The Effects of Gut Microbiome of Pregnant Mice on Palate Development in Fetal Mice

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Abstract

Background

Cleft palate (CP) is one of the most common congenital birth defects in the craniofacial region, recent studies have shown that the occurrence of cleft palate is closely related to maternal health. Pregnant women with an imbalance in their gut microbiota might be at risk not only for their health but also for their fetuses. However, few reports focus on the relationship between the occurrence of CP on embryos and gut microbiota of their mothers.

Results

In this study, we used retinoic acid (RA) to induce cleft palate model for E10.5 mice, then at E16.5, the feces of 5 RA-treated pregnant mice and 5 control pregnant mice were respectively collected for metagenomics analysis. The results showed that compared with the control group, *Lactobacillus* in the gut microbiome of pregnant mice in the RA group was significantly increased. GO, KEGG and CAZy analysis of differentially expressed unigenes demonstrated the most abundant metabolic pathway in different groups, such as TCA cycle, lipopolysaccharide biosynthesis, and histidine metabolism.

Conclusions

Our findings indicated that changes in maternal gut microbiome could affect fetal palatal development, which might be related to changes in *Lactobacillus* and metabolic disorders. These results provide a new idea and direction for the pathogenesis of the cleft palate.

Background

Cleft palate (CP) is one of the most common congenital birth defects in the craniofacial region, with an average occurrence of 1/1000 newborns around the world [1]. It is universally acknowledged that CP has a connection with genetic background and environmental factors. At present, many studies found that there was a relationship between the occurrence of CP and behavioral maternal gestational conditions, including gestational diabetes, gestational obesity, and gestational smoking [2–5]. Moreover, it has been shown that the microbiota can be changed between pregnant women and neonates [6].

The “microbiota” consists of microbiota communities on the mucosal surface and lumen of the respiratory tract, gastrointestinal (GI) tract, urinary tract, and reproductive tract. The GI tract has the greatest density of microbiota, defined as the “gut microbiota” [7, 8]. Gut microbiota is essential for digesting food, producing short-chain fatty acid, synthesizing vitamins, and protecting the mucosal. To a large extent, host metabolism and immune response were due to the interaction between host cells and the gut microbiota [9]. The imbalance of the intestinal microbiota was mainly manifested by an increase

in harmful bacteria and a decrease in beneficial bacteria. Microbiota imbalances were associated with several underlying diseases, for instance, metabolic syndrome, allergic diseases, some kinds of cancer, and neurological diseases [10–12].

However, the dangers of disrupted gut flora are not limited to these. Recent studies found that pregnant women with an imbalance in gut flora might be at risk not only for their health but also for their fetuses. During pregnancy, maternal gut environment could finetune energy homeostasis, which was a key factor to prevent metabolic syndrome for offspring [13]. In addition, the mother's gut flora was also a source of immunity for offspring. The neonatal mice lacking the ability to produce IgG could be protected against enterotoxigenic Escherichia coli infection by the mother's natural IgG antibody to Escherichia coli, which was transmitted through the placenta or breast milk [14]. For the past few years, the emergence of the metagenomic could associate the microflora with genes, thereby better illustrating the mechanism of regulation of diseases by microflora disorders [15]. For example, maternal gut microbiome is proved to be a vital signal in developing brain neurons through microbial-regulated metabolites, which promote fetal-thalamic cortex axons [16].

All of these studies provided the best evidence for the relationship between the gut microbiome of pregnant women and the health of their offspring. Pereira et al. [17] manifested that the intestinal bacteria of children with CP changed before and after palatoplasty. Nevertheless, little information has been focused on the relationship between the occurrence of CP on embryos and gut microbiota in pregnant women, especially the mechanisms involved. However, it is difficult to obtain such pregnant and embryo samples. According to embryology and etiology studies, mice are an easily available animal with a high farrowing rate and a high degree of homologous genetic sequence to humans (99%), which is recognized as a suitable animal model for most studies [18]. Nowadays, many studies have shown that retinoic acid (RA) gavage is the most common method for inducing cleft palate model except knockout mice, which could cause the highest incidence of CP in mice on embryo day (E) 10–12 [19, 20]. Therefore, we selected E10.5 pregnant mice to construct the fetal cleft palate model by intragastric administration of RA and collected faeces samples of all pregnant mice at E16.5 for metagenomics analysis. Our works aimed to provide a new direction for the pathogenesis of CP.

Results

Successful construction of RA-induced cleft palate model

E10.5 pregnant mice were used to construct the fetal cleft palate model by intragastric administration of RA (100 mg/kg). At E16.5, when the normal palate was primarily formed, the palate of fetal mice in the control group was normally fused, and the incidence of cleft palate in the RA group was approximately 98% (38/39). In the palate shelf tissue and histological sections of control E16.5 embryos, the opposed palatal shelves had come into contact and fused during normal development (Fig. 1a, c). In the meanwhile, RA-treated palatal shelves remained small in volume, failed to rise and fuse with the contralateral side, and were vertically oriented beside the tongue (Fig. 1b, d). Simultaneously, at E16.5, the

RA-induced fetal mice had a smaller and narrower tongue than the normal group which were accordant with previously reports [21].

Biodiversity of the pregnant mice microbiome had no difference between RA and control groups

To detect the composition and structure of the microbial community in RA-treated pregnant mice and controls, we conducted the analyses of alpha and beta diversity of the microbiome in the pregnant mice faeces samples. Alpha diversity reflects the diversity of intestinal microflora in individuals and does not involve the comparison between individuals. Results on alpha diversity verified that compared with the control group, the indices of observed species, Shannon, Simpson, and Chao1 were no differences in the RA group (Fig. 2a-d). Beta diversity is adopted to illustrate phylogenetic differences in microbial communities between the diseased and controls. This method can present the bacterial difference between two groups based on the distance. PCA analysis failed to demonstrate the significant difference in distribution between the two groups, with the principal components of 91.5% and 5.65% (Fig. 2e). As for PCoA analysis, which is conducted using weighted UniFrac distances based on the OTU level. The result of PCoA revealed that there was a similar bacterial environment between controls and RA-treated pregnant mice (Fig. 2f). ANOSIM analysis also showed that there was no clear difference in composition and structure of bacteria between the controls and RA-treated pregnant mice with R statistic was − 0.04 and *p* value 0.52 ($p > 0.05$).

The expression of Lactobacillus was increased in RA group

Subsequently, the relative abundance of microbial taxa at the phylum, class, order, family genus, and species levels were confirmed. Among them, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* occupied the main position in both two groups at the phylum level. (Fig. 3a, b). At the family and genus levels, *Lactobacillales* and *Lactobacillaceae* were the most abundant in the RA group (Fig. 4a, b), other up-regulation bacteria were listed in Table S1 and Table S2. At the species level, the expression of *Lactobacillus intestinalis*, *Lactobacillus paragasseri*, *Lactobacillus sp. ASF360* and *Lactobacillus amylovorus* were increased in the RA group (Fig. 4c), other abundant bacteria were listed in Table S3.

To further explore the influence of bacteria on the control and RA group, the Linear discriminant analysis effect size (LEfSe) method was used, with loglinear discriminant analysis (LDA) > 2 labeled based on OTU level, which considered the statistical significance and biological correlation. This method revealed the influence of significantly different bacteria on the two groups. *Lactobacillales*, *Lactobacillaceae*, and *Lactