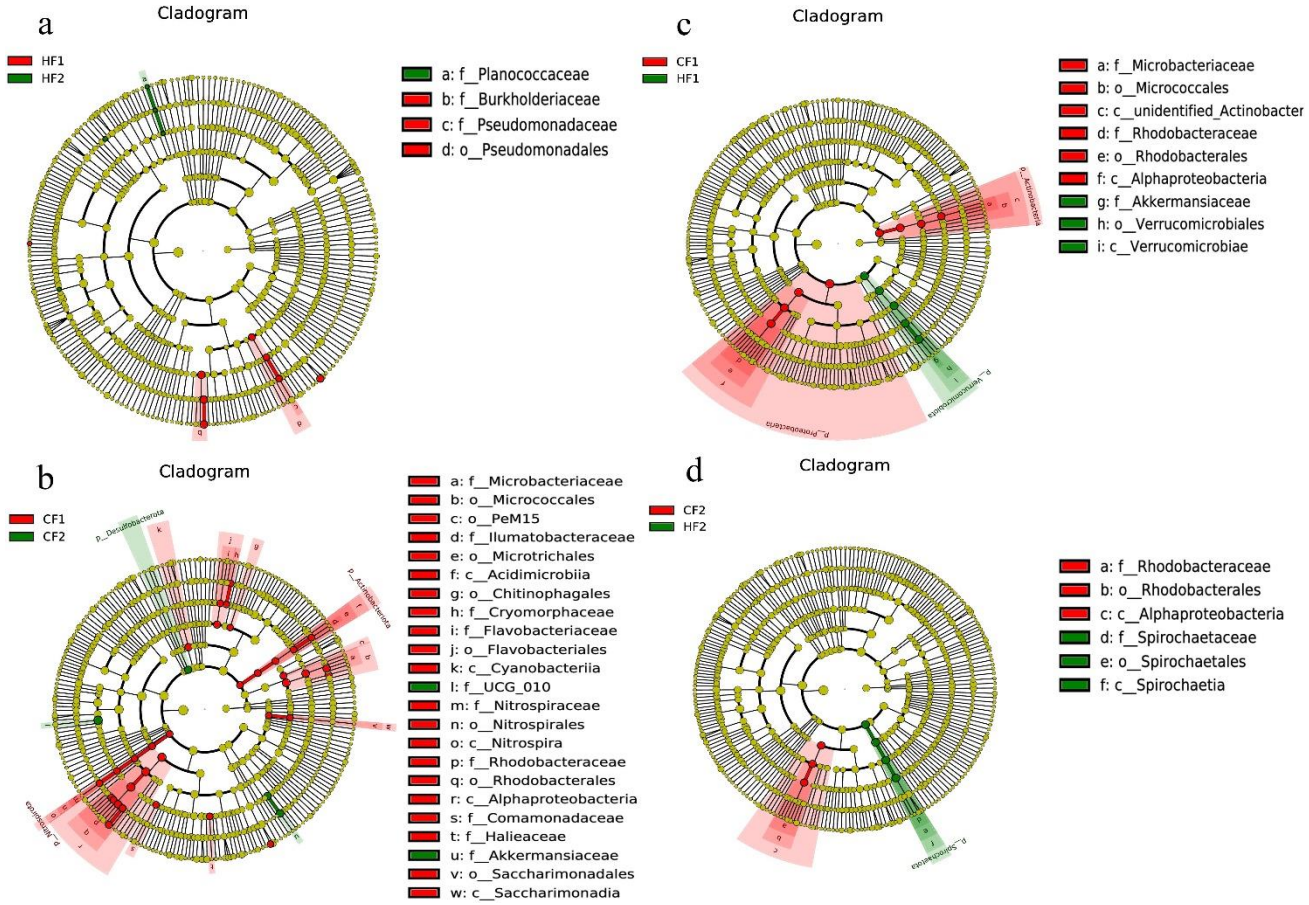

Fig. 4: LEFSe analysis showing the significant ($P<0.05$) changes in the bacterial structure at different taxa. (a) HF1 vs HF2. (b) CF1 vs CF2. (c) HF1 vs CF1. (d) HF2 vs CF2.

