Animal Nutriomics

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Multi-omics analysis reveals the dominant intestinal microbial strains and metabolites related to the reproductive performance in pregnant sows

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Research Article

Cite this article: Ye Q, Luo T, Han L, Chen Y, Hu Y, Jiang H, Xu X, Yan X (2024) Multi-omics analysis reveals the dominant intestinal microbial strains and metabolites related to the reproductive performance in pregnant sows. *Animal Nutriomics* **1**, e5, 1–12. https://doi.org/10.1017/anr.2024.7

Received: 1 January 2024
Revised: 5 March 2024
Accepted: 10 March 2024

Keywords:
litter sizes; steroid hormones; microbiota; metabolomics; sows

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Abstract

Gut microbiome changed dramatically during pregnancy and played important roles in metabolic status and reproductive endocrinology in mammals. However, investigating the functional microbiota and metabolites to improve the reproductive performance and understanding the host–microbiota interaction are still arduous tasks. This study aims to reveal the dominant strains and metabolites that improve the reproductive performance. We analyzed the fecal microbiota composition and metabolic status of higher yield Chinese pig breed Meishan (MS) sows and lower yield but widespread raised hybrid pig breed Landrace × Yorkshire (L × Y) sows on days 28 and 100 of gestation. Results showed that MS sows had higher litter sizes and steroid hormone level but lower short-chain fatty acid level in feces. Fecal metabolomic analysis revealed that MS sows showed a different metabolic status compared with L × Y sows both at early and late pregnancy, which enriched with phenylpropanoid biosynthesis, bile secretion, steroid hormone biosynthesis, and plant secondary metabolite biosynthesis. In addition, 16S rDNA and internal transcribed spacer sequencing indicated that MS sows showed different structures of microbiota community and exhibited an increased bacterial α-diversity but non-differential fungal α-diversity than L × Y sows. Moreover, we found that the litter sizes and bacteria including *Sphaerochaeta, Solibacillus, Oscillospira, Escherichia–Shigella, Prevotellaceae_UCG-001, dgA-11_gut_group*, and *Bacteroides*, as well as fungi including *Penicillium, Fusarium, Microascus, Elutherascus*, and *Heydenia* both have positive association to the significant metabolites at the early pregnancy. Our findings revealed significant correlation between reproductive performance and gut microbiome and provided microbial and metabolic perspective to improve litter sizes and steroid hormones of sows.

Introduction

Intestinal microbiota could undergo significantly changes during pregnancy, which is of remarkable importance to host health through affecting host's metabolism, immune system, and behavior in mammals (Chen et al. 2021; Koren et al. 2012; Mohajeri et al. 2018; Rooks and Garrett 2016; Vuong et al. 2017). For instance, feces in humans displayed lower diversity of microbiota but higher abundance of *Proteobacteria* and *Actinobacteria* at the third trimester and contributed to an increased adiposity (Koren et al. 2012). Moreover, the higher productive capacity sows could exhibit a lower microbial richness at the late pregnancy but display a higher microbial diversity after parturition than the lower productive capacity sows (Shao et al. 2019). These studies suggest that host's metabolism and biochemical parameters during pregnancy are concerned to maternal intestinal microbiota in mammals (Tian et al. 2020). It is well-known that the intestinal microbiota could be shaped by many factors including diet, feeding environment, host genetics, and physiological state (Rothschild et al. 2018). However, further studies are needed to reveal the functional microbiota in improving the reproductive performance and therefore to elucidate the host–microbiota interaction and metabolic regulation.

Meishan (MS) sows are a Chinese indigenous breed that are characterized by its prolificacy and produced an average of more than three piglets per litter than European breeds (Hernandez et al. 2014; Xu et al. 1998). Recent studies suggested that MS sows of higher prolificacy had increased intestinal microbial diversity and steroid hormones contents than lower prolificacy

ones (Jiang et al. 2019). The steroid hormones contribute to the maintenance of pregnancy, as its key roles in regulating endometrial proliferation and receptivity to reduce embryonic loss during early pregnancy (Ye et al. 2021). Recently, it was reported that gut microbiota is vitally important in modulating reproductive endocrinology via interacting with steroid hormones and insulin (Qi et al. 2021). Moreover, it is consumed that gut microbiota could regulate sex hormones synthesis and, conversely, that sex hormones also alter the structure and diversity of gut microbiota (Insenser et al. 2018). Our previous studies found that L × Y gilts transplanted with fecal microbiota of MS gilts during the ovary-dependent period had larger area of endometrial gland, suggesting that gut microbiota may be conducive to uterine development (Xu et al. 2021). However, the difference of microbiota composition between MS sows and L × Y sows during pregnancy and which functional microbiota regulating embryo development and steroid hormones synthesis are still unclear.

Metabolic regulation is critically important to facilitate embryo survival during early pregnancy; moreover, it also affects the placental nutrient transport and fetal development during late pregnancy, which ultimately influence pregnancy efficiency in mammals (Armistead et al. 2020; Ye et al. 2021). We hypothesized that the different levels of reproductive performance between MS sows and L × Y sows are closely related to gut microbiota composition and metabolism. The aim of the present study was to reveal the dominant intestinal microbial strains and metabolites that are responsible for embryo survival during pregnancy. We collected fresh feces of MS sows and L × Y sows during early and late pregnancy and compared gut microbiota composition and metabolic status of these two breeds via multi-omics technology including 16S rRNA sequencing, internal transcribed spacer (ITS) sequencing, and metabolomics.

Materials and methods

Animal ethics statement

All protocols of sows in the present experiments were approved by the Scientific Ethics Committee of Huazhong Agricultural University (approval number: 202311010004) under the recommendations of the Guide for the Care and Use of Laboratory Animals Monitoring Committee of Hubei Province, China.

Experimental design of sows and sample collection

A total of 42 sows including 21 Landrace × Yorkshire crossbred (L × Y) primiparous sows and 21 MS sows were used in this study. L × Y sows with similar genetic background and body condition were housed individually in a gestation stall from the Dabeinong sow farm in Huangpi country, Wuhan city, Hubei province. MS sows with similar genetic background were fed in Taihu pig breeding farm located at Guannan country, Lianyungang city, Jiangsu province. All the L × Y sows and MS sows were fed the fortified corn–soybean meal gestation diets which were formulated to meet or exceed the nutrient recommendations of the National Research Council (Tables S1 and S2). After farrowing, the numbers of total piglets born (litter size) and live piglets born (live litter size) per litter were recorded. On the morning of day 28 and day 100 of gestation, the fresh feces samples from these 42 sows were individually collected and stored in liquid nitrogen. Fecal samples were grouped as followed: L × Y_E and L × Y_L: fecal samples of L × Y sows at day 28 (L × Y_E) and day 100 (L × Y_L) of gestation; MS_E and MS_L: fecal samples of MS sows at day 28 (MS_E) and day 100 (MS_L) of gestation.

Microbial genomic DNA extraction

The total microbial including bacterial and fungal genomic DNA of fecal samples was extracted using TIANamp stool DNA kit (Tiangen, Beijing, China). Briefly, 0.20 g feces were suspended in buffer solution containing proteinase K and 0.25 g grinding bead. Then, the suspension was homogenized sufficiently and incubated at 70°C for 15 min. After vortex, the homogenate solution was centrifuged to obtain the supernatant and added 10 μL RNase A incubating at 25°C for 5 min to remove the RNA. Next, the supernatant added buffer solution was incubated for 5 min in ice and centrifuged to obtain the supernatant. Microbial genomic DNA was eluted using elution buffer at room temperature for 5 min and then quantified with fluorometer and determined DNA integrity using gel electrophoresis.

