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Succession of rumen microbiota and metabolites across different reproductive periods in different sheep breeds and their impact on the growth and development of offspring lambs

Yuzhu Sha[1], Xiu Liu[1], Xiongxiong Li[1], Zhengwen Wang[2], Pengyang Shao[1], Ting Jiao[2], Yanyu He[3] and Shengguo Zhao[1*]

Abstract

Background The microbiota and metabolites in the gastrointestinal tracts of female animals at different reproductive periods are very important to the growth, development, and health of themselves and their offspring. However, the changes in the gastrointestinal microbiota and metabolites throughout reproductive period of different sheep breeds and their effects on the growth and development of offspring lambs are still unclear. Hence, this study presents an assessment of the reproductive hormone levels, immune levels, rumen microbiota, and metabolites in Hu sheep and Suffolk ewes at different reproductive periods and their effects on the growth and development of offspring lambs.

Results Hu sheep and Suffolk during non-pregnancy, pregnancy, and lactation were used as the research objects to determine reproductive and immune indexes of ewes at different periods, analyze rumen microbiome and metabolome, and track the growth performance and development of offspring lambs. The results showed that the reproductive hormone and immune levels of Hu sheep and Suffolk underwent adaptive changes across different reproductive periods. Compared with non-pregnancy, the microbial energy metabolism and lipid metabolism function decreased during Hu sheep pregnancy, and energy metabolism function decreased during lactation. In Suffolk, energy metabolism, glycan biosynthesis, and metabolism function were enhanced during pregnancy, and the metabolism of cofactors and vitamins was enhanced during lactation. *Prevotella* increased in Suffolk during pregnancy and lactation ($P < 0.05$) and was positively correlated with the birth weight and body size of the lambs ($P < 0.05$). Moreover, the abundances of *Butyrivibrio* and *Rikenellaceae_RC9_gut_group* during pregnancy were positively correlated with the intestinal immunity of the offspring lambs ($P < 0.05$), thereby regulating the intestinal immunity level of the lambs. Metabolomic analysis revealed that the protein digestion, absorption, and amino acid metabolism of Hu sheep were enhanced during pregnancy, which provided amino acids for the growth and development of pregnant ewes and fetuses and was significantly correlated with the birth weight, body size, and intestinal immunity of lambs ($P < 0.05$). Simultaneously, there was an increase in acetate and propionate during the pregnancy and lactation period

*Correspondence:
Shengguo Zhao
zhaosg@gsau.edu.cn
Full list of author information is available at the end of the article

of both Hu sheep and Suffolk, providing energy for ewes during reproductive period. Moreover, the microbiota during the lactation period was significantly correlated with the milk quality and lambs daily gain ($P < 0.05$).

Conclusions This study revealed the characteristic succession changes in the rumen microbiota and its metabolites at different reproductive periods in sheep breeds and their regulation of reproductive hormone and immune levels and identified their potential effects on the growth and development of offspring lambs. The findings provide valuable insights into the health and feeding management of different sheep breeds during the reproductive stage.

Keywords Reproductive period, Rumen microbiota, Metabolites, Lambs, Immune function

Introduction

The reproductive performance of sheep is a key indicator for assessing their productivity, with the health and management of ewes during the reproductive stage being crucial factors influencing their production capabilities. During different reproduction period, ewes undergo adaptive changes in their physiological metabolism. These changes significantly impact various aspects, such as energy metabolism requirements, hormone levels, health, and fetal growth and development [1–4]. In recent years, with the in-depth study of microbiomes and metabolomics, the involvement of the gut microbiota and its metabolites in the reproductive period regulation in ewes has been increasingly revealed [3, 5]. They are considered fully functional endocrine organs [5] that influence distant organs and metabolic pathways. Studies have shown that the microbiota plays an important role in the reproductive endocrine system by interacting with estrogen, androgen, insulin, and other hormones [5]. Studies on the gut microbiota and metabolites of goats have shown that there is a significant correlation between the levels of reproductive hormones and the microbiota, and that their metabolites, such as *Family_XIII_AD3011_group* and other microbiota, influence the levels of progesterone (PROG) and estradiol (E2) [6]. In addition, the composition of the gut microbiota varies at different periods of reproduction, especially in the third trimester, when microbial changes lead to changes in host metabolism, immunity, and hormone levels necessary to support healthy pregnancy and fetal development [7]. The study revealed a specific ecological succession of the gut microbiota in goats during non-pregnancy, pregnancy, and lactation, resulting in a reduced abundance of gut bacteria in the *Family_XIII_AD3011_group* during pregnancy [6]. In addition, changes in rumen microbiota and its metabolites in lactating dairy cows also affect milk production and quality. In the rumen of lactating dairy cows with high milk protein production (MPY), the number of several *Prevotella* increases, which helped to improve the biosynthesis of branchable chain amino acids [8]. Our previous studies revealed that the abundance of the rumen microbiota and its metabolites during different reproductive periods differed among small-tailed Han sheep, and there was a significant correlation between the abundance of these microbes and the body's immune system. Additionally, the abundance of *Prevotella* during pregnancy significantly increased, the abundance of *Fibrobacter* during lactation significantly increased, and rumen microbial carbohydrate metabolism, glucose biosynthesis, and metabolic functions significantly increased during pregnancy, indicating that different microbiota and its metabolites are involved mainly in regulating maternal energy metabolism and immune levels during pregnancy and lactation [9].

During pregnancy, metabolic changes associated with energy homeostasis protection are critical for fetal development, future metabolic fate, and maternal health [3]. In the third trimester, microbial changes lead to differences in metabolic, immune, and hormonal levels, thus guaranteeing healthy maternal pregnancy and good fetal development [7]. For example, the metabolite short-chain fatty acids (SCFAs) can penetrate the placental barrier and play a crucial role in the development, growth, and immunity of fetal organs [3, 10–12]. Studies have shown that pregnancy increases the concentration of acetate and propionate, which are involved in metabolic processes in the body [3]. Acetate is a major SCFA in pregnant women and their infants [12], and a decrease in the maternal pro-inflammatory factors TNF-α and IL-1β during pregnancy is associated with butyrate [13]. In addition, studies have shown that maternal gut microbes and its metabolites in the reproductive period play a role in the development of an infant's innate immune system and postnatal adaptability in addition to regulating maternal metabolism [14, 15]. These studies have shown that maternal microbes at different reproductive periods play an important role in the growth and development of offspring and the immune system. However, the current research on gut microbes and its metabolites in sheep at different reproductive periods is limited to a single breed, and it is still unclear whether there are similarities and differences between different breeds of sheep. The reproductive performance of sheep is determined by the host genome at the time of birth, and reproductive traits are regulated at the gene level. The microbiota, the second genome of the host, also participates in regulating host traits [8,

16]. Therefore, this study selected high-reproduction Hu sheep (with a reproductive rate of 220%) and meat-type Suffolk (with a reproductive rate of 150%) as the research objects to further analyze the changes in the succession of the rumen microbiota and its metabolites, as well as changes in the reproductive hormones and immune levels of sheep breeds with different production directions at different reproductive periods, and to explore the effects of these changes on the growth and development of offspring lambs. The aims of this study were as follows: (a) To reveal the succession of rumen microbiota and its metabolites in different reproductive periods of different breeds of sheep, (b) to explore the effects of rumen microbiota and its metabolites on reproductive hormone levels and immune levels at different reproductive periods, and (c) to analyze the effects of microbiota and its metabolites on the growth and development of offspring lambs during pregnancy and lactation. The above results will provide a reference for the healthy feeding and management of different breeds of sheep during non-pregnancy, pregnancy, and lactation.

Results

Dynamic changes in blood reproductive hormones and immune levels in different reproductive periods

In this study, the reproductive hormone and immune levels of the two breeds of sheep at different reproductive periods were detected (Fig. 1), and it was found that progesterone (PROG), estradiol (E2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were significantly increased during pregnancy and lactation ($P < 0.05$; Fig. 1a). Among them, progesterone (PROG) and estradiol (E2) increased first and then decreased with the change of reproductive period, and the hormone level of Hu sheep during the whole reproductive period was higher than that of Suffolk. In addition, the immunoglobulins (IgA, IgG, and IgM) in different reproductive periods of Hu sheep showed a pattern of initially decreasing and then increasing, with levels during the lactation period significantly higher than those in other period ($P < 0.05$; Fig. 1b). Among the Suffolk, the rangeability in immunoglobulins at different reproductive periods was small, and the IgA and IgG levels were significantly lower

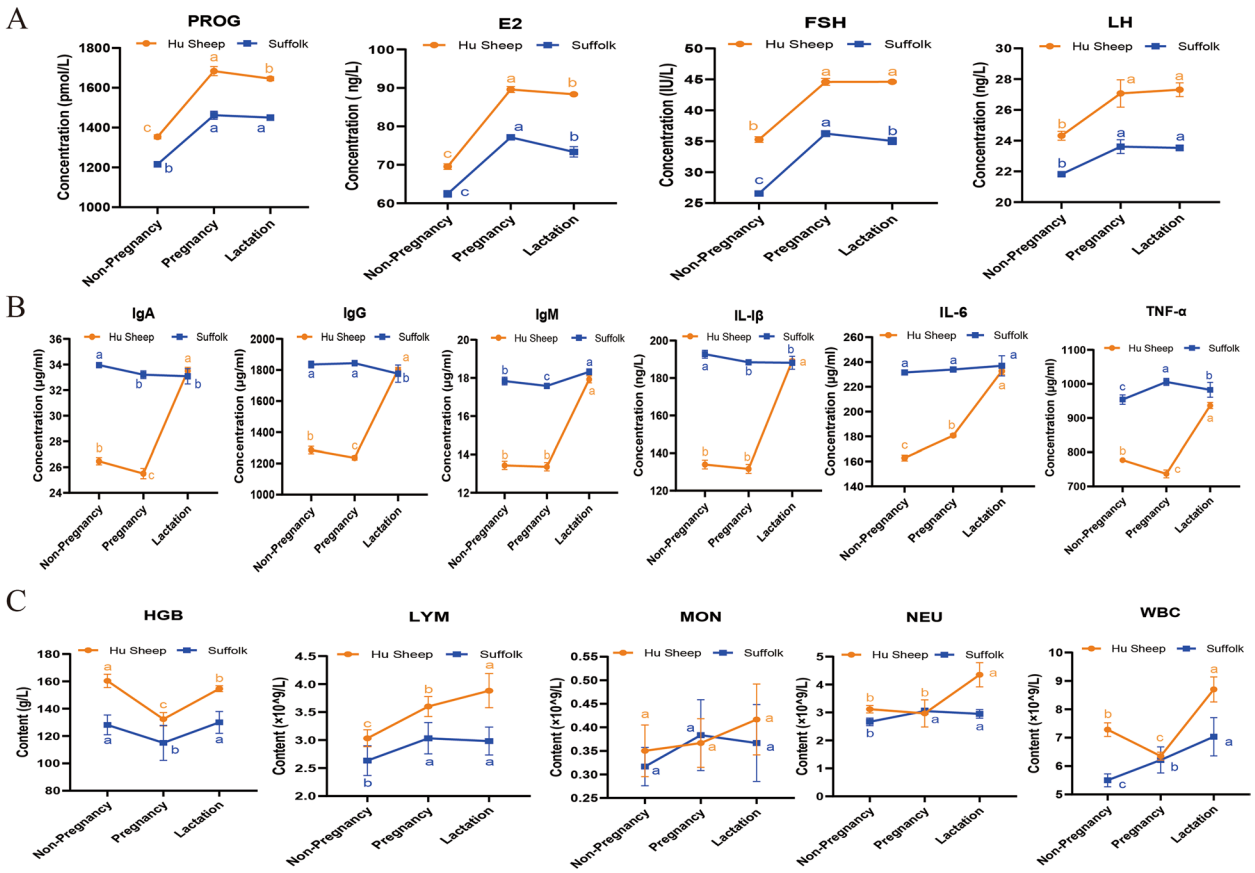

Fig. 1 Analysis of blood reproductive hormones and immune indexes in different reproductive periods of sheep breeds. **A** Reproductive hormone indicators. **B** Immunoglobulins and immune factors. **C** Blood physiological indicators. Different lowercase letters on the line chart represent significant differences ($P < 0.05$)