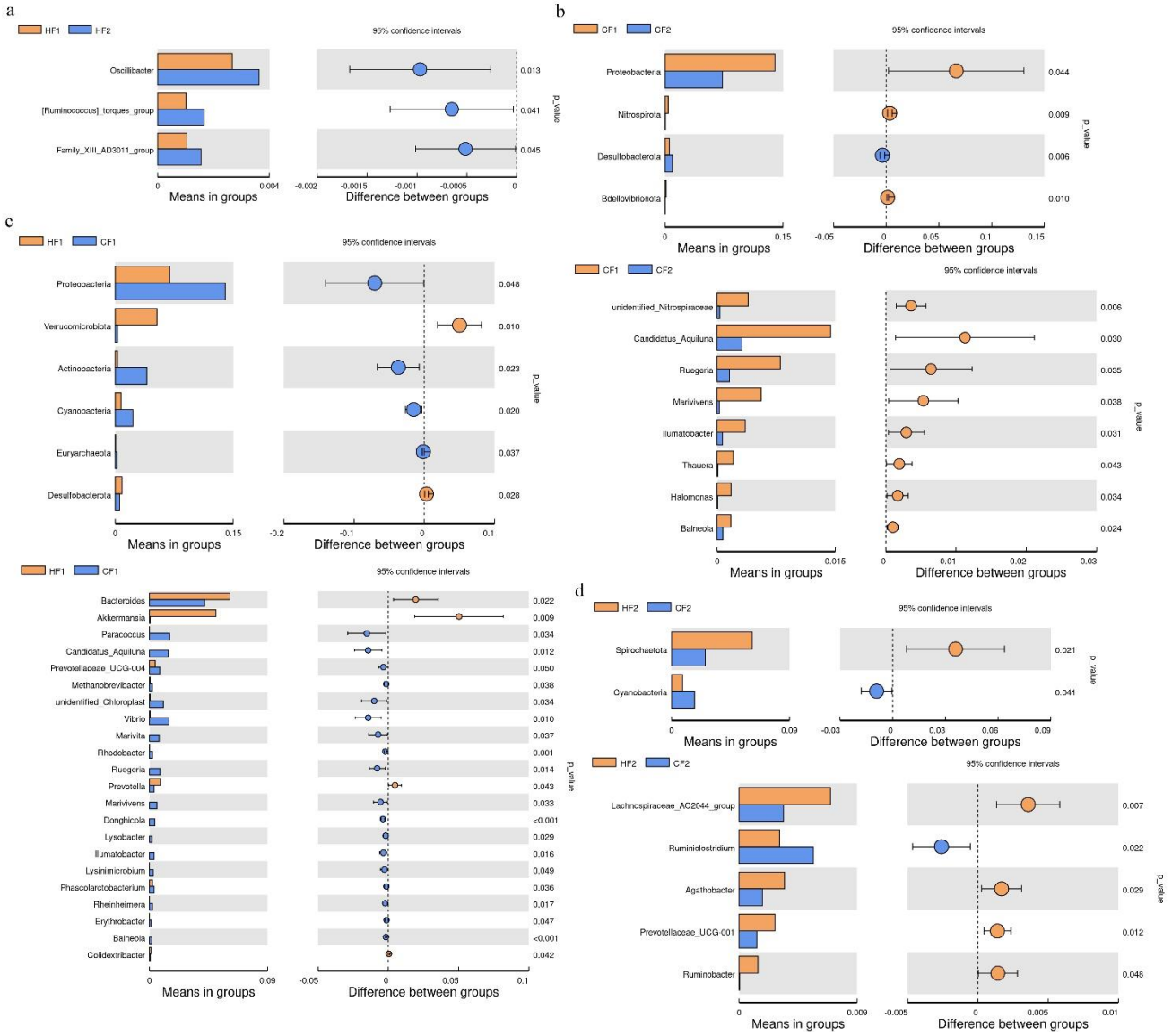

Fig. 5: T-test analysis showing the significant changes in the bacterial structure at the phylum and genus levels. (a) HF1 vs HF2. (b) CF1 vs CF2. (c) HF1 vs CF1. (d) HF2 vs CF2.

DISCUSSION

Small-tailed Han sheep come from low-altitude areas and are known for their extremely high reproduction rate. Relying on its strong fattening ability, low-fat content, strong disease resistance, and stable genetic performance, it is widely raised in China. These sheep possess excellent characteristics not only because of their genetics but also possibly related to gut microbiota (Zhang *et al.*, 2018).

The intestinal microbiota is known as the second genome in mammals, occupying an important position in animal physiological health. The microbiota has multiple effects on nutrition metabolism, growth, development, and immunoregulation in animals (Wu, 2022). At present the digestive tract microbiota of multiple animals has been deeply explored including sheep, cattle, and other ruminants.

In the present research, *Firmicutes* and *Bacteroidota* were the primary phyla detected in rectal fecal samples of small-tailed Han sheep and it is following the distribution of microbiota in the rectum detected by Zhang *et al.* (2018). This result was similar to other ruminant animals as well such as *Bos mutus* and *Pantholops hodgsonii* (Ma *et al.*, 2019; Li *et al.*, 2022).