16S rRNA and ITS genes amplification and high-throughput sequencing

The 16S rRNA and ITS genes amplification and high-throughput sequencing of feces samples were performed at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The V3–V4 hypervariable region of the 16S rRNA gene was amplified with the barcode fusion primers (338F: 5′-ACTCCTACGGGAGGC AGCAG-3′, 806R: 5′-GGACTACHVGGGTWTCTAAT-3′). The primer sequences for ITS region amplification were 5′-GCATCG ATGAAGAACGCAGC-3′ (forward) and 5′-TCCTCCGCTTATT GATATGC-3′ (reverse). The purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA). After demultiplexing, the resulting sequences were quality filtered with fastp and merged with FLASH (v1.2.11). Then, the high-quality sequences were denoised using DADA2 plugin in the Qiime2 pipeline. DADA2 denoised sequences are usually called amplicon sequence variants (ASVs). Taxonomic assignment of ASVs was performed using the naive Bayes consensus taxonomy classifier implemented in Qiime2 and the SILVA 16S rRNA and ITS database. Bioinformatic analysis of the fecal microbiota was carried out using the Majorbio Cloud platform (https://cloud.majorbio.com).

Metabolomic analysis

The polar metabolite profiling in fecal samples was detected at Majorbio using liquid chromatography-tandem mass spectrometry (Prasad et al. 2011). Briefly, 50 mg feces were weighed and then extracted using 400 μL methanol:water (4:1, v/v) solution with 0.02 mg/mL L-2-chlorophenylalanin as internal standard. The mass spectrometric data were collected using a UHPLC-Q Exactive HF-X Mass Spectrometer (Thermo Scientific, Waltham, MA) equipped with an electrospray ionization source operating in either positive or negative ion mode. The significant different metabolites were determined based on the variable importance in the projection (VIP) obtained by the OPLS-DA (Orthogonal Projections to Latent Structures-Discriminant Analysis) model (VIP $>$ 1.0) and the p-value of student's t-test ($p < 0.05$) and fold change (MS vs L × Y) higher than 1.30 or lower than 0.77. The significant different metabolites were mapped into biochemical pathways based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database search (http://wwwgenome.jp/kegg/).

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Quantification of short-chain fatty acid (SCFA) profiling

SCFAs in feces were quantified with gas chromatography (Xu et al. 2021). Briefly, 1 g feces were weighted, dissolved, and homogenized in 1 mL methanol. Then, the homogenized solution was centrifuged to obtain the supernatant and diluted (5:1, v/v) with 25% metaphosphoric acid at 4°C overnight. Finally, the supernatant was obtained after centrifugation at 12,000 g for 10 min at 4°C and used for SCFA detection with a gas chromatography (GC Trace 1300, Thermo Fisher Scientific, USA).

Measurement of steroid hormones

Steroid hormones including estradiol and progesterone in feces were measured using commercially available radioimmunoassay (RIA) kits (Beijing North Institute of Biotechnology). Briefly, 0.2 g feces were weighted, dissolved, and homogenized in 1 mL 80% ethanol. After incubation at 70°C for 15 min and vortex for 1 min, the homogenized solution was centrifuged to obtain the supernatant. Then, 0.5 mL 80% ethanol was added into the residue and vortex for 1 min. The supernatant was obtained and dried in a vacuum concentrator using nitrogen. The residue was stored at −20°C or redissolved in phosphate buffer to detect the estradiol or progesterone levels using iodine [^{125}I] estradiol RIA kit or iodine [^{125}I] progesterone RIA kit.

Statistical analysis

All results were expressed as means ± SEM (Standard Error of the Mean). Differences were considered statistically significant if $p < 0.05$. Statistical analysis using two-sided Student's *t*-test and plotting of data were performed using GraphPad Prism 9.0 (Graph Pad, San Diego, CA), as indicated in the figure legends. The significant different genera and characteristic genera were respectively identified using Wilcoxon rank-sum test and linear discriminant analysis effect size analysis. Bar plots and correlations between differentially presented bacterial taxa and the concentrations of fecal metabolites, steroid hormones, and litter sizes were calculated by Spearman correlation analysis.

Results

MS sows have higher reproductive performance but lower fecal SCFA levels compared with L × Y sows

As shown in Fig. 1A, we recorded the litter size and live litter size of 21 MS sows and 21 L × Y sows and collected their fecal samples at the early (day 28) and late (day 100) pregnancy to analyze the microbiome and detect the levels of progesterone (P_4), estradiol (E_2), and SCFAs. The results showed that MS sows had significantly higher litter size and live litter size than the L × Y sows (Fig. 1B and C). In addition, the fecal progesterone and estradiol levels of MS sows at the early pregnancy were significantly higher than that in the L × Y sows (Fig. 1D and E), and the fecal progesterone level in MS sows at the late pregnancy was significantly higher than that in the L × Y sows (Fig. 1D). SCFAs synthesized by microorganisms were suggested to be important regulatory factors contributing to its biological function (Koh et al. 2016). However, we found that the fecal SCFA levels of MS sows were significantly lower than that in the L × Y sows (Fig. 1F).

Metabolite profiling difference between MS sows and L × Y sows

We used nontargeted metabolomic analysis to investigate the difference in fecal metabolites and its metabolism pathways. PLSDA (Partial Least Squares Discriminant Analysis) was used to identify the pattern of difference in metabolites between the MS sows and L × Y sows both at the early and late pregnancy. As shown in Fig. 2A, we found that MS sows had a distinct metabolism signature compared to the L × Y sows both at the early and late pregnancy (MS_E vs L × Y_E, MS_L vs L × Y_L). A total of 3172 metabolites were detected and could be successfully annotated, of which 208 metabolites in MS_E group were significantly higher than that in L × Y_E group, and 281 metabolites in MS_E group were significantly lower than that in L × Y_E group (Fig. 2B). In addition, 67 metabolites in MS_L group were significantly higher than that in L × Y_L group, and 213 metabolites in MS_L group were significantly lower than that in L × Y_L group (Fig. 2C). Figure 2D and E showed the expression profile and VIP of the top 50 different metabolites of MS_E versus L × Y_E and ME_L versus L × Y_L. We found that a considerable part of metabolites (pointed by the red arrow) in MS sows were significantly higher than that in L × Y sows at both the early and late pregnancy, such as epothilone B, destruxin E, eltoprazine, N-eicosapentaenoyl aspartic acid, 3,4-dimethyl-5-pentyl-2-furannonanoic acid, rhodinyl phenylacetate, germacrone-13-al, rubraflavone D, rubschisandrin, pentolame, and diethylthiophosphate. In addition, a considerable part of metabolites (pointed by the blue arrow) in MS sows were significantly lower than that in L × Y sows at both the early and late pregnancy.

In order to systematically reveal the modulation of intestinal metabolism in these two pig breeds, the KEGG pathways enriched by the significant different metabolites were showed in Fig. 2F and G. Phenylpropanoid biosynthesis was the top one pathway that both enriched at the early and late pregnancy. Previous researchers have indicated that phenylpropanoid biosynthesis is an important pathway for the production of plant secondary metabolites (Ma et al. 2018), and we found that biosynthesis of plant secondary metabolites and bile secretion were also enriched at both the early and late pregnancy (Fig. 2F and G). In addition, arginine and proline metabolism, glycerophospholipid metabolism, sphingolipid signaling pathway, retrograde endocannabinoid signaling, and steroid hormone biosynthesis that have been reported to play key regulatory roles for embryo implantation and uterine receptivity in the previous studies (Wu et al. 2013; Ye et al. 2021) were enriched as significant pathways between MS and L × Y sows at the early pregnancy. Moreover, GnRH signaling pathway was also enriched at the early pregnancy for the comparison of MS and L × Y sows.