in the lactation (33.08 μg/ml, 1776.60 μg/ml) than in the non-pregnancy (33.96 μg/ml, 1835.41 μg/ml; $P < 0.05$), which is just the opposite of the Hu sheep. While the IgM level was significantly increased in the Suffolk lactation (18.32 μg/ml; $P < 0.05$). Analysis of immune factors at different reproductive periods showed that IL-1β and IL-6 significantly increased during the lactation period in Hu sheep (189.07 ng/L, 232.63 μg/ml; $P < 0.05$), with relatively small changes in different reproductive periods of Suffolk. TNF-α decreased first and then increased in different breeding period of Hu sheep, reaching the highest level in lactation. In Suffolk, the opposite was true, showing first an increase and then a decrease. Furthermore, the hemoglobin (HGB) content of the two breeds decreased first and then increased, with the lowest content during pregnancy, which was 132.33 g/L (Hu sheep) and 115.00 g/L (Suffolk), respectively (Fig. 1c). The total lymphocyte count (LYM) significantly increased during the pregnancy and lactation period, and the total white blood cell (WBC) decreased first and then increased in different reproductive periods of Hu sheep and increased significantly in Suffolk with the change of reproductive period, and the highest value was reached in lactation period ($P < 0.05$; Fig. 1c). There was no significant difference in monocyte count (MON) at different reproductive periods between the two sheep breeds ($P > 0.05$). The number of neutrophils (NEU) in the lactation period of Hu sheep was significantly increased ($4.35 \times 10^{\wedge 9}$/L; $P < 0.05$), while the number of NEUs in the pregnancy ($3.05 \times 10^{\wedge 9}$/L) and lactation period ($2.95 \times 10^{\wedge 9}$/L) of Suffolk was significantly decreased ($P < 0.05$; Fig. 1c).

Changes in rumen microbiota succession at different reproductive periods

PCoA showed differences in rumen microbes between Hu sheep and Suffolk at different reproductive periods (Fig. S1). A total of 9559 ASVs were detected in Hu sheep, and 10,558 ASVs were detected in Suffolk (Fig. S2, Table S1). Alpha-diversity analysis revealed that the Shannon index of Hu sheep decreased with the change of pregnancy (4.78) and lactation (4.60) (Fig. 2a), with a significant decreased during the lactation period ($P < 0.05$). However, there was no significant difference in Shannon index of Suffolk sheep ($P > 0.05$; Fig. 2d). The Simpson index in Hu sheep was significantly lower during the lactation period (0.92) than during the other period ($P < 0.05$; Fig. 2b). In Suffolk, the Simpson index was significantly lower during the pregnancy (0.93) and lactation (0.93) period than during the non-pregnancy (0.95) ($P < 0.05$; Fig. 2c). Microbial species composition analysis revealed that the abundance of the dominant phylum Firmicutes was greatest during the non-pregnancy among the Suffolk (55.85%) and decreased during the pregnancy (42.91%) and lactation (45.38%) (Fig. 2e). In contrast, in Hu sheep, the abundance Firmicutes during the pregnancy (55.62%) and lactation (54.37%) was significantly greater than that during the non-pregnancy (43.97%) ($P < 0.05$). Compared with non-pregnancy, the Bacteroidetes in Hu sheep were significantly decreased in pregnancy (37.46%) and lactation (38.53%) ($P < 0.05$), while the Bacteroidetes in Suffolk were significantly increased in pregnancy (46.09%) and lactation (45.51%) ($P < 0.05$). Genus-level analysis revealed that the dominant genus *Prevotella* showed relatively small differences across the three reproductive period in Hu sheep (Fig. 2f), but in Suffolk, it increased during the pregnancy (16.38%) and lactation (15.76%). Furthermore, *Succiniclasticum* was more abundant in Hu sheep than in Suffolk across all reproductive period, and the abundance of *Succiniclasticum* increased during the pregnancy and lactation. In contrast, *Rikenellaceae_RC9_gut_group* abundance decreased during gestation (8.11%) and lactation (6.35%) with the change of reproductive cycle, while in Suffolk, it significantly increased during the pregnancy (9.75%; $P < 0.05$). LEfSe analysis revealed biomarkers for different reproductive periods (Fig. 2g). Further, differential microbial KEGG functional analysis revealed that energy metabolism and lipid metabolism decreased and cell motility increased during pregnancy, and energy metabolism decreased during the lactation period in Hu sheep (Fig. 3a, b). In Suffolk during the pregnancy, there was an increased in energy metabolism, glycan biosynthesis and metabolism, nucleotide metabolism, replication and repair, and metabolism of cofactors and vitamins, and membrane transport and signal transduction decreased during Suffolk pregnancy. During the lactation period, there is an increased in metabolism of cofactors and vitamins, nucleotide metabolism, and drug resistance: antimicrobial (Fig. 3c, d).

Changes in the rumen microbial metabolic profile at different reproductive periods

The rumen microbial metabolic profiles of different breeds of sheep at the reproductive stage differed (Fig. 4a, Table S2). The top 10 differentially abundant metabolites with the largest absolute values of log2FC were selected for radar analysis, and the differential metabolites at different breeding stages of each breed of sheep were found (Fig. S3). Further, differentially abundant metabolite KEGG functional enrichment analysis revealed that during the pregnancy in Hu sheep (Fig. 4e1, e2, e3), functions such as protein digestion and absorption; biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid; mineral absorption, glycine, serine, and threonine metabolism; and alanine, aspartate, and glutamate metabolism were upregulated. In the pregnant Suffolk (Fig. 4f1, f2, f3), there

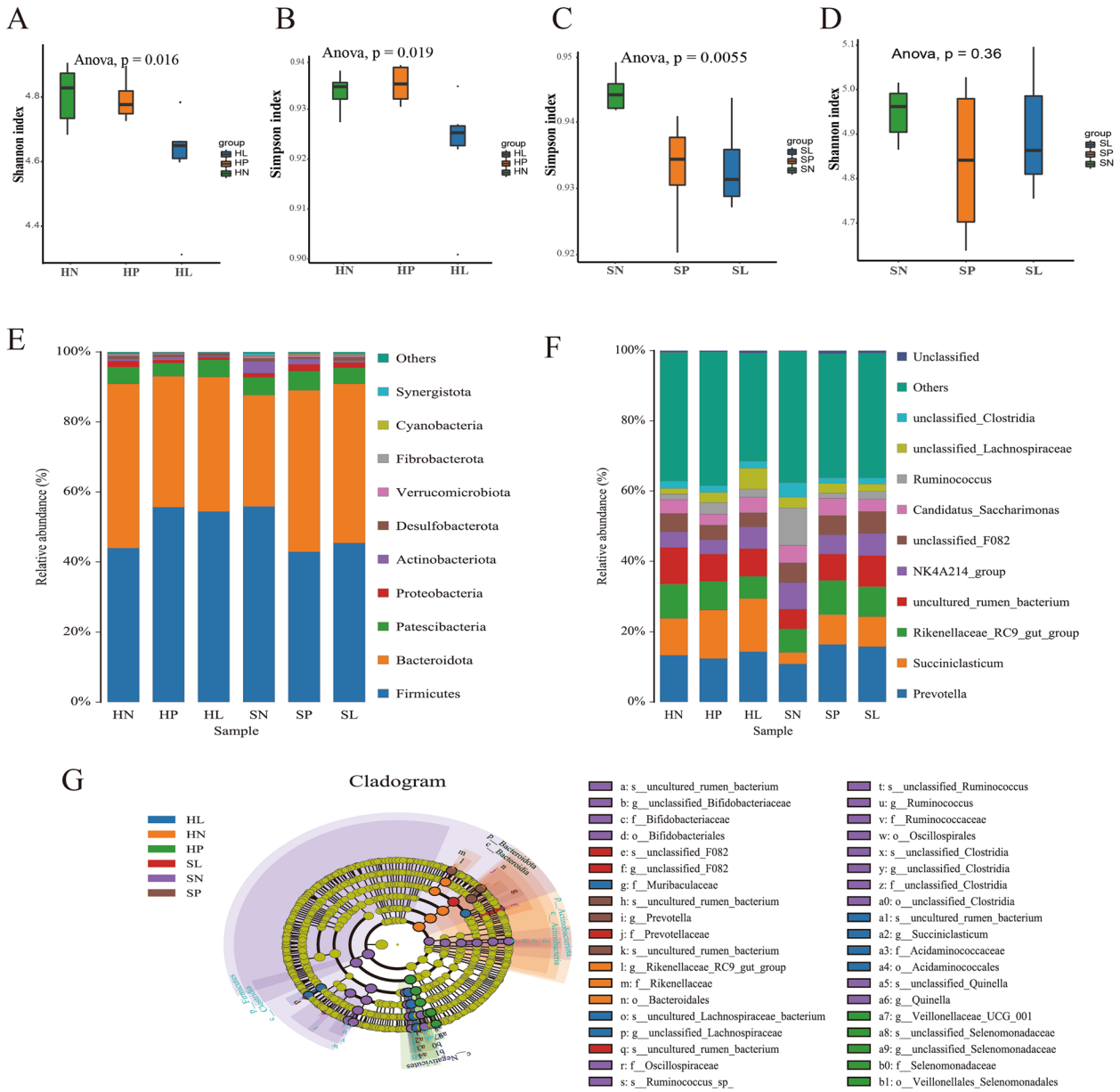

Fig. 2 Rumen microbial diversity and composition analysis of different breeds of sheep at reproductive stage. **A**, **B**, **C**, **D** Microbial diversity indicators, Shannon, Simpson indices of Hu sheep, and Suffolk. **E**, **F** Microbial composition at the level of phylum and genus. **G** LEfSe analysis. Note: HN, HP, and HL represent the non-pregnancy, pregnancy, and lactation period of Hu sheep respectively. SN, SP, and SL respectively represent the non-pregnancy, pregnancy, and lactation period in Suffolk

is a downregulation of functions including valine, leucine, and isoleucine biosynthesis and valine, leucine, and isoleucine degradation, and propanoate metabolism decreased. Additionally, ABC transporters were enriched in Hu sheep and Suffolk during pregnancy period. Compared to the nonpregnant period, during the pregnancy and lactation period in Hu sheep, there was an enrichment of functions such as the biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid, citrate cycle (TCA cycle), and pyruvate metabolism, with the enrichment being particularly pronounced during the pregnancy period compared to the lactation period. ABC transporters and nicotinate and nicotinamide metabolism were enriched during lactation. Compared to those in the nonpregnant period, during the lactation period in the Suffolk, functions such as pyruvate metabolism, valine, leucine, and isoleucine degradation, carbohydrate digestion and absorption, starch and sucrose metabolism, ABC transporters, and galactose