Firmicutes play an important role in degrading fibers and cellulose in ruminants (Thoetkiattikul *et al.*, 2013), and Bacteroides can promote digestion and increase the utilization rate of complex carbohydrates (Spence *et al.*, 2006). In addition, the abundance of *Proteobacteria* was relatively increased in the high-density group, whereas there was a decrease in *Firmicutes* and *Bacteroidota*. The abundance of *Proteobacteria* in group CF1 was significantly higher than that in group CF2. The same phenomenon of microbial changes was observed preceding neonatal necrotizing enterocolitis (Wang *et al.*, 2024). Besides, phylum Proteobacteria contains extensive conditional pathogens, including *Salmonella* and *Escherichia coli* (Chen *et al.*, 2022), implying that high-density feeding of pregnant ewes exerts adverse effects on their young ones. Furthermore, the Firmicutes-to-Bacteroidetes ratio was observed statistically significant. This ratio was higher in the high-density group than the control group in the ewes, and the gap became more pronounced over time. Previous studies reported that this

ratio turned higher in obese populations of mice and the abundance of endotoxin-producing Proteobacteria was increased (Ley *et al.,* 2006; Chang *et al.,* 2015). The similar changes in the microbial community in sheep rectum may be a sign of metabolic health problems.

At the genus level, multiple alternations of intestinal microbiota were observed. The increase or decrease of certain bacteria hinted that excessive housing density during pregnancy may have adverse effects on ewes and offspring. The abundance of Bacteroides was higher in the control groups of the ewes or lambs. Bacteroides can transmit nutrients and beneficial metabolites to other intestinal residents through various complex metabolic mechanisms in the alimentary canal (Zafar and Saier, 2021). Bacteroides were highly recognized to be commensals, mutualists, and beneficial organisms and were the key to the immunological healing of cancer (Zafar and Saier, 2021).

From the information shown in Fig. 5, it can be seen that the abundance of Rikenellaceae_RC9_gut_group was higher in the control group of offspring. It is reported that the antibacterial Rikenellaceae_RC9_gut_group enriched in the fecal microbiota of Tibetan pigs after adding compound probiotics in feed (Yanguang *et al.,* 2021). As a widely recognized bacterium responsible for the degradation of dietary fiber, Succinivibrio was detected enricher in the control group of ewes, and the difference was more obvious when the young sheep was 4 months old. UCG-005 participating in the process of cellulose degradation and starch digestion in animals was also detected higher in the control group. Bacteria generally believed beneficial to the body mentioned above are relatively few in the high-density group, which may imply that the ability of small-tailed Han sheep in the high-density group to resist diseases will decrease and health can be affected to a certain extent. It was reported that Pseudomonadaceae has a strong ability to produce spoilage products, such as ammonia, resulting in rotten and spoiled meat products, vegetables, and other foods, and some species are pathogenic to humans or animals. In sheep, there is a certain correlation between the abundance of Pseudomonas on the skin of sheep and wool decay, which reduces the health and production performance of sheep (Norris *et al.,* 2008).

The famous *Pseudomonas aeruginosa* in the Pseudomonas family is a pathogenic microorganism, and it has been reported that multiple infections are caused by drug-resistant *Pseudomonas aeruginosa*. Carbapenem-resistant *Pseudomonas aeruginosa* became one of the bacterial families that posed the greatest threat to human health on the WHO list of antibiotic-resistant key pathogens in 2017 (Azam and Khan, 2019). The increase in the abundance of Pseudomonas in HF1 suggests the possibility of adverse health effects. Significance analysis such as LEFSe and T-test analysis uncovered that 13 taxa and 38 taxa were significantly altered between groups HF1 and HF2, CF1 and CF2, respectively. *Ralstonia pickettii*, being an opportunistic pathogen, which significantly increased in the HF1 group, has been reported to cause more widespread infections. In populations with low immunity, infection is more likely to occur, and this bacterium has also been proven to survive in different disinfectants (Ryan and Adley, 2014).