Intestinal microbiota composition and dominant strains between MS sows and L × Y sows

To further examine the differences in the microbiota composition and abundance between MS and L × Y sows, 16S rRNA and ITS gene sequencing of fecal samples were performed, which showed a satisfactory sequencing depth of bacterial and fungal DNA in Fig. 3A and B, respectively. On the whole, there were clear segregation of the bacteria (Fig. 3C) and fungi (Fig. 3D) structure between MS_E and L × Y_E, as well as MS_L and L × Y_L. The α-diversity indices of bacteria such as Sobs, ACE (Abundance-based Coverage Estimator), Chaos, and Shannon indices of MS_E were markedly lower than L × Y_E, whereas Simpson and coverage indices of

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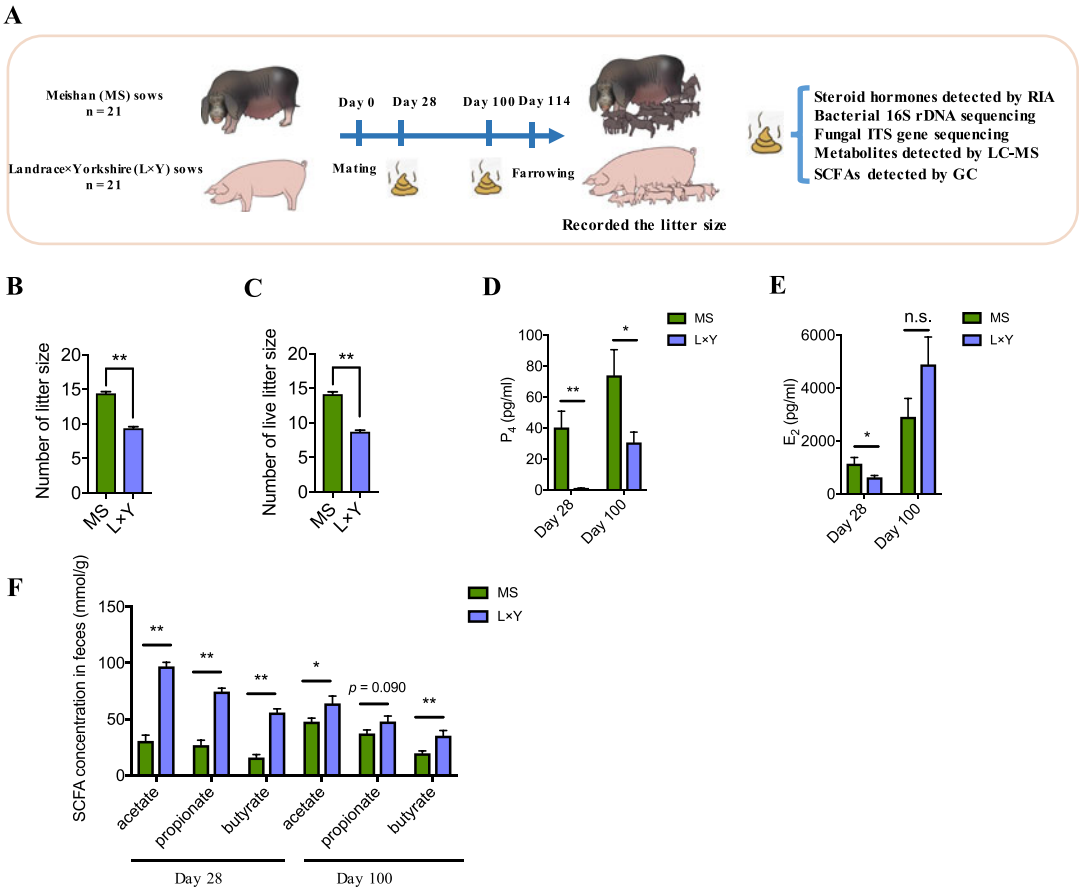

Figure 1. MS sows have higher reproductive performance but lower fecal SCFA levels compared with L × Y sows. (A) Study design for the whole experiment. Each group comprised 21 sows. (B–C) The number of litter size (B) and live litter size (C) of MS sows and L × Y sows ($n = 21$). (D–F) The levels of P_4 (D), E_2 (E), and SCFAs (F) in feces of MS sows and L × Y sows at the early (day 28) and late (day 100) pregnancy ($n = 21$). *$p < 0.05$, **$p < 0.01$. n.s. not significant.

MS_E were markedly higher than L × Y_E (Fig. 3E). Moreover, the α-diversity indices of bacteria such as Sobs, ACE, and Chaos indices of MS_L were markedly lower, whereas coverage index was markedly higher than L × Y_L, with Shannon and Simpson indices have no difference between MS_L and L × Y_L (Fig. 3E). Additionally, the α-diversity indices of fungi such as Simpson index of MS_E were markedly higher than L × Y_E, whereas the coverage index of MS_E was markedly lower than L × Y_L, with Sobs, ACE, Chaos, and Shannon indices have no difference for MS_E vs L × Y_E and MS_L vs L × Y_L (Fig. 3F). The bacteria were dominated by two phyla including *Firmicutes* and *Bacteroidota* (Fig. 3G) and five genera including *Treponema, Terrisporobacter, Lachnospiraceae_XPB1014_group, Escherichia–Shigella*, and *Christensenellaceae_R-7_group* (Fig. 3H). The fungi were dominated by two phyla including *Neocallimastigomycota* and *Ascomycota* (Fig. 3I) and four genera including *Piromyces, Kazachstania, Dipodascus*, and *Aspergillus* (Fig. 3J).

Next, db-RDA (Distance-based Redundancy analysis) was applied to illustrate the distribution of the SCFA level and reproductive performance including litter size, live litter size, and the levels of P_4 and E_2 based on microbiota genus levels. We found that the spots belonging to MS_E group and L × Y_E group showed clear segregation (Fig. 4A), which suggested an important regulation of intestinal bacteria composition at the early pregnancy on the SCFA level and reproductive performance. Moreover, the angles of arrows between the reproductive performance including litter size, live litter size, and the levels of P_4 and E_2 were acute angles, which were consistently showed in the bacteria and fungi view both at the early and late pregnancy (Fig. 4A–D), suggesting a positive correlation of these reproductive performance in sows from the perspective of microbial regulation during pregnancy. However, the angles between the arrows of reproductive performance and SCFAs were obtuse angles (Fig. 4A–D), indicating a negative correlation between the reproductive performances and SCFAs from the perspective of microbial regulation during pregnancy.

The different genera of bacteria and fungi were showed in Fig. 5. For bacteria, we found that MS sows showed significantly higher abundance of *Treponema, Escherichia–Shigella, Solibacillus, dgA-11_gut_group, Bacteroides*, and *Prevotellaceae_UGC-001* at the early pregnancy (Fig. 5A) and significantly higher abundance of *Treponema, Rikenellaceae_RC9_gut_group, NK4A214_group, Ruminococcus, Prevotelaceae_UGC-003, Bacteroides, Alloprevotella, Family_XIII_AD3011_group, Sphaerochaeta, Oscillospira, Oscillibacter*, and *Fibrobacter* at the late pregnancy than L × Y sows (Fig. 5B). For fungi, MS sows showed significantly higher abundance of *Heydenia, Eleutherascus, Fusarium, Ciliophora, Penicillium, Microascus, Wallrothiella, Cleistothelebolus, Meyerozyma*, and *Coprinopsis* at the early pregnancy (Fig. 5C) and significantly higher abundance of *Fusarium, Scopulariopsis, Wallrothiella, Meyerozyma, Kodamaea*, and *Komagataella* at the late pregnancy than L × Y sows (Fig. 5D).