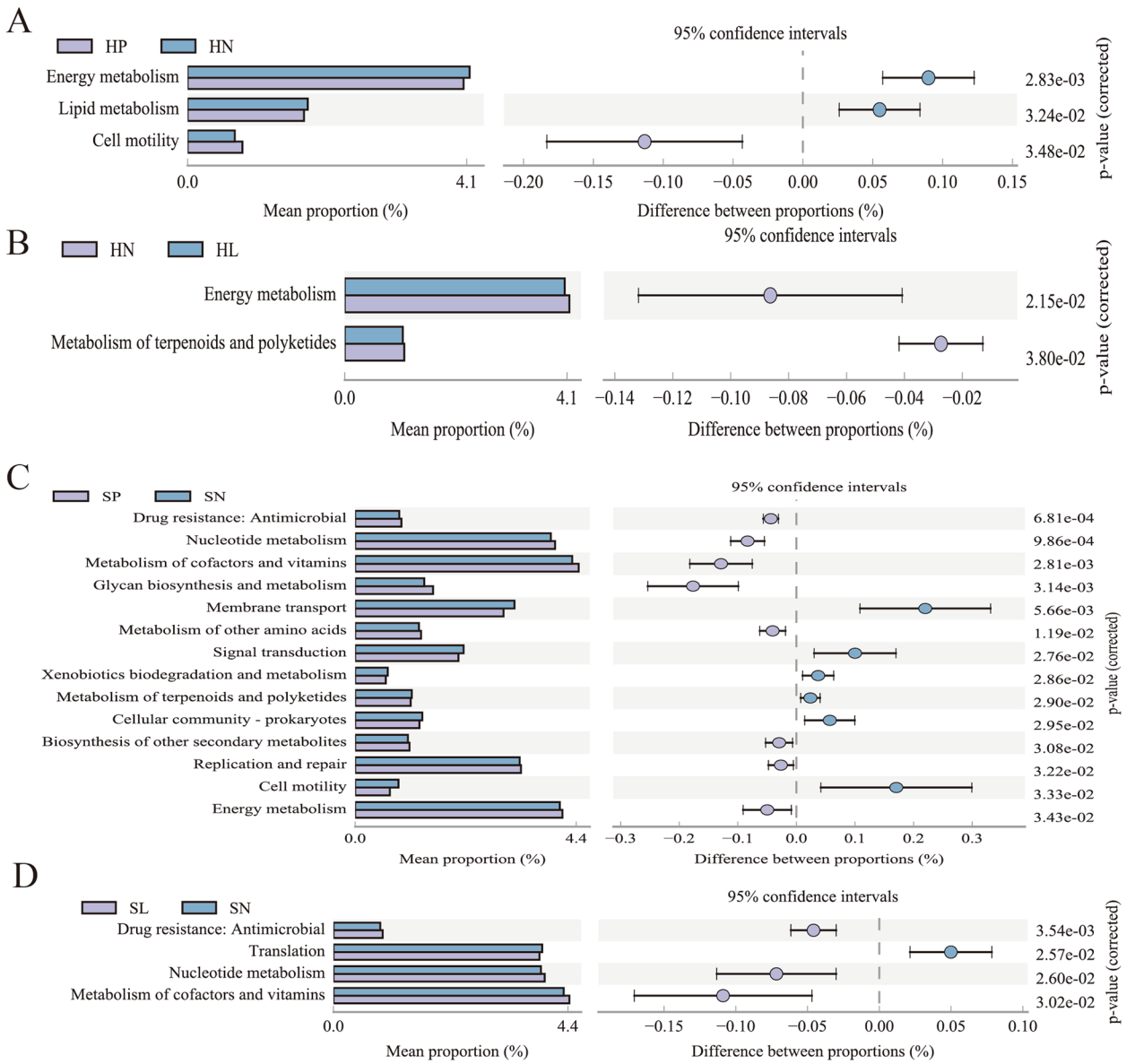

Fig. 3 Rumen microbial function through PICRUSt2 analysis of different breeds of sheep at different reproductive periods. Note: HN, HP, and HL represent the non-pregnancy, pregnancy, and lactation period of Hu sheep respectively. SN, SP, and SL respectively represent the non-pregnancy, pregnancy, and lactation period in Suffolk

metabolism were downregulated. In addition, with the physiological changes during pregnancy and lactation, the contents of acetate and propionate increased in lake sheep and Suffolk sheep ($P < 0.05$; Fig. 4b, c), and the contents were highest in lactation. The A/P ratio (acetate/propionate) was significantly greater during the lactation period than the during the nonpregnant period in both Hu sheep and Suffolk ($P < 0.05$). When comparing Hu sheep and Suffolk, it was found that acetate was greater in Suffolk, while during pregnancy period, the levels of propionate and butyrate were greater in Hu sheep (12.43 mM, 7.39 mM) than in Suffolk (11.14 mM, 6.52 mM). The NH_3-N content in both breeds of sheep was significantly greater during the pregnancy period than during the other period ($P < 0.05$, Fig. 4d), and the NH_3-N content in Hu sheep was significantly greater than that in Suffolk ($P < 0.05$).

Microbe-metabolite interactions at different reproductive periods are related to reproductive hormones and immune levels

There was a strong correlation between different microbiota (genus level) and different metabolites in the reproductive

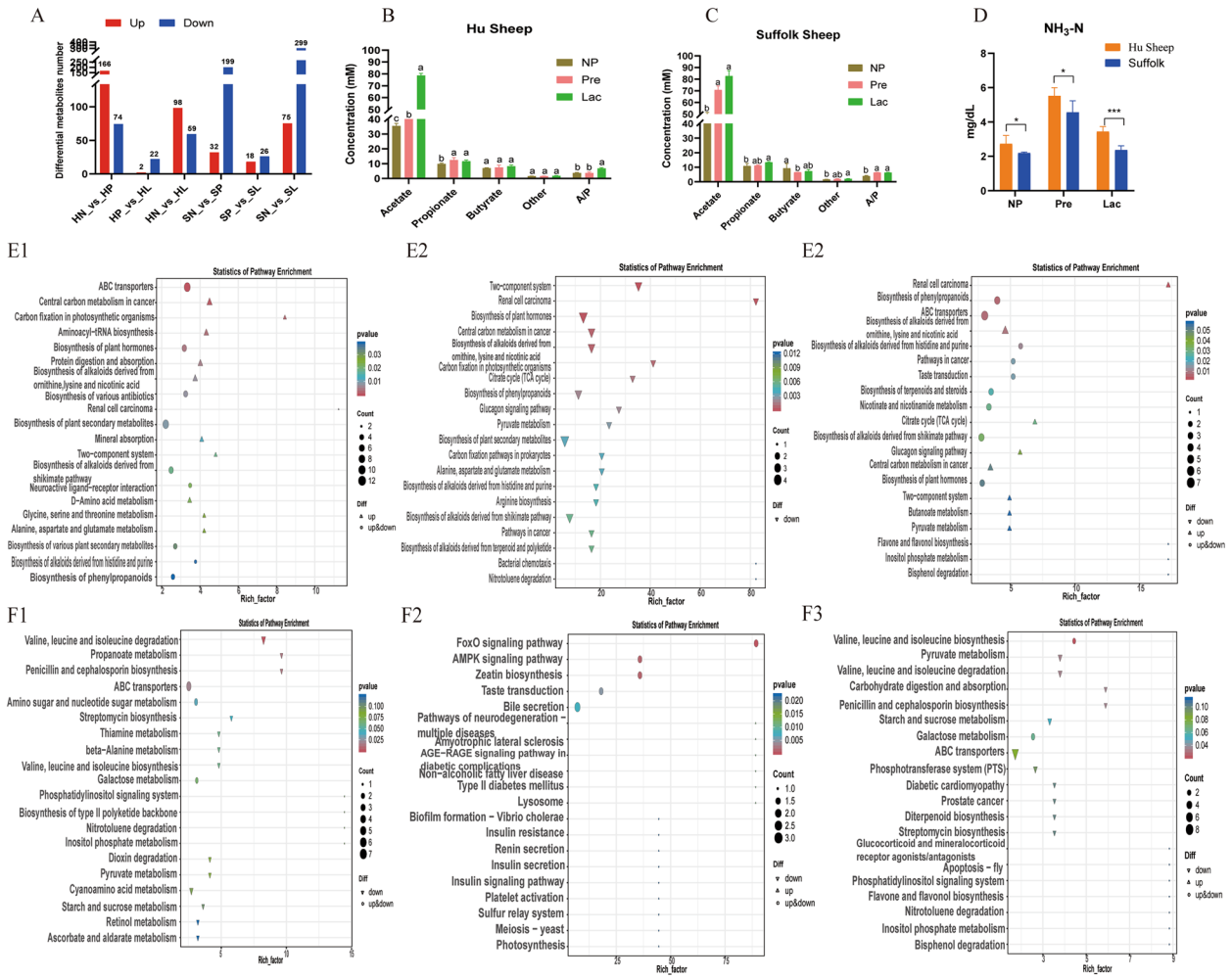

Fig. 4 Analysis of rumen microbial metabolic profile at reproductive period of different breeds of sheep. **A** Differential metabolite quantity statistic. **B** Ruminal SCFA concentration of Hu sheep. **C** Ruminal SCFA concentration of Suffolk. **D** Concentration of ruminal ammonia nitrogen content. **E1**, **E2**, **E3** KEGG functional enrichment map of differential metabolites of HN_vs_HP, HP_vs_HL, and HN_vs_HL, respectively. **F1**, **F2**, **F3** KEGG functional enrichment map of differential metabolites of SN_vs_SP, SP_vs_SL, and SN_vs_SL, respectively

period of Hu sheep and Suffolk (Table S3), such as *Prevotellaceae*, *Alloprevotella*, *Prevotellaceae_NK3B31_group*, *Prevotellaceae_UCG_004*, *Christensenellaceae_R_7_group*, which is strongly associated with metabolites (D-glucosaminate-6-phosphate, CDP-DG (a-17:0/5-iso PGF2VI), calcidiol, alpha-isopropylmalate, Arg-Arg-Leu-Pro, etc. al) during pregnancy and lactation (Fig. S4). *Prevotella* showed a significant positive correlation with acetate ($r = 0.53$, $P < 0.001$), isovalerate ($r = 0.46$, $P < 0.01$), and isobutyrate ($r = 0.55$, $P < 0.001$), while NH_3-N showed a positive correlation with *Succiniclasticum* ($r = 0.50$, $P < 0.01$). And butyrate was significantly positively correlated with *Ruminococcus* ($r = 0.45$, $P < 0.05$) (Fig. S4g, h). In addition, changes in the rumen microbiota and its metabolites at different reproductive periods are correlated with reproductive hormone and immune levels. The levels of LH, FSH, PROG, and E2 were significantly positively correlated with those of *Succiniclasticum* and *unclassified_Prevotellaceae* ($P < 0.05$, Fig. 5a, b) and significantly negatively correlated with those of *Ruminococcus* and *Candidatus_Saccharimonas* ($P < 0.05$). Moreover, *Butyrivibrio* was significantly negatively correlated with the immune indicators IgG ($r = -0.56$, $P < 0.001$), IgA ($r = -0.39$, $P < 0.05$), TNF-α ($r = -0.54$, $P < 0.001$), IL-1β ($r = -0.39$, $P < 0.05$), and IL-6 ($r = -0.37$, $P < 0.05$). Microbial metabolites also showed correlations with serum indicators (Fig. 5c). Notably, the reproductive hormone indicators LH, FSH, PROG, and E2 were significantly positively correlated with L-allo-threonine, L-cis-3-amino-2-pyrrolidinecarboxylic acid, nicotyrine, and CDP-DG(PGF2alpha/i-22:0) ($P < 0.05$) and significantly negatively correlated with 4-(butylamino)