In addition, some studies have also demonstrated a correlation between the increase in *Ralstonia picketii* in the intestine and obesity, suggesting the possibility of a connection between this bacterium and metabolic diseases (Udayappan *et al.,* 2017). Ruegeria enriched obviously in group CF1. It is reported that the abundance of Ruegeria turned higher in the intestines of mice with long-term intake of sucralose (Daims and Wagner, 2018; Zheng *et al.,* 2022). However, it was unclear whether the enrichment of *Ruegeria* in the rectum of ruminants represented adverse factors. In the CF1 group, several genera of the family Rhodobacteraceae displayed higher abundance than in the CF2 group. It was reported that according to the origin of the family, it is mainly composed of aquatic bacteria that grow in the ocean (Pujalte *et al.,* 2014). In addition, *Acidimicrobiia* and *Nitrospira* are mostly discovered in water bodies and soil and have a more specific function (Daims and Wagner, 2018; Yuan-Qiu *et al.,* 2020). The causation of the increased abundance of these bacteria in the small-tailed Han sheep gut and the pros and cons they represent need to be further explored.

Increased genus *Oscillibacter* in group HF2 was considered to be related to starch degradation (Lin *et al.,* 2021). Besides, it has a good chance to be a genus able to produce short-chain fatty acids (SCFAs) such as butyrate, which is an important reference indicator for screening "next-generation probiotics". It has also been proven to have a positive effect on certain metabolic diseases (Yang *et al.,* 2021). By coincidence, genus *Akkermansia,* which increased in CF2, was positively connected with the amelioration of multiple metabolic diseases (obesity, diabetes, fatty liver, etc.) and was regarded as possessing the potential to become a probiotic (Bani *et al.,* 2022). Furthermore, enriched Ruminococcaceae-UCG-010 and UCG-005 (genus level) in the offspring control group were interrelated with the degradation of starch and fiber of ruminants. In Aohan Fine-Wool Sheep, Ruminococcaceae-UCG-010 was the major bacterial species in the posterior part of the intestine, where it degrades cellulose and produces SCFAs (Ma *et al.,* 2022). Among the bacterial communities that have undergone significant changes as mentioned above, bacteria that may cause health problems enriched in the high-density group while bacteria considered to have the potential to become probiotics decreased compared to the control group. This result highlighted a negative outcome that ewes were raised with high density during pregnancy. Multiple studies showed that the composition and structure of digestive tract microbiota are not only influenced by geographical differences but also by age groups (Dominguez-Bello *et al.,* 2011; Jabeen *et al.,* 2023). The growth of fetuses in the uterus is influenced by various factors and the maternal factor is the primary one. The development of the fetus in the uterus can affect the state of the body after birth, including the structure and development of intestinal microorganisms (Li *et al.,* 2021). The nutritional and physiological status of ewe vertically affects the growth and development of the newborn animals, regulating the immune system and gut microbiota of offspring (Vandenplas *et al.,* 2020). In addition, breast milk is crucial for the original development of newborn animals, being the most influential external factor in the development of the infant

microbiome, contributing to the establishment of the initial gut microbiota (Chen *et al.*, 2018; Liu *et al.*, 2022). It was known that the gut microbiota of newborns comes from the maternal microbiome during childbirth and lactation (Liu *et al.*, 2019). As a result, excessive density feeding of ewes during pregnancy not only has adverse effects on themselves but also transmits the adverse effects to offspring. Reducing prenatal stocking density has a profound economic impact on the animal husbandry industry, increasing fixed costs of facility construction. As a result, increasing the density of breeding stock can reduce the cost of facilities to some extent. However, the current study implied that this action may impact negatively on the health of farm animals, which leading to loss of economic benefits ultimately.

Conclusions: In conclusion, herein the research uncovered the response of gut microbiota in offspring after high-density rearing of pregnant ewes. The results found that the number of possibly harmful new bacterial taxa increased in high-density groups including *Proteobacteria* and *Ralstonia pickettii*, while the abundance of some probiotics like *Bacteroides*, Ruminococcaceae-UCG-010, and UCG-005 was decreased as compared to control group. Hence, our study provides new suggestions for the healthy breeding and improvement of breeding benefits of small-tail Han sheep.

Ethics approval: This research was conducted with the approval of the ethics committee of Linyi University and Nanjing Agricultural University (NJAU.No20220520108).

Data availability statement: All raw sequence data were deposited in the NCBI Sequence Read Archive and BioSample database (PRJNA972175).

Competing interests: No

Authors contribution: MJW, SJL, and SMY: research idea and methodology. MJW, ZQH, SJL, QG, JJL, YY, and JYY: reagents, materials, and analysis tools. MJW and SJL: writing – original draft and preparation. SJL, MHA, BOA, and SMY: writing – review and editing. SJL and SMY: visualization and supervision.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (NO. 31902203), Innovation Ability Training Program for doctoral students in Hebei Province (CXZZBS2023075). The authors extend their appreciation to the Researchers Supporting Project number (RSP2025R191), King Saud University, Riyadh, Saudi Arabia.

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