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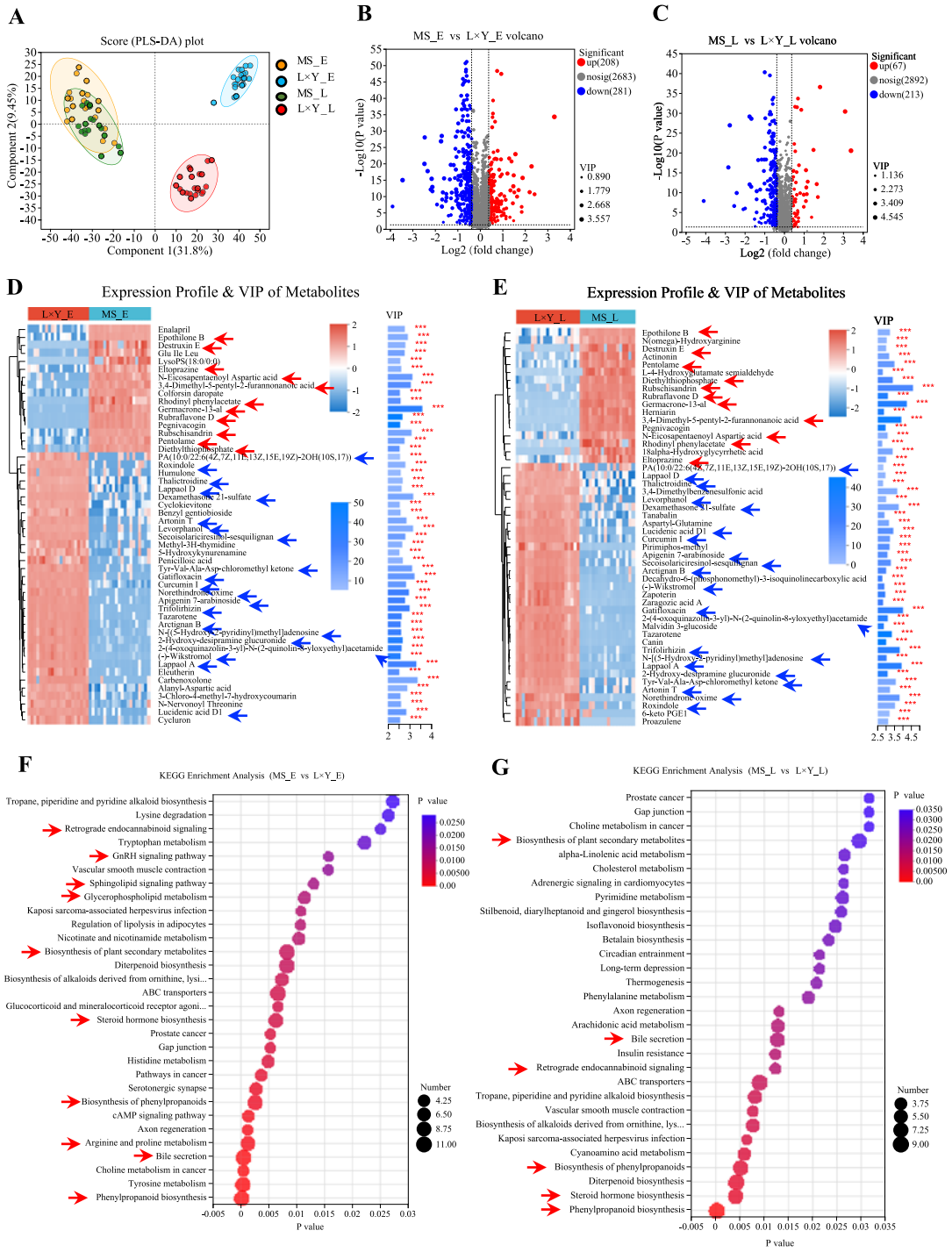

Figure 2. Metabolite profiling difference between Meishan sows and L × Y sows during pregnancy. Each group comprised 21 sows. (A) The score plot of PLSDA in fecal samples. (B–C) The volcano plots of the metabolites in fecal samples at the early pregnancy (B) and the late pregnancy (C). Red dots represent upregulated metabolites, blue dots represent downregulated metabolites, and gray dots represent not significant different metabolites. (D–E) The expression profile and VIP of the top 50 metabolites at the early pregnancy (D) and the late pregnancy (E) in fecal samples. (F–G) The KEGG pathway enrichment analysis for the different metabolites at the early pregnancy (F) and the late pregnancy (G). Abbreviations: MS_E, Meishan sows at the early pregnancy; MS_L, Meishan sows at the late pregnancy; L × Y_E, Landrace × Yorkshire sows at the early pregnancy; L × Y_L, Landrace × Yorkshire sows at the late pregnancy.

Meanwhile, the characteristic genera of bacteria and fungi using at the early and late pregnancy were showed in Fig. 6. We found that MS sows were featured by 11 genera of bacteria including *Solibacillus, Escherichia–Shigella, Treponema, dgA-11_gut_group, Prevotellaceae_UGC-001, Acinetobacter, Bacteroides, Sphaerochaeta, Oscillospira, Comamonas*, and *Clostridium_sensu_stricto_3* at the early pregnancy (Fig. 6A) and by two genera (including *Treponema* and *Turicibacter*) at the late pregnancy (Fig. 6B). In addition, MS sows were featured by 13 genera of fungi at the early pregnancy and 9 genera at the late

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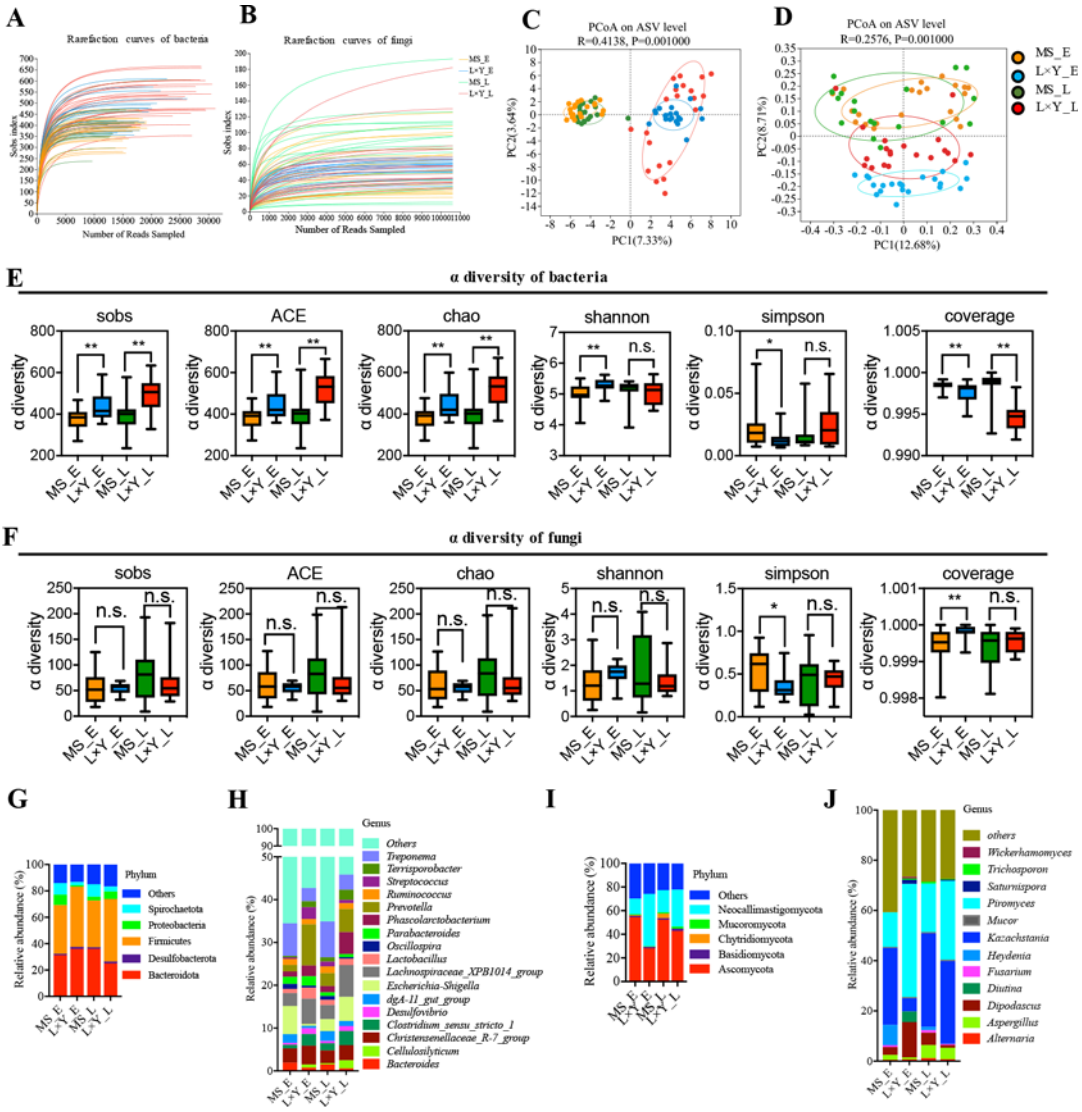