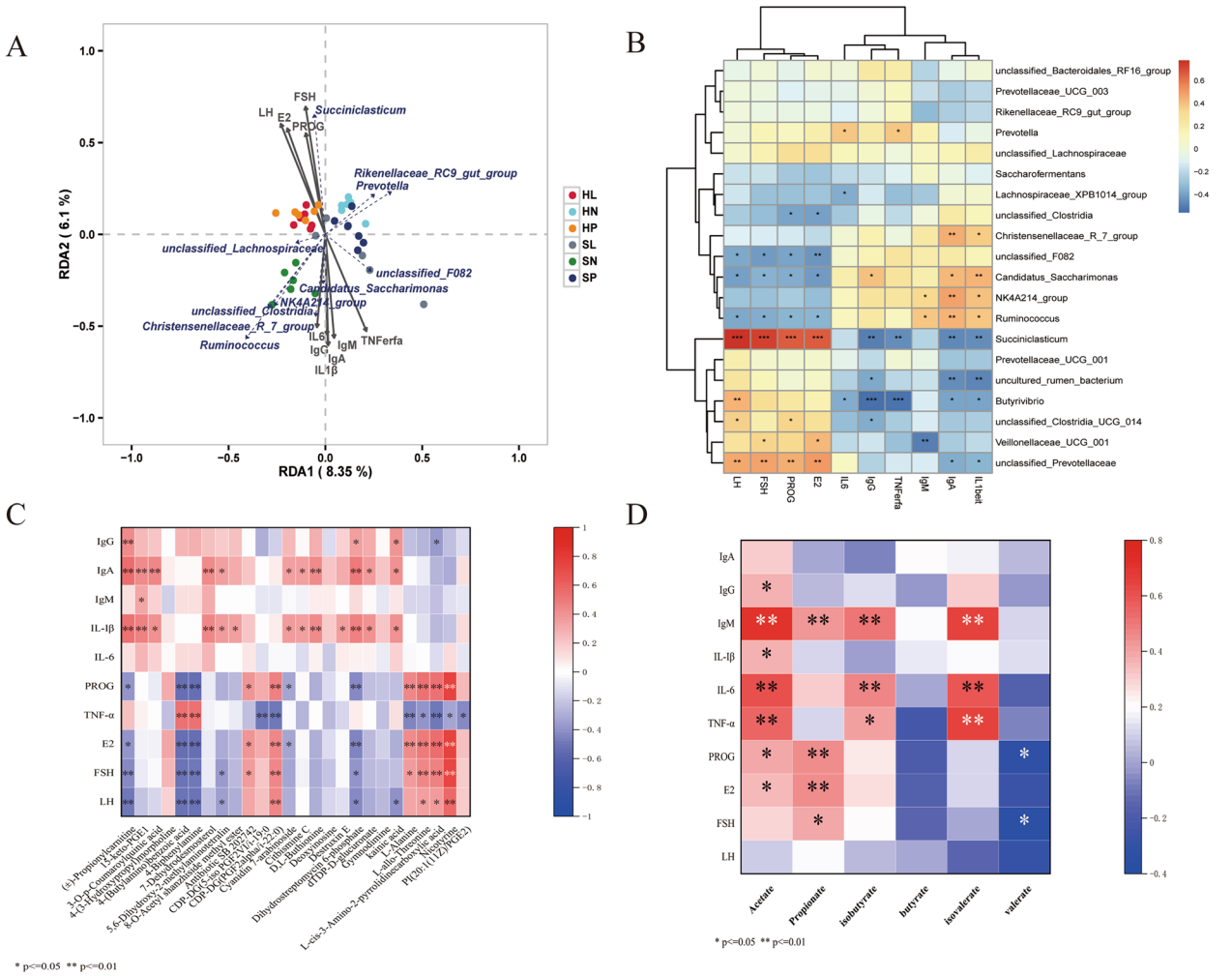

Fig. 5 Correlation analysis of rumen microbiota and metabolites with reproductive hormones in reproduction period. **A** RDA analysis of microbiota and reproductive hormones and immune indicators. **B** Heat map analysis of microbial correlations with reproductive hormones and immune indicators. **C** Heat maps of correlations between metabolites and reproductive hormones and immune indicators. **D** Heat maps of SCFA correlation with reproductive hormones and immune markers. Note: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

benzoic acid and 4-biphenylamine ($P < 0.05$). Additionally, (±)-propionylcarnitine, dihydrostreptomycin 6-phosphate, D,L-buthionine, 7-dehydrodesmosterol, and 15-keto-PGE1 were significantly positively correlated with the IgA and IL-1β ($P < 0.05$). 4-(Butylamino) benzoic acid ($r = 0.58$, $P < 0.05$) and 4-biphenylamine ($r = 0.52$, $P < 0.05$) were significantly positively correlated with TNF-α. SCFAs also exhibited correlations with reproductive hormones and immune indicators (Fig. 5d). Specifically, acetate and propionate were significantly positively correlated with PROG and E2 ($P < 0.05$), while propionate was significantly positively correlated with FSH ($P < 0.05$), and valerate was significantly negatively correlated with FSH and PROG ($r = 0.45$, $P < 0.05$). Acetate was significantly positively correlated with the TNF-α ($r = 0.52$, $P < 0.01$), IL-6 ($r = 0.61$, $P < 0.01$), IL-1β ($r = 0.37$, $P < 0.05$), IgM ($r = 0.71$, $P < 0.01$), and IgG ($r = 0.46$, $P < 0.05$); isovalerate was significantly positively correlated with TNF-α ($r = 0.67$, $P < 0.01$), IL-6 ($r = 0.51$, $P < 0.01$), and IgM ($r = 0.57$, $P < 0.05$).

The rumen microbiota and its metabolites during pregnancy are related to the development and immunity of newborn lambs

The birth weight (*BW* = 4.18 kg), body diagonal length (*Bdl* = 31.25 cm), and chest circumference (*CC* = 37.38 cm) of Suffolk lambs were significantly higher than those of Hu sheep lambs (*BW* = 2.90 kg, *Bdl* = 26.79 cm, *CC* = 34.25 cm; $P < 0.05$, Fig. 6a, b). And an analysis of immune factors in the feces of newborn lambs revealed that the intestinal IgA of Suffolk lambs (21.09 μg/ml) was significantly greater than that of Hu sheep lambs (20.02 μg/ml,

$P < 0.05$, Fig. 6c1), while the intestinal IgG of Hu sheep lambs (1035.58 μg/ml) was significantly greater than that of Suffolk lambs (964.51 μg/ml; $P < 0.05$, Fig. 6c2). IL-1β, IL-6, and TNF-α levels were significantly greater in the Suffolk lambs than in the Hu sheep lambs ($P < 0.05$ Fig. 6c4, c5, c6). Further analysis of the correlation revealed significant positive correlations between *Prevotella* abundance and birth weight ($r = 0.73$, $P < 0.01$), body diagonal length ($r = 0.63$, $P < 0.05$), TNF-α ($r = 0.76$, $P < 0.01$), IL-1β ($r = 0.65$, $P < 0.05$), and IgA ($r = 0.72$, $P < 0.01$; Fig. 6d, e). Chest circumference was significantly negatively correlated with *Veillonellaceae_UCG_001*

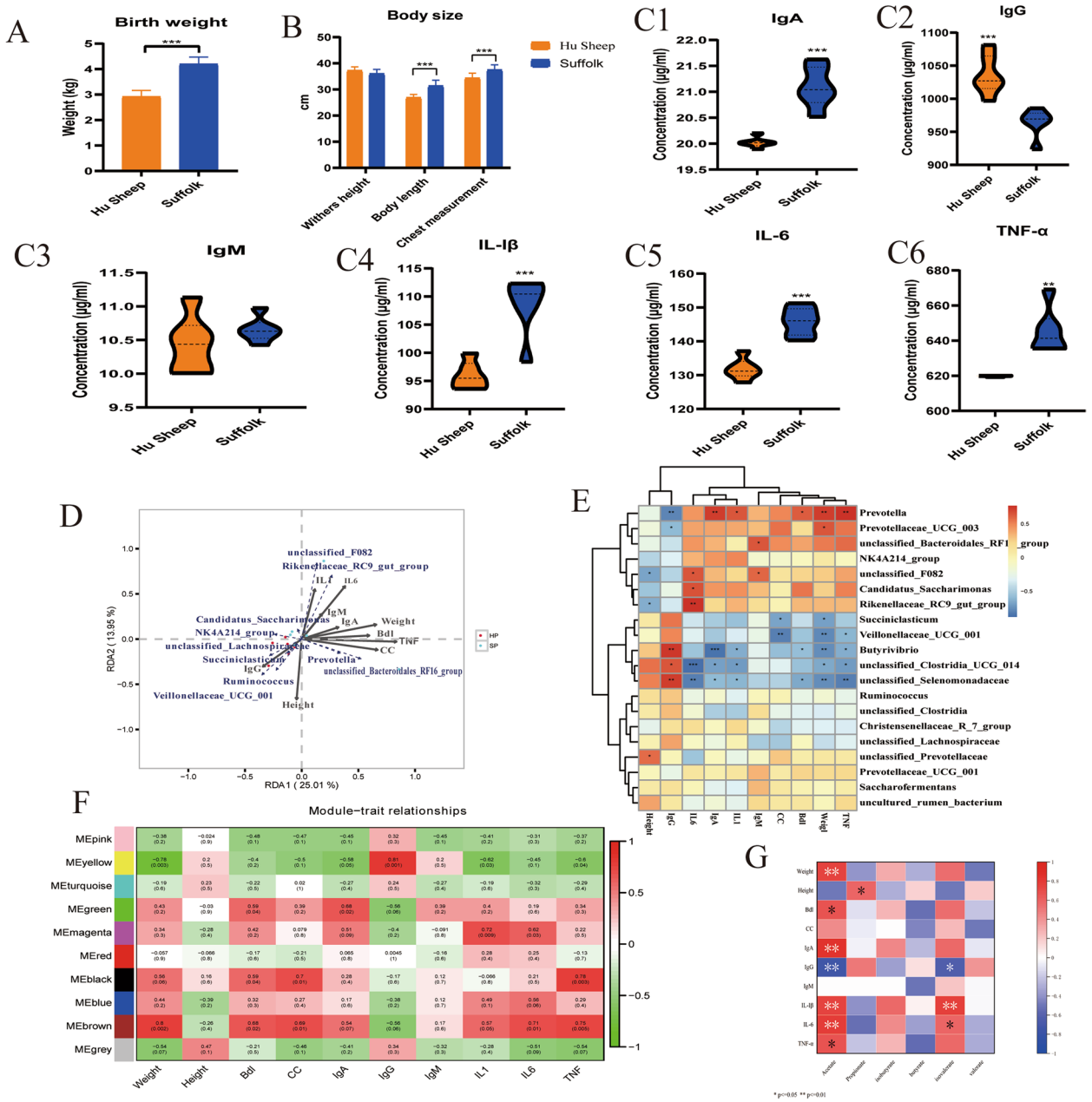

Fig. 6 Correlation analysis of ewe's ruminal microbiota and metabolites during pregnancy with developmental and immune indexes of newborn lambs. **A** Birth weight of newborn lambs. **B** Body size of newborn lambs. **C1**, **C2**, **C3**, **C4**, **C5**, **C6** Immunoglobulin and immune factor concentrations in lamb intestinal feces. **D** RDA analysis of ewe microbiome during pregnancy and body size and immune indices of newborn lambs. **E** Heat maps of the correlation between pregnant ewe microbiome and body size and immune indices of newborn lambs. **F** WGCNA analysis of metabolites in ewes during pregnancy and body size and immune indices of newborn lambs. **G** Heat map of correlation between SCFA in ewes during pregnancy and body size and immune indices in newborn lambs. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

($r = -0.81$, $P < 0.01$) and *Succiniclasticum* abundance ($r = -0.68$, $P < 0.05$). *Butyrivibrio* exhibited a significant positive correlation with IgG ($r = 0.74$, $P < 0.05$) and a significant negative correlation with IgA ($r = -0.85$, $P < 0.05$). IL-6 was significantly positively correlated with *Rikenellaceae_RC9_gut_group* ($r = 0.77$, $P < 0.05$). WGCNA reveals a significant positive correlation ($r > 0.5$, $P < 0.05$) between BW, Bdl, CC, TNF-α, IL-6, and the metabolite module MEbrown (Fig. 6f, Table S4). The identified metabolites included prenol lipids such as 3-(4-isopropylphenyl) propanal, pyrimidines and their derivatives such as 3-methylcytosine, linoleic acid and its derivatives such as (6Z,9Z,12Z)-octadecatrienoic acid, amino acids, peptides, and analogs such as o-(2-fluoroethyl)-l-tyrosine, benzoic acid and its derivatives like 4-(butylamino) benzoic acid, and biphenyls and their derivatives like 4-biphenylamine. The newborn lamb intestinal IgG showed a significant positive correlation with the MEyellow module ($P < 0.05$), such as hydroxy acids and their derivatives such as 3-dehydroquinate, benzene and its substituted derivatives such as 2-amino-4,6-dinitrotoluene, amino acids, peptides, and analogs like Z-Gly-Gly-Arg-AMC and hydroxyethyl glycine, as well as carbonyl compounds such as 3-acetyl-2,5-dimethylfuran. IgA showed a significant positive correlation with the MEgreen module, including metabolites such as benzoic acid and its derivative cannabigerolate, carbohydrates, and the carbohydrate conjugate beta-D-lactose. In addition, rumen SCFAs during pregnancy of ewe were correlated with lamb growth and immunity, and acetate was significantly positively correlated with BW ($r = 0.82$, $P < 0.01$) and Bdl ($R = 0.78$, $P < 0.05$) of newborn lambs ($P < 0.05$, Fig. 6g), as well as a significant positive correlation with IgA ($r = 0.80$, $P < 0.01$), IL-1β ($r = 0.65$, $P < 0.01$), IL-6 ($r = 0.76$, $P < 0.01$), and TNF-α ($r = 0.54$, $P < 0.05$). Additionally, propionate was significantly positively correlated with body height ($r = 0.52$, $P < 0.05$), while IgG was significantly negatively correlated with acetate ($r = -0.73$, $P < 0.01$) and isovalerate ($r = -0.47$, $P < 0.05$).

The rumen microbiota and its metabolites of ewe in lactation were correlated with milk quality and lambs daily gain

Milk quality analysis during the lactation revealed that the milk protein (5.33%) and casein (4.55%) levels were greater in Suffolk than in Hu sheep, while the milk fat content was significantly greater in Hu sheep (9.64%) than in Suffolk (8.99%) ($P < 0.05$, Fig. 7a). The average daily gain (ADG) of Suffolk lambs (199.54 g) was significantly higher than that of Hu sheep lambs (164.17 g) ($P < 0.05$, Fig. 7b). These lamb phenotypic differences were related to the changes of rumen microbiota and its metabolites in lactating ewes. *Prevotella*, Prevotellaceae, and Rikenellaceae were positively correlated with lactose, milk protein, and ADG (Fig. 7c). *Succiniclasticum* and *Christensenellaceae_R_7_group* were positively correlated with milk fat. Furthermore, WGCNA showed that the metabolite module MEred was significantly positively correlated with milk fat ($r > 0.8$, $P < 0.001$; Table S5, Fig. 7e), while it was significantly negatively correlated with lactose and ADG ($r > 0.6$, $P < 0.05$). Within the MEred module, metabolites such as ketones and their derivatives (4-(4-methylcyclohexyl)-4-oxobutanoic acid), fatty acids and conjugates (2-hydroxy-2-ethylsuccinic acid), carbohydrates and their conjugates (beta-D-lactose), fatty alcohol (methyl-12-gingerdiol), amino acids, peptides, and (N-(gamma-glutamyl) ethanolamine) were identified. Additionally, MEgreen showed a significant negative correlation with ADG ($r = -0.81$, $P < 0.001$), and within this module, metabolites such as carbonyl compounds (3-ketosphingosine) and triterpenoids (ganoderic acid eta) were identified. In addition, SCFAs were correlated with dairy quality and daily gain of lambs (Fig. 7d), and there were significant positive correlations among acetate, propionate, isovalerate, valerate, and casein ($r > 0.5$, $P < 0.05$). Isovalerate also showed a significant positive correlation with protein ($r > 0.5$, $P < 0.05$), and propionate and isovalerate were significantly negatively correlated with milk fat ($P < 0.05$).

Discussion

In this present study, by integrating the microbiome of the second genome and its metabolome, the succession changes of rumen microbiotas and their metabolites in different reproductive periods of sheep breeds with different production directions were investigated, and the effects of these changes on the growth and development of offspring lambs were also discussed. Ruminant animals exhibit dynamic changes in hormone levels, immune status, gut microbiota, and its metabolites across different reproductive periods. In this study, we observed increased reproductive hormone levels during pregnancy in both sheep breeds. Notably, the reproductive hormone levels in Hu sheep were greater than those in Suffolk. Hu sheep, which have a high reproductive rate, generally give birth to more than two lambs per pregnancy [17, 18]. In contrast, Suffolk, a meat-type breed, typically have a greater probability of giving birth to a single lamb, which may be related to genetic background and variations in reproductive hormone levels. However, changes in hormone levels can also lead to changes in the immune level of ewes, among which IgA related to intestinal epithelial immunity [19] decreases in the Hu sheep and Suffolk pregnancy period, with Hu sheep presenting lower IgA levels than Suffolk, indicating that Hu sheep may be more prone to intestinal diseases during pregnancy period. Therefore, more attention can be

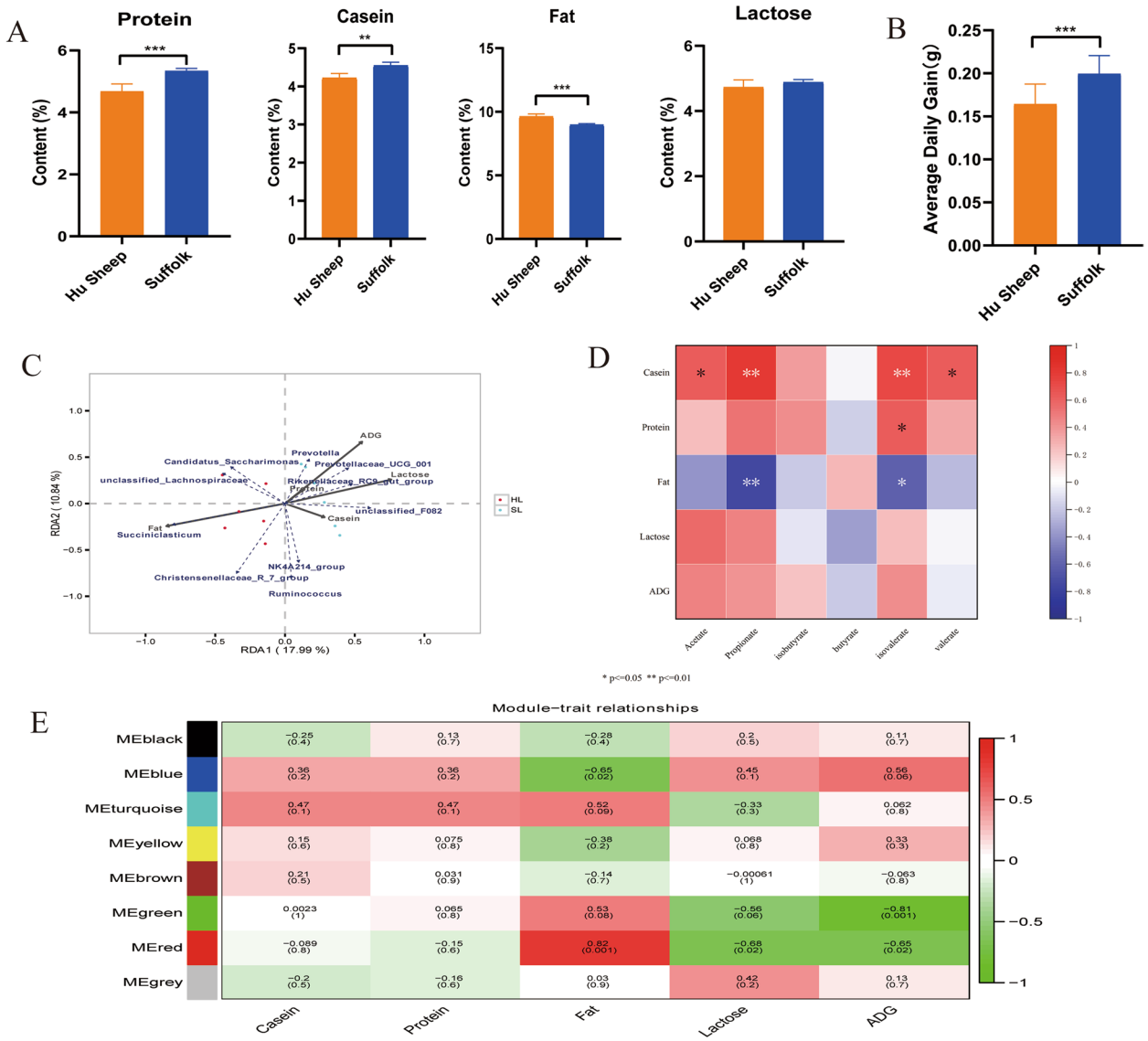

Fig. 7 Correlation analysis of ewe's ruminal microbiota and metabolites with milk quality and daily gain of lambs during lactation. **A** Milk quality analysis of Hu sheep and Suffolk. **B** Average daily gain of lambs during the ewes' lactation period. **C** RDA analysis of ruminal microbiome and milk quality and average daily gain of lamb. **D** The correlation between SCFA and milk quality and average daily weight gain of lambs during the lactation period. **E** WGCNA analysis of ruminal metabolites and milk quality of ewes in lactation and average daily gain of lambs. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

paid to the intestinal health of ewes during pregnancy, and relevant feeding and management strategies can be formulated to ensure a healthy pregnancy. Furthermore, some studies have shown that the IgG concentration decreases during pregnancy and increases gradually during lactation [20], consistent with the results obtained for the Hu sheep in this study. During lactation, ewes may transfer part of their IgG into milk while increasing their immunity [21], thus improving the intestinal immunity of Hu sheep lambs. We also found that the intestinal IgG content in the feces of Hu sheep lambs was greater than that Suffolk lambs, and it was proved that IgG plays a role in the intestinal tract of lambs through breast milk. Highly active IgM increases during the lactation period in Suffolk, and studies have reported that IgM can clear pathogens from the body [22], ensuring the health of ewes and the quality of their milk. It has been reported that the IL-1 family is related to the intestinal immune barrier [23, 24], which is influenced by genetic factors and microbiota [25]. This study revealed that the immune

factors IL-1β and IL-6 increased during lactation in Hu sheep, but the changes were not significant in Suffolk. The upregulation of immune factors during lactation in Hu sheep may indirectly improve the intestinal immunity of offspring lambs through milk suckling. Furthermore, the levels of IL-1β, IL-6, and TNF-α in the intestinal feces of Hu sheep lambs were lower than those Suffolk lambs. In addition, human studies have shown that pregnant women are prone to iron deficiency anemia during pregnancy [26]. In this study, the HGB of Hu sheep and Suffolk decreased during pregnancy, and the immune system of mothers during pregnancy changed to adapt to fetal development [27], resulting in increased LYM in Hu sheep and Suffolk during pregnancy and lactation. Therefore, in the sheep, pregnancy and lactation period should pay attention to the supply of nutrients, such as vitamins and iron, to prevent the occurrence of iron deficiency anemia during pregnancy leading to abortion or lactation milk shortage. In addition, these changes in reproductive hormone and immune levels also lead to changes in the intestinal microbiota at different reproductive periods [6]. In this study, the Shannon and Simpson indices of microbial diversity in lactation of Hu sheep decreased, and the Simpson indices of pregnancy and lactation in Suffolk decreased, indicating that the rumen microbial diversity of sheep decreased during pregnancy and lactation, as also observed by Grice [28]. Firmicutes, which encode enzyme genes related to energy metabolism [29], increased during pregnancy and lactation in Hu sheep but decreased in Suffolk. However, the abundance of Bacteroidota [30, 31], related to the degradation of carbohydrates and protein, was lower during the pregnancy and lactation of Hu sheep, and the F/B ratio (Firmicutes/Bacteroidetes) was greater than that of Suffolk, indicating that the absorption efficiency of energy substances [32, 33] is greater during the pregnancy and lactation of Hu sheep to meet the energy needs of the ewe and the fetus; this may be related to the common birth of double and single lambs in Hu sheep and Suffolk respectively. In addition, this study revealed that the abundance of *Prevotella* increased in Suffolk during pregnancy and lactation and was greater than that in Hu sheep. *Prevotella* can ferment fiber to produce acetate [34, 35], and the abundance of *Succiniclasticum* increases during pregnancy and lactation of Hu sheep and greater than that in Suffolk, providing energy for ewes and ensuring the normal development of fetuses and lambs. The plant polysaccharide degrading bacteria *Rikenellaceae_RC9_gut_group* [36, 37] decreased during pregnancy and lactation in Hu sheep but increased during Suffolk pregnancy, indicating that the ability to degrade plant polysaccharides was enhanced during Suffolk pregnancy. In the future, these microbiotas can be manipulated to change the energy metabolism and absorption efficiency of sheep during pregnancy and lactation, thereby improving reproductive performance. KEGG functional analysis revealed that energy metabolism and lipid metabolism decreased during pregnancy and lactation in Hu sheep, while cell motility increased. In Suffolk, energy metabolism, glycan biosynthesis and metabolism, metabolism of cofactors, and vitamins increased during pregnancy, consistent with previous research results [9]. These microbial functions ensure the high-energy metabolic requirements of Suffolk ewes during pregnancy to meet the growth and development needs of the fetus, revealing the potential reason for which Suffolk lambs are larger than Hu sheep lambs at birth, but this needs to be further verified. Overall, our results showed that the rumen energy metabolism and lipid metabolism function of multi-fetal Hu sheep were low during pregnancy and lactation, and further attention should be paid to this aspect in daily feeding and management, so as to supplement sufficient energy substances in the diet to ensure healthy pregnancy and lactation of Hu sheep.