Figure 3. MS sows have distinct intestinal microbiota composition during pregnancy. Each group comprised 21 sows. (A–B) The rarefaction curves of bacteria (A) and fungi (B) for MS sows and L × Y sows. (C–D) The PCoA on ASV level of bacteria (C) and fungi (D) for MS sows and L × Y sows. (E–F) The α-diversity of bacteria (E) and fungi (F) for MS sows and L × Y sows. (G–H) The phylum (G) and genus (H) levels of bacteria for MS sows and L × Y sows. (I–J) The phylum (I) and genus (J) levels of fungi for MS sows and L × Y sows.

pregnancy (Fig. 6D). These results showed that the pattern of fecal microbiota including bacteria and fungi at both early and late pregnancy were different between MS sows and L × Y sows.

Correlation analysis of intestinal microbiota and metabolites related to reproductive performance

We selected the overlap of differential genera and characteristic genera of the bacteria and fungi and then used Spearman correlation analysis to investigate the correlation between abundance of the top 50 differential metabolites and key microbiota and reproductive performance (including litter size, live litter size, P_4, and E_2) at the early and late pregnancy (Fig. 7). At the early pregnancy, there were 19 metabolites that were positively correlated with the reproductive performance (including litter size, litter size, P_4, and E_2) and seven genera of bacteria including *Sphaerochaeta, Solibacillus, Oscillospira, Escherichia–Shigella, Prevotellaceae_UCG-001, dgA-11_gut_group*, and *Bacteroides* (Fig. 7A) as well as five genera of fungi including *Penicillium, Fusarium, Microascus, Elutherascus*, and *Heydenia* (Fig. 7B). At the late pregnancy, there were 18 metabolites that were positively correlated with the litter size, live litter size, and P_4 whereas negatively correlated with E_2 (Fig. 7C and D). In addition, the 18 metabolites were positively correlated with four genera of bacteria including *Bacteroides, Sphaerochaeta, Oscillospira*, and *Prevotellaceae_UCG-004* (Fig. 7C) as well as six genera of fungi including *Kodamaea, Fusarium, Meyerozyma, Scopulariopsis, Wallrothiella*, and *Komagataella* (Fig. 7D).

Discussion

Chinese MS pig is highly prolific breed, farrowing >3 live piglets per litter than European pig breeds (Hernandez et al. 2014; Xu et al. 1998). Previous studies suggested that the larger litter size of MS sows partly results from higher embryonic survival (Huang et al. 2015). In pigs, 30–50% of embryos are lost during early

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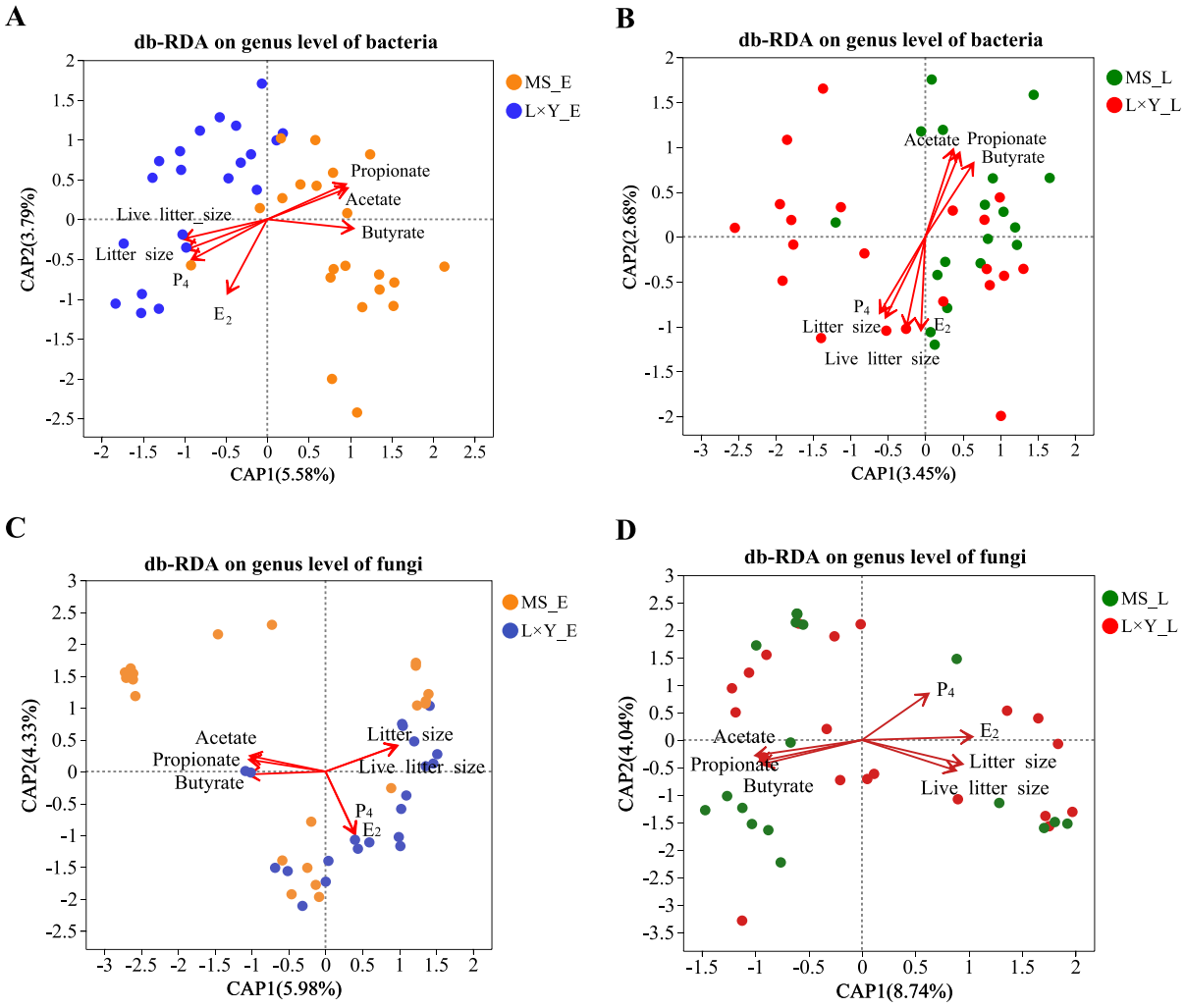