The animal intestinal microbiota plays a functional role in the host through metabolite production [38]. In this study, the rumen microbial metabolic profiles of two sheep breeds showed adaptive changes at different reproductive periods. In both Hu sheep and Suffolk during pregnancy and lactation, several metabolites related to prostaglandin were identified, such as CDP-DG (PGF2alpha/i-22:0), PI(20:1(11Z)/PGE2), and CDP-DG(5-iso PGF2VI/i-19:0). Studies in humans have shown that prostaglandin is involved in inflammation in pregnancy tissues and plays a crucial role in the process of parturition [39]. In addition, some amino acid-related metabolites (L-alanine, L-allo-threonine) were also found during the pregnancy of Hu sheep, and fetuses during pregnancy required more amino acids for protein synthesis [40], thus affecting fetal development. This highlighted the fact that Hu sheep may require more amino acids than Suffolk during pregnancy to meet the developmental needs of multiple fetuses. Therefore, it is recommended to supplement sufficient amino acid diet during pregnancy of Hu sheep. KEGG functional analysis revealed that protein digestion, absorption, and amino acid metabolisms, such as the metabolism of lysine, glycine, serine, and threonine, and the metabolism of alanine, aspartate, and glutamate were upregulated in Hu sheep during pregnancy, providing amino acids for the growth and development of mothers and fetuses during pregnancy [40]. Moreover, propanoate metabolism decreased during Suffolk pregnancy, which was also confirmed by the lower propionate content in pregnant Suffolk than in pregnant Hu sheep. ABC transporters are highly functional during pregnancy in Hu sheep and

Suffolk, and members of the ABC transporter family can restrict the entry of exogenous substances into the placenta and maintain placental function and normal fetal development [41]. Citrate cycle and pyruvate metabolism are enriched during pregnancy and lactation in Hu sheep. Some studies have reported that pyruvate is an important energy substance in the early embryonic development of mammals [42] and participates in the citric acid cycle for energy supply in order to meet the energy requirements for the growth and development of ewe and fetus during pregnancy. In addition, the metabolic functions of nicotinate and niacinamide during lactation are enhanced, and they participate in energy metabolism, glucose metabolism, and fatty acid synthesis [43] to meet the energy requirements of lactation Hu sheep. During the lactation period in Suffolk, carbohydrate digestion and absorption, starch and sucrose metabolism, and ABC transporter and galactose metabolism are downregulated, which may affect lactation traits. Therefore, in the feeding and management of lactation period, we should tend to pay attention to digestion and metabolism to ensure normal lactation. In addition, acetate and propionate increased in Hu sheep and Suffolk during pregnancy and lactation, with the highest concentrations reached during lactation, consistent with previous research results [9], thereby providing energy for ewe during pregnancy and lactation [44]. Propionate and butyrate in the rumen of pregnant Hu sheep were greater than those Suffolk. As propionate during pregnancy determines fetal development and metabolism [3], Hu sheep may require more propionate to achieve fetal development. There was an increase in NH_3-N during the pregnancy period in both Hu sheep and Suffolk, consistent with the findings of Close et al. [45]. During pregnancy, the increased demand for nitrogen sources can be attributed to the proliferation of reproductive tissues, and the synthesis of proteins must meet the growth and development needs of the fetus. Interestingly, the NH_3-N content was greater in Hu sheep than in Suffolk, indicating that Hu sheep carrying two lambs would require more NH_3-N to meet their nitrogen source needs. Therefore, it is necessary to supplement sufficient nitrogen sources during pregnancy to meet the development of ewe and fetus during pregnancy.

There were interactions between the rumen microbiota and its metabolites at different reproductive periods. Studies have reported that Prevotellaceae is related to the intestinal barrier and plays a role in intestinal homeostasis [46, 47]. In this study, *Prevotella* was positively correlated with acetate, as *Prevotella* is known to ferment fiber into acetate [34, 35], while propionate is positively correlated with *Succiniclasticum*, which is a propionate-producing bacterium [48]. The interaction of these microbiota and its metabolites is also closely related to reproductive hormone and immune levels [5, 6, 9, 49]. In this study, LH, FSH, PROG, and E2 were significantly positively correlated with *Succiniclasticum*, and propionate was significantly positively correlated with PROG, E2, and FSH. These results indicate that the variation in reproductive hormone levels is closely related to the variation in *Succiniclasticum* abundance, thus affecting the metabolic level (propionate change) in ewe reproductive period. A decrease in the maternal proinflammatory factors TNF-α and IL-1β during pregnancy is reportedly related to butyrate [13], which is consistent with the results of this study. In this study, other metabolites in the rumen were also correlated with reproductive hormones and immune indices, such as CDP-DG(PGF2alpha/i-22:0), acetate, and isovalerate. SCFAs are not only responsible for maintaining maternal homeostasis during pregnancy but also participate in regulating immune function [3]. Together, these results suggest that the changes of microbiota and its metabolites at different reproductive periods are related to the levels of reproductive hormones and immunity of ewe. However, the mechanism of reproductive hormone-microbe-metabolite interactions is still unclear and needs to be further verified in animal models. This will help to understand the functional role of rumen microbiota in physiological regulation at different reproductive stages of ruminants. The maternal intestinal microbiota and its metabolites during pregnancy and lactation play a crucial role in the healthy development of offspring [49–52]. In this study, *Prevotella* and acetate during pregnancy were positively correlated with lamb birth weight and body size. *Prevotella* abundance was higher in Suffolk sheep than in Hu sheep during pregnancy. Pregnant Suffolk ewes may produce more acetate through a greater abundance of *Prevotella*, thus providing more energy to the ewe and fetus and resulting in greater birth weight and body size in Suffolk lambs than in Hu sheep lambs. However, how *Prevotella* and acetate in pregnant ewe affect fetal development through the placenta remains to be further verified. In addition, the abundance of *Butyrivibrio* in pregnant ewe was positively correlated with the level of intestinal fecal IgG in lambs. The maternal microbiome is involved in regulating the establishment of fetal immunity during pregnancy [49, 50]. SCFAs can increase the number of T cells [53], which activate B cells in turn promoting the production of antibodies. In this study, the abundances of both *Butyrivibrio* and butyrate in Hu sheep during pregnancy are greater than those in Suffolk, resulting in greater IgG content in the intestine of newborn Hu sheep lambs. In addition, *Butyrivibrio* is negatively correlated with IgA. Studies have shown that butyrate and acetate can be converted into each other [54], indicating that *Butyrivibrio* may also participate in

acetate production. Acetate can induce the generation of IgA by regulating the interaction between intestinal epithelial cells and immune cells [19]. In this study, the acetate content of Suffolk ewes during pregnancy was greater than that of Hu sheep, resulting in greater intestinal IgA in Suffolk lambs, which could reduce the probability of intestinal inflammation in newborn lambs [55]. Studies have shown that maternal infection with pathogens can regulate fetal immunity by increasing the level of IL-6, which crosses the placental barrier, promotes the intestinal T-cell response, and enhances the intestinal protective immunity of the fetus [56]. In this study, the *Rikenellaceae_RC9_gut_group* in ewes showed a significant positive correlation with IL-6 in lambs. Fibrolytic Rikenellaceae [36, 37] may affect the intestinal immunity of newborn lambs through the production of SCFAs. By WGCNA, several metabolites associated with the birth weight, body size, and intestinal immunity of newborn lambs, such as pyrimidine and pyrimidine derivatives 3-methylcytosine, linolenic acid and its derivatives (6Z,9Z,12Z)-octadecatrienoic acid, amino acids, peptides, and analogues o-(2-fluoroethyl)-L-tyrosine, were identified. Pyrimidine and its derivatives are essential components of DNA synthesis and for normal fetal development [57]. The intake of linolenic, a precursor of docosahexaenoic acid (DHA), is related to the fetal nervous system development [58]. The fetus needs to synthesize its own protein through metabolites such as amino acids supplied by the mother to support cell proliferation and tissue development. These metabolites were found in greater proportion in Suffolk than in Hu sheep during pregnancy, resulting in greater birth weight and body size in newborn Suffolk lambs. Therefore, the feeding and management of pregnant ewe should pay attention to the supplement of amino acids to ensure the development of the fetus.

Furthermore, the maternal microbiota and its metabolites during lactation period have long-term effects on the health and growth of offspring. In this study, the abundances of *Prevotella* and Prevotellaceae in lactating ewes were positively correlated with lactose, lactalbumin, and lamb daily gain. In dairy cows, *Prevotella* is linked to the microbial protein synthesis pathway (BCAA biosynthesis) [8, 59], which can serve as precursor for milk protein synthesis in the mammary gland. In the feeding and management of sheep during lactation period, the milk quality can be affected by regulating the abundance of *Prevotella*, and then the daily gain of lambs can be affected. This study also showed that compared with those in Hu sheep, the ruminal concentrations of acetate and propionate in Suffolk lactation were greater, resulting in higher levels of protein, lactose, and casein in Suffolk milk than in Hu sheep milk; this results in Suffolk lambs having a larger body size than Hu sheep lambs. In addition, *Succiniclasticum* and *Christensenellaceae_R_7_group* were positively correlated with milk fat, and these genera were involved in propionate and butyrate production [48, 60, 61]. Studies have shown that growth and lactation performance can be improved by increasing the abundance of the rumen *Christensenellaceae_R_7_group* [62, 63]. This study proved that the relative abundances of *Succiniclasticum* and *Christensenellaceae_R_7_group* in the rumen were greater in Hu sheep than in Suffolk, and the milk fat of Hu sheep was also greater than that of Suffolk. These results indicate that some metabolic pathways were regulated through the relative abundances of *Prevotella*, *Rikenellaceae*, *Succiniclasticum*, and *Christensenellaceae_R_7_group* in the lactation period, which may affect the composition of milk, further influencing the daily gain of nursing lambs. In future studies, the growth and development of lambs can be influenced by regulating the abundance of these bacteria in lactation. In addition, WGCNA revealed that keto acid and its derivative 4-(4-methylcyclohexyl)-4-oxobutanoic acid, fatty acid and conjugate 2-hydroxy-2-ethylsuccinic acid, carbohydrate and carbohydrate conjugate beta-D-lactose, and fatty alcohol methyl-12-gingerdiol are positively correlated with milk fat and negatively correlated with lactose and lamb daily gain. Lactose is very important for growth and is the main energy source for infant development [64]. In this study, both the lactose content in Suffolk milk and the daily gain of lambs were higher than those of Hu sheep, and the levels of 4-(4-methylcyclohexyl)-4-oxobutanoic acid, 2-hydroxy-2-ethylsuccinic acid, beta-D-lactose, methyl-12-gingerdiol in Suffolk milk were also lower than those in Hu sheep. These metabolites may affect the lactose synthesis pathway in mammary glands and thereby affect the daily gain of lambs.

Conclusion

In conclusion, the study identified the successive characteristics of the rumen microbiota and its metabolites in different breeds of sheep during non-pregnancy, pregnancy, and lactation and revealed the effects of these changes on the ewe's own metabolism and on the growth and development of offspring lambs (Fig. 8). Some microbial communities and its metabolites related to birth weight, body size, and intestinal immunity of newborn lambs were identified, such as *Prevotella*, *Butyrivibrio*, *Rikenellaceae_RC9_gut_group*, pyrimidine, linolenic acid and its derivatives, amino acids, and peptides. These changes enhance energy metabolism, glycan biosynthesis, and metabolism in the pregnancy period of Suffolk. Moreover, the changes of acetate, propionate, butyrate, and amino acids in the rumen upregulated the functions related to protein

digestion, absorption, and amino acid metabolism. And the pathways related to ABC transporters, citrate cycle, and pyruvate metabolism are enriched, providing amino acids and energy for the growth and development of pregnant ewe and fetuses. In addition, *Prevotella*, Prevotellaceae, Rikenellaceae, *Succiniclasticum*, and *Christensenellaceae_R_7_group* were significantly correlated with milk quality and daily gain of lambs. These bacteria participate in nutritional regulation by producing metabolites, which may affect the quality of milk, and then affect the daily gain of lactating lambs. These findings provide a deeper understanding of the potential effects of rumen microbiota and its metabolites during pregnancy and lactation on the growth and development of offspring lambs and provide a reference for the healthy feeding and management of different breeds of sheep during the non-pregnancy, pregnancy, and lactation.

Methods

Experimental design and sample collection

For this study, healthy 10-month-old Hu sheep and Suffolk ewes (n = 60/breed) were selected as research subjects, all of whom had reached sexual maturity. All sheep were allowed to feed and drink freely under unified feeding and management conditions during the test cycle.

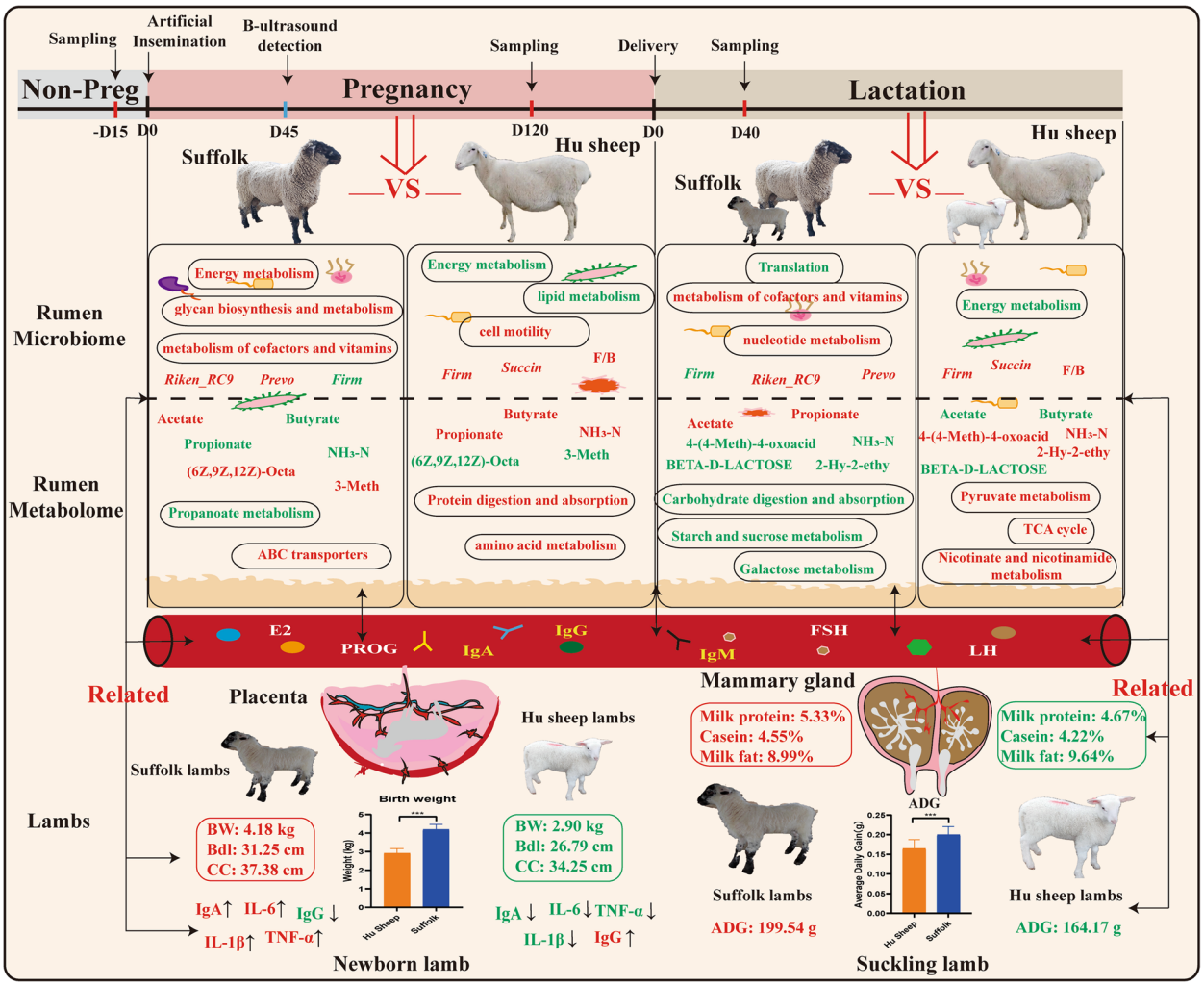

Fig. 8 Graphical summary of the effects of ruminal microbiota and its metabolites on the growth and development of offspring lambs at different reproductive periods of Hu sheep and Suffolk breeds. The two breeds of sheep in pregnancy period and lactation period were compared respectively. The red font represented upregulation of bacterial flora, metabolites, and KEGG function, while the green font represented downregulation. Firm, Firmicutes; Riken_RC9, *Rikenellaceae_RC9_gut_group*; Succin, *Succiniclasticum*; Prevo, *Prevotella*; F/B, Firmicutes/Bacteroidota; (6Z,9Z,12Z)-Octa, (6Z,9Z,12Z)-octadecatrienoic acid; 3-Meth, 3-methylcytosine; 4-(4-Meth)-4-oxoacid, 4-(4-methylcyclohexyl)-4-oxobutanoic acid; 2-Hy-2-ethy, 2-hydroxy-2-ethylsuccinic acid; BW, birth weight; Bdl, body diagonal length; CC, chest circumference; ADG, average daily gain

To eliminate technical bias and increase the resolution of changes related to animal physiological state, all sheep selected in this study were sourced from the same pasture (Guanghe County Tuteng Animal Husbandry Co., Ltd., Gansu Province, China), the diet composition remained consistent throughout the experiment, and the entire study process was strictly standardized. The trial was launched in January 2022, and artificial insemination was conducted on all experimental sheep (first insemination). The period before estrus was defined as the non-pregnancy, and the 15th day before artificial insemination was considered as the sampling time point of the non-pregnancy. The experimental sheep were injected with pregnant mare serum and prostaglandin (PG) to induce synchronized estrus. Artificial insemination was performed following estrus induction, and pregnancy status was examined 45 days after insemination using ultrasonography. Of the 120 sheep tested, only 112 were confirmed to be pregnant, and the whole reproductive cycle (non-pregnancy, pregnancy, and lactation) was tracked and detected. Blood samples and rumen fluid samples (collected before morning feeding) were obtained from the ewes during the non-pregnancy (Np, 15 days before mating), pregnancy period (Pr, 120 days after pregnancy), and lactation period (Lp, 40 days after delivery), respectively. A total of 3 mL of whole blood (non-anticoagulant tube) was collected through the jugular vein and centrifuged at 3000 r/min for 15 min. The serum was separated, transferred to a frozen storage tube, placed in a liquid nitrogen tank, and returned to the laboratory and stored at −80 °C. At the same time, whole blood was collected from the anticoagulant tube to measure physiological indices. A rumen fluid sampler with a gastric tube was used to enter the rumen through the mouth, and 50 mL of rumen fluid was extracted from ewes at different reproductive periods, immediately filtered with four layers of sterile gauze, stored in 5 mL sterile frozen tubes, numbered, placed in liquid nitrogen, and returned to the laboratory for storage at −80 °C. Lambing status was tracked and recorded throughout the process. According to the lambing records, six ewes each of Hu sheep (double lambing) and Suffolk (single lambing) were randomly selected. Microbial 16S rRNA sequencing and metabolite determination (metabolome, SCFAs, and NH_3-N) were performed on rumen fluid samples at different reproductive periods. Reproductive hormone and immune indices were measured in the blood of ewes at different reproductive periods, and milk was manually collected 40 days after lactation for milk quality determination. The birth weight and body size of the offspring lambs were measured, and the lambs were followed up until weaning (45 days). Intestinal feces of newborn lambs (0–3 days) were collected to determine immune factors, and fresh fecal samples were collected after lactation in the early morning.

Reproductive hormone, immune index, and physiological index measurements

Blood reproductive hormone index level was quantitative determination using the sheep progesterone (PROG) ELISA Kit (F3911-A), sheep luteinizing hormone (LH) ELISA Kit (F3921-A), sheep follicle-stimulating hormone (FSH) ELISA Kit (F3906-A), and sheep estradiol (E2) ELISA Kit (F3907-A). Blood and fecal immune indicators were quantitative determination using the sheep immunoglobulin A (IgA) ELISA Kit (F72006-A), sheep immunoglobulin G (IgG) ELISA Kit (F3902-A), sheep immunoglobulin M (IgM) ELISA Kit (F72008-A), sheep interleukin-1β (IL-1β) ELISA Kit (F3895-A), sheep interleukin-6 (IL-6) ELISA Kit (F3894-A), and sheep tumor necrosis factor-α (TNF-α) ELISA Kit (F72110-A). All assay kits were purchased from Shanghai Fanke Wei in China, and the procedures strictly followed the instructions provided. Detection and analysis were conducted using a Thermo 3020 microplate reader. The operation procedure was as follows: the prepared sample and standard product were added to the enzyme-labeled plate, the plate was reacted for 30 min at 37 °C, the plate was washed five times, the enzyme-labeled reagent was added, the plate was allowed to react for 30 min at 37 °C, the plate was wash five times, color developing solutions A and B were added, the color was developed at 37 °C for 10 min, and the stop solution was added. The absorbance (OD value) of each well was measured at wavelength of 450 nm with blank hole zeroing, the OD value was read within 15 min, and, finally, the concentration was calculated. Physiological indicators such as hemoglobin (HGB), total white blood cell (WBC), neutrophils (NEU), lymphocytes (LYM), and monocytes (MON) count were measured using the GRT-6008 (ANIMAL) fully automatic blood cell analyzer.