Figure 4. The db-RDA analysis demonstrated the distribution of the reproductive performance and SCFA level based on microbiota composition. (A–B) The distribution of the reproductive performance and SCFA level based on the genus levels of bacteria at the early (A) and late (B) pregnancy. (C–D) The distribution of the reproductive performance and SCFA level based on the genus levels of fungi at the early (C) and late (D) pregnancy. The arrows indicate the litter size, live litter size, progesterone (P_4), estradiol (E_2), acetate acid, propionate acid, and butyrate acid levels, and their contributions to the explanation of the sample difference are shown by the arrow length. The angle between the arrows represents the positive correlation (<90°) or negative correlation (>90°) among the reproductive performance and SCFA level.

pregnancy, and it had been indicated that Chinese MS pigs had a 20–30% greater fetal survival than the European pig breeds (Huang et al. 2015). It was reported that the maternal metabolic status and gut microbiota were associated with the level of steroid hormones (Chen et al. 2021; Jiang et al. 2019), which was known to coordinate uterine receptivity and contribute to the embryo survival (Cha et al. 2012). In addition, SCFAs, the critical product of gut microbial fermentation from undigested dietary carbohydrates, have been recognized as important mediators between microbiota and host physiology (Koh et al. 2016). These findings suggested an important regulation of intestinal microbiota and metabolism on reproductive performance via modulating endocrinology system.

Herein, the intestinal metabolomic analysis and microbiota sequencing of MS sows and L × Y sows during pregnancy were integrated to digest the potential pattern of microbiome regulating the litter size and steroid hormone synthesis. Our results suggested that MS sows showed a different metabolic status compared with L × Y sows both at the early and late pregnancy, which enriched with phenylpropanoid biosynthesis, bile secretion, steroid hormone biosynthesis, and plant secondary metabolites biosynthesis. Moreover, MS sows showed different microbiota community structure compared with L × Y sows and suggested a decreased bacterial α-diversity but non-differential fungal α-diversity. In addition, we found positive correlation between the litter sizes and steroid hormones and bacteria including *Sphaerochaeta, Solibacillus, Oscillospira, Escherichia–Shigella, Prevotellaceae_UCG-001, dgA-11_gut_group*, and *Bacteroides* as well as fungi including *Penicillium, Fusarium, Microascus, Elutherascus*, and *Heydenia* at the early pregnancy. Furthermore, we also found positive correlation between the litter sizes and progesterone and bacteria including *Bacteroides, Sphaerochaeta, Oscillospira*, and *Prevotellaceae-UGC-04* as well as fungi including *Kodamaea, Fusarium, Meyerozyma, Scopulariopsis, Wallrothiella*, and *Komagataella* at the late pregnancy. Pigs of different breeds did not have the same age, diet, and housing environment. The gut microbiota formation in these two breeds of sows was the result of a combination of various factors as mentioned above. Our study first integrated the bacteria and fungi and metabolite data of MS sows and L × Y sows during gestation and revealed the

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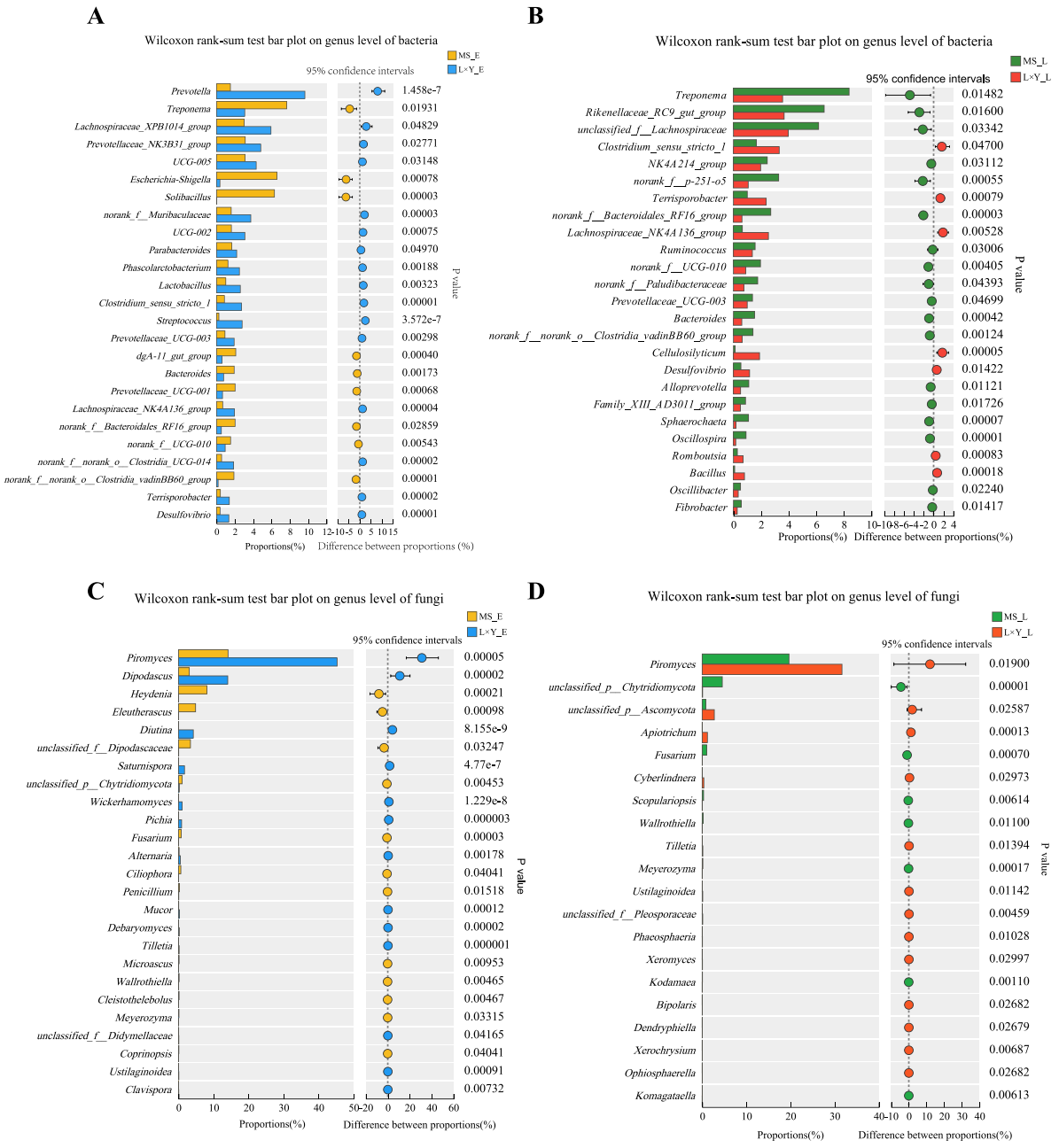

Figure 5. The significant different microbiota between MS sows and L × Y sows during pregnancy. Each group comprised 21 sows. (A–B) The top 25 of different genera of bacteria between MS sows and L × Y sows at the early (A) and late (B) pregnancy. (C–D) The top 25 of different genera of fungi between MS sows and L × Y sows at the early (C) and late (D) pregnancy.

underling microorganisms and metabolites that could influence the reproductive performance.

Previous studies have been explored the relationship between gut bacteria and reproductive performance in sows (Chen et al. 2021; Shao et al. 2019; Uryu et al. 2020). Uryu et al. (2020) reported that sows with high-reproductive performance exhibited similar α-diversity but increased abundances of *Treponema, Ruminococcus, Collinsella, Fibrobacter, Phascolarctobacterium, Rummeliibacillus, Butyricicoccus, Bulleidia, Oribacterium, Blautia, Sphaerochaeta*, and *Peptococcus* than sows with low-reproductive performance (Uryu et al. 2020). Chen et al. (2021) reported that sows with high-reproductive performance revealed increased α-diversity and butyrate-producing genera than sows with low-reproductive performance (Chen et al. 2021). In addition, the relative abundance of *Turicibacter* and *Ruminococcaceae_UGC-014* in feces of sows at the early pregnancy had positive correlation with litter size, and the relative abundance of *Clostridium_sensu_stricto_1, Turicibacter, Terrisporobacter, Christensenellaceae_R-7_group*, and *Escherichia–Shigella* at the late pregnancy exhibited positive correlation with litter size (Chen et al. 2021). At the late pregnancy, Shao et al. (2019) reported that sows with high-productive capacity had lower microbial richness but higher abundance of *Prevotellaceae_NK3B31_group, Alloprevotella, Prevotella-2, Fibrobacter*, and *Sphaerochaeta* in

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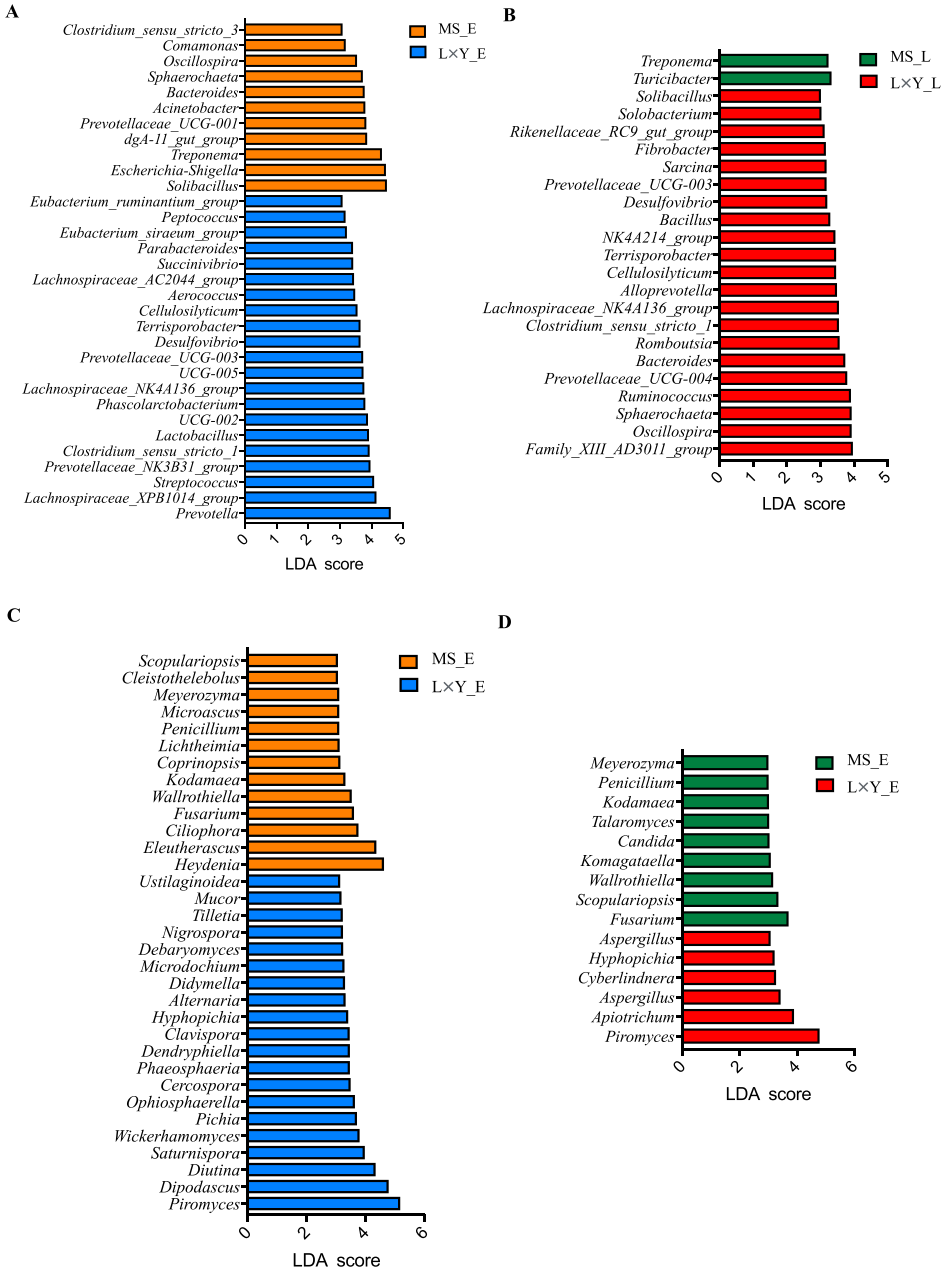

Figure 6. The characteristic microbiota between MS sows and L × Y sows during pregnancy. (A–B) The characteristic genera of bacteria in MS sows and L × Y sows at the early (A) and late (B) pregnancy. (C–D) The characteristic genera of fungi in MS sows and L × Y sows at the early (C) and late (D) pregnancy.

feces than sows with low-productive capacity (Shao et al. 2019). In our results, MS sows exhibited increased level of *Treponema, Escherichia–Shigella, Solibacillus, dgA-11_gut_group, Bacteroides*, and *Prevotellaceae_UCG-001* at the early pregnancy and higher abundance of *Treponema, Rikenellaceae_RC9_gut_group, NK4A214_group, Prevotellaceae_UCG-003, Bacteroides, Alloprevotella, Sphaerochaeta, Oscillospira, Oscillibacter*, and *Fibrobacter* at the late pregnancy than L × Y sows.

During gestation, gut microbes play a vital role in the function and health (such as nutrient absorption, metabolism, immune system, and endocrinology system) and are also affected by the physiological state of their host (Mohajeri et al. 2018; Qi et al. 2021; Vuong et al. 2017). In humans, the composition of intestinal flora in pregnant women during the first trimester was similar to that of nonpregnant women, and significant changes were existed in the composition of intestinal flora in the third trimester, with increasing abundance of inflammatory-related bacteria, such as *Actinobacteria* and *Proteobacteria* (Koren et al. 2012). In pigs, previous studies found that dietary supplementation with *Bacillus subtilis* PB6 during late gestation could shorten piglet birth intervals, reduce oxidative stress, and improve the gut health of sows (Zhang et al. 2020). Kong et al. (2016) found that the colonic flora α-diversity of Huanjiang sows decreased significantly with the progression of gestation, and the relative abundance of *Firmicutes* increased but the abundance of *Bacteroidetes* decreased in the distal colon contents (Kong et al. 2016). However, Zhou et al.