Determination of SCFAs, *ammonia* nitrogen, and milk quality

The rumen SCFAs content at different reproductive periods was determined by GC-2010 Plus Gas Chromatograph. The internal standard method was used with 2-ethylbutyric acid (2EB) as the internal standard. Chromatography was performed on an AT-FFAP (50 m × 0.32 mm × 0.25 μm) capillary column. The temperature of the column was kept at 60 °C for 1 min, then increased to 115 °C at 5 °C /min without reservation, and then increased to 180 °C at 15 °C /min. The temperature of the detector was 260 °C, and the temperature of the inlet was 250 °C. The NH_3-N content was determined according to the method of Chaney et al. [65]. The milk protein, lactose,

casein, and milk fat of Hu sheep and Suffolk were determined by MilkoScan FT1.

Microbial 16S rRNA sequencing

Rumen microbiome DNA was extracted using the MN NucleoSpin 96 Soil bacterial DNA extraction kit (Omega, Shanghai, China). The conserved region of nucleotides encoding bacterial ribosomal RNA is mainly the 16S region. PCR was used to amplify the V3–V4 regions of the highly variable region of the 16S rRNA gene using the forward primer 338F:5′-ACTCCTACGGGA GGCAGCA-3′ and reverse primer 806R:5′-GGACTA CHVGGGTW TCTAAT-3′. The forward and reverse 16S primers were tailed with sample-specific Illumina index sequences to allow for deep sequencing. PCR was performed in a total reaction volume of 10 μl: 5–50 ng of DNA template, 0.3 μl of forward primer (10 μM), 0.3 μl of reverse primer (10 μM), 5 μl of KOD FX Neo Buffer, 2 μl of dNTP (2 mM each), 0.2 μl of KOD FX Neo, and finally ddH2O up to 20 μL. After initial denaturation at 95 °C for 5 min, 20 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 40 s, and a final step at 72 °C for 7 min. The amplified products were purified with an Omega DNA purification kit (Omega Inc., Norcross, GA, USA) and quantified using Qsep400 (BiOptic, Inc., New Taipei City, Taiwan, ROC). The amplicon library was paired-end sequenced (2 × 250) on an Illumina NovaSeq 6000 (Beijing Biomarker Technologies Co., Ltd., Beijing, China). Using FLASH v1.2.11 software, reads of each sample were spliced according to the minimum overlap length of 10 bp and the maximum mismatch ratio allowed in overlap area of 0.2. The splicing sequence obtained was the RawTags data. Trimmomatic v0.33 software filtered the RawTags obtained by splicing, and the parameter was set to a 50-bp window. If the average quality value in the window is lower than 20, the back-end base is cutoff from the window, and the Tags whose length is less than 75% of the length of Tags after quality control are filtered; high-quality Tags data can be obtained. Using UCHIMEv4.2 software, the chimera sequences were identified and removed to obtain the final EffectiveTags. Clean reads then were conducted on feature classification to output an ASVs (amplicon sequence variants) by dada2 [66], and the ASVs counts less than 2 in all samples were filtered. Based on the Naive Bayes classifier in QIIME2 [67], the SILVA database [68] (release 138.1) was used to classify and label ASV, and the confidence threshold was 70%. Using the QIIME2 2020.6 software [67], the assessment of alpha-diversity indices (ACE, Chao1, Simpson, and Shannon indices) was performed on the samples to study the species diversity within individual samples. The alpha-diversity index was tested for normal distribution before ANOVA (Shapiro-Wilk, SW). The microbial species composition of different taxonomic levels (phylum, class, order, family, genus, species) was obtained by using SILVA database (release 138.1) [68]. The PCoA diagram of the corresponding distance samples is obtained according to the distance matrix algorithm (binary Jaccard). After normalized species abundance, Kruskal-Wallis rank-sum test was performed, and then linear discriminant analysis (LDA) was used to obtain different biomarker species at different reproductive periods ($P < 0.05$, LDA value > 4) [69]. Nonparametric factorial Kruskal-Wallis (KW) sum-rank test was used to detect the features of significant abundance difference by Python LEfse package analysis, and the group with significant abundance difference was found. Linear discriminant analysis (LDA) was then used to estimate the magnitude of the influence of each component (species) abundance on the differential effect. Additionally, PICRUSt software was used for functional prediction of 16S rRNA sequencing data based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) [70] (parameters: -s study_seqs.fna -i study_seqs.biom -o picrust2_out_pipeline -p 1). First, it is necessary to standardize the generated Feature abundance table. Then, the KEGG information corresponding to the Feature can be obtained through the corresponding ID of each Feature, so as to calculate the abundance of the KEGG and obtain the Pathway from the information of the KEGG database.

Metabolome assay

The metabolites in the rumen contents of sheep at different reproductive periods were detected by liquid chromatography-mass spectrometry. After the rumen contents were thawed at room temperature, 100-μL samples were weighed each time, and 500 μL of extraction solution containing an internal standard (1000:2) was added (methanol-acetonitrile volume ratio = 1:1, internal standard concentration = 2 mg/L) and vortexed for 30 s. Then, the mixture was ultrasonicated in an ice water bath for 10 min, left at −20 °C for 1 h, and centrifuged at 4 °C for 15 min (12,000 rpm). Then, 500 μL of the supernatant was removed from the EP tube, the extract was dried in a vacuum concentrator, and 150 μL of the extract solution (acetonitrile-water volume ratio: 1:1) was added to the dried metabolites for redissolution. The mixture was swirled for 30 s, ultrasonicated in an ice water bath for 10 min, and centrifuged at 4 °C for 15 min (12,000 rpm). Finally, 120 μL of the supernatant was removed into a 2-mL injection bottle, and each sample was mixed with 10 μL to form QC samples for machine detection. The LMS system for metabolomics analysis consisted of a Waters ACQUITY I-Class PLUS ultrahigh performance liquid phase tandem Waters Xevo G2-XS QTof high-resolution mass spectrometer. The ACQUITY UPLC HSS

T3 column (1.8 μm, 2.1 × 100 mm) was purchased from Waters. The samples were eluted in positive ion mode (ESI+) and negative ion mode (ESI−) with a mobile phase consisting of water and 5% acetonitrile, 0.1% formic acid as solvent A, and 0.1% acetonitrile and 0.1% formic acid as solvent B at flow rates of 0.35 mL/min and 400 μL/min, respectively. Subsequent mobile phase (A:B) elution gradient is as follows: 98%: 2% during 0–0.25 min, 2%: 98% during 10.0–13.0 min, 98%: 2% during 13.1–15.0 min, followed by ion source temperature: 150 °C, and a desolvent temperature of 500 °C. The flow rates of the backflow and desolvent gas were 50 L/h and 800 L/h, respectively. The original data collected by MassLynx V4.2 were processed by the Progenesis QI software for peak extraction, peak alignment, and other data processing operations. Using the original data collected by MassLynx V4.2, the Progenesis QI software was used to do data processing operations such as peak extraction and peak alignment, and based on the Progenesis QI software online METLIN database and Biomark's self-built library for identification, the theoretical fragment identification was also carried out (the mass number deviation of parent ion is 100 ppm, and the mass number deviation of fragment ion is less than 50 ppm) [71]. After normalizing the original peak area information with the total peak area, the follow-up analysis was performed. The identified compounds are searched for classification and pathway information in KEGG [72], HMDB [73], and LIPID MAPS databases [74]. To calculate and compare the difference multiples, *T*-test was used to calculate the difference significance *P*-value of each compound, the R language package ropls was used to perform OPLS-DA modeling [75], and 200 times permutation tests were performed to verify the reliability of the model. When $Q2Y > 0.5$, the model was regarded as effective, and when $Q2Y > 0.9$, the model was considered excellent (parameters: cross-verification fold 7; number of replacement tests 200). The VIP value of the model was calculated using multiple cross-validation. The method of combining the difference multiple, the *P*-value and the VIP value of the OPLS-DA model, was adopted to screen the differentially abundant metabolites. The screening criteria were $FC > 2$, $P\text{-value} < 0.01$, and $VIP > 1$. The quantitative values of differentially abundant metabolites were calculated to calculate the corresponding ratios, and the top 10 metabolites with the largest absolute values of log2FC were selected for radar display. KEGG pathway enrichment analysis was performed for differentially abundant metabolites [76], and the top 20 items with the most annotated differentially abundant metabolites in the pathway were selected to construct a map of the enrichment points.

Data analysis

According to the method of McHardy et al. [77], the microbiome and metabolome were jointly analyzed, and PCoA was used to reduce the dimension of the microbiome (genus level) and metabolome respectively. Firstly, the distance matrix was calculated by the quantitative matrix of microorganism and metabolite respectively. The distance algorithm of microbiome was Bayesian distance, the distance algorithm of metabolome was Euclidean distance, and PCoA was used to sort the distance. The coordinates of characteristic axes in the PCoA results of microbiome and metabolome were extracted, and Procrustes analysis was performed to compare the similarity and variation between microbiome and metabolome [78]. Metabolic data were dimensionally reduced by weighted gene co-expression network analysis (WGCNA) [79], and metabolites were divided into different metabolite clusters, with the expression of metabolite clusters represented by the median content in the same cluster. Pearson correlation analysis was conducted with microbiota [80], heat maps were drawn, and the correlation analysis results were screened based on $|CC| > 0.8$, and $CCP < 0.05$. Then, the frequency of occurrence of metabolite clusters/microbiota was counted, and the correlation result table of metabolite clusters/microbiota with top 30 frequency was drawn. In addition, Spearman correlation test was used for correlation analysis, and the statistical significance level was $P < 0.05$. IBM SPSS Statistics 26.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. One-way analysis of variance (ANOVA) was used to analyze the levels of reproductive hormones, immune indexes, SCFAs, and NH_3-N in rumen fluid of Hu sheep and Suffolk at different reproductive periods. The results were expressed as "mean ± standard deviation." The birth weight, body size, fecal immune index, and milk quality of Hu-sheep lambs and Suffolk lambs were tested by independent sample *T*-test.

Abbreviations

SCFAs	Short-chain fatty acids
PROG	Progesterone
E2	Estradiol
LH	Luteinizing hormone
FSH	Follicle-stimulating hormone
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1β	Interleukin-1β
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor-α
HGB	Hemoglobin
WBC	White blood cells
NEU	Neutrophils
LYM	Lymphocytes
MON	Monocytes
KEGG	Kyoto Encyclopedia of Genes and Genomes
WGCNA	Weighted gene co-expression network analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01892-z.

Supplementary material 1: Fig. S1: Microbial PCoA analysis in different breeding period of sheep. Fig. S2: ASV Venn diagram of Hu sheep and Suffolk sheep. Fig. S3: Radar distribution maps of the top 10 differential metabolites with the largest absolute log2FC values for HN_vs_HP, HN_vs_HL, SN_vs_SP and SN_vs_SL, respectively. Fig. S4: Interaction analysis of rumen microbiota and their metabolites at different reproductive period.

Supplementary material 2: Table S1: ASV analysis.

Supplementary material 3: Table S2: Differential metabolites.

Supplementary material 4: Table S3: The relationship between different bacterial flora (genus level) and different metabolites at different reproductive period.

Supplementary material 5: Table S4: Pregnancy module metabolites.

Supplementary material 6: Table S5: Lactation module metabolites.

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Authors' contributions
Y.S., X.L., Y.H., and S.Z. conceived and designed the research. Y.S., X.L., and Z.W. collected samples and performed the experiment. Y.S. and X.L. analyzed the data. P.S. and T.J. helped with the bioinformatics and statistical analysis. Y.S. wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data sets presented in this study can be found in the NCBI Sequence Read Archive(SRA) under accession numbers PRJNA1060421.

Declarations

Ethics approval and consent to participate
All studies involving animals were carried out in accordance with the regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004), and sample collection protocols were approved by the Livestock Care Committee of Gansu Agricultural University (Approval No. GAU-LC-2020-27).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730070, China. [2]College of Pratacultural Science, Gansu Agricultural University / Key Laboratory for Grassland Ecosystem, Ministry of Education / Sino-US Grassland Animal Husbandry Sustainable Development Research Center, Lanzhou 730070, China. [3]School of Fundamental Sciences, Massey University, Palmerston North 4410, New Zealand.

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