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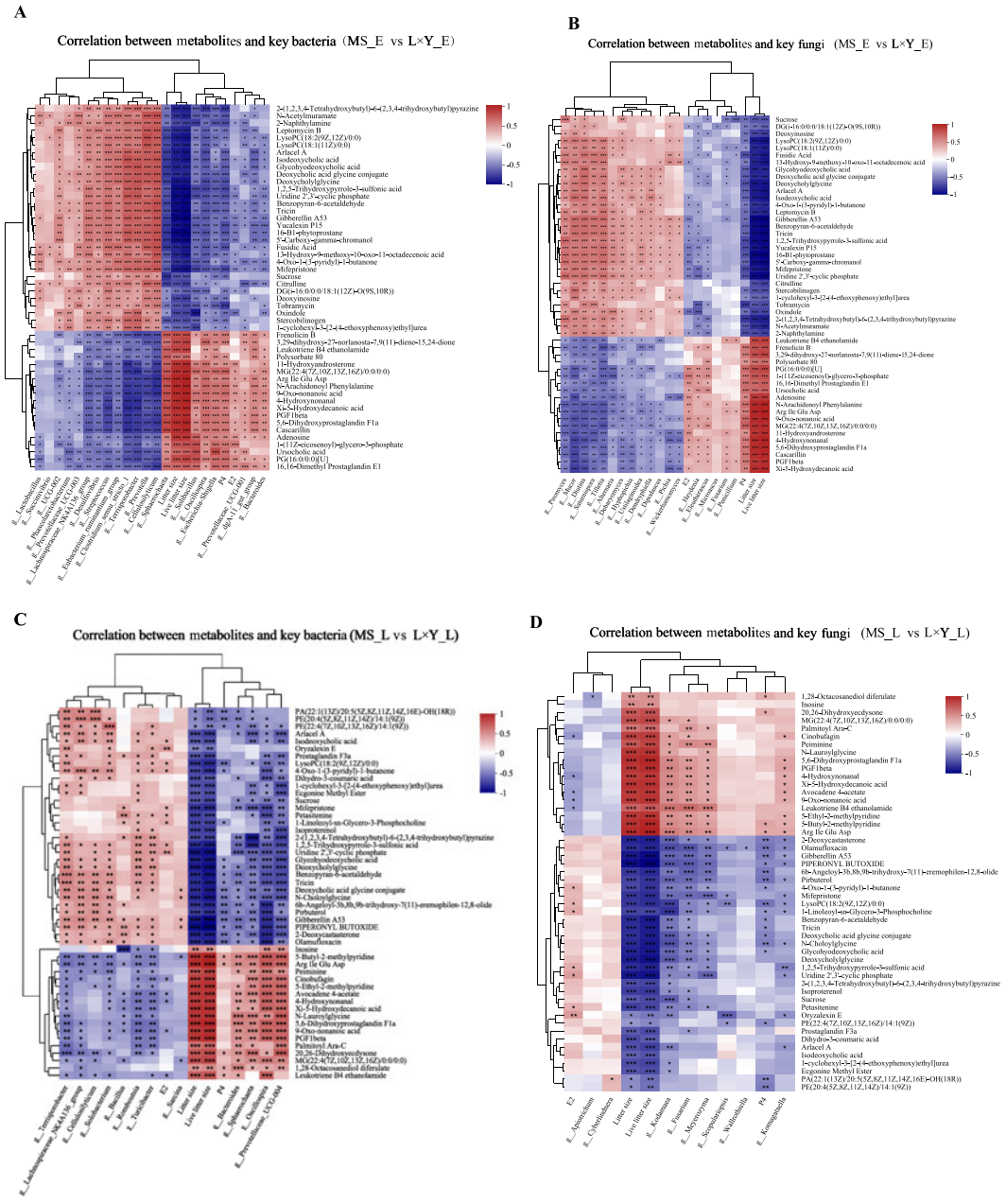

Figure 7. The correlation analysis between top 50 of different metabolites and key microbiota and reproductive performance during pregnancy. (A–B) The correlation analysis between different metabolites and key genera of bacteria (A) and fungi (B) at the early pregnancy. (C–D) The correlation analysis between different metabolites and key genera of bacteria (C) and fungi (D) at the late pregnancy.

(2017) pointed out that compared with the first and second trimester of pregnancy, the perinatal fecal flora α-diversity (Chao index) and relative abundance of *Bacteroidetes* were significantly increased, while the relative abundance of *Firmicutes* was significantly decreased (Zhou et al. 2017). In our results, the bacterial α-diversity (including Sobs, ACE, and Chaos indices) of L × Y sows at the late pregnancy was significantly increased compared with the early pregnancy, with no significant changes of MS sows between the early and late pregnancy. In addition, the fungal α-diversity of MS sows and L × Y sows was not significantly changed from the early to late pregnancy. The reasons for the different changes in microbial diversity and composition may be related to the diet composition, genetic background, parity of sows, and the location and time of sample collection (Kong et al. 2016; Liu et al. 2019).

Previous studies have showed that the richness of intestinal microbiota during pregnancy is significantly positively correlated with dietary fiber intake, which is associated with the production of SCFAs (Gomez-Arango et al. 2018; Röytiö et al. 2017). In pigs, high-nutrient diets (high-energy and high-protein but low-fiber diets) during pregnancy could increase the relative abundance of *E. coli* in the proximal colon (Kong et al. 2016), while maternal diets supplemented with 1.5% inulin could increase the relative abundance of SCFA production-related bacteria, such as *Oscillospira* and *Eubacterium hallii* in feces (Zhou et al. 2017). In rats, maternal

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high-fat diets could lead to an increase in the ratio of *Firmicutes* to *Bacteroides* (Mann et al. 2018). Moreover, the relative abundance of *Proteobacteria* was significantly positively correlated with cholesterol and monounsaturated fatty acid intake (Mandal et al. 2016). In our results, the levels of SCFAs in feces of MS sows were lower, even though the litter sizes and steroid hormones were higher than that in L × Y sows. These results were discrepancy with the previous studies which declared a beneficial effect of dietary fiber on the litter size at farrowing, with increased levels of fecal steroid hormones and SCFAs during late gestation (Jiang et al. 2019; Zhuo et al. 2020). The different changes of SCFA level could attribute to the difference of diets and its fiber composition (Han et al. 2023). Importantly, our results suggested that there were other potential key metabolites that play a key role in fetal survival and steroid hormone synthesis and ultimately affect the litter size of sows. In the metabolome results, the enrichment pathways of bile acid metabolism, arginine and proline metabolism, glycerophospholipid and sphingolipid metabolism, and endogenous cannabinoid metabolism further deepened our hypothesis, considering their important roles proved in regulating oocytes and embryo development in mammals (Wu et al. 2013; Ye et al. 2021).

In summary, our findings demonstrated that MS sows had larger litter sizes and higher level of steroid hormones but lower level of SCFAs in feces than L × Y sows during gestation. The metabolic status and the microbiota (including bacteria and fungi) community structure differed significantly between MS sows and L × Y sows both at the early and late pregnancy. Our results first integrated the intestinal bacterial and fungal and metabolites of these two species of sows to provide microbial and metabolic perspective to improve the reproductive performance in pig production.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/anr.2024.7.

Acknowledgements. Qianhong Ye and Xianghua Yan contributed to the experimental design and project management; Qianhong Ye, Yifan Hu, Yuwen Chen, Haoyi Jiang, and Xiaojian Xu contributed to sample collection; Qianhong Ye, Tingting Luo, and Longshan Han analyzed data; Qianhong Ye wrote the paper; Qianhong Ye and Xianghua Yan revised the paper. All authors have read and approved the final manuscript. We thank Yan lab's members for helpful discussions and critical reading of the manuscript. This study was funded by the National Natural Science Foundation of China (31925037 and 32202700), the National Key R&D Program of China (2022YFD1300405), the National Key Laboratory of Agricultural Microbiology (AML2023B06), and the Project 2662023DKPY002 supported by the Fundamental Research Funds for the Central University. None of the authors have any potential conflicts of interest related to this article to declare.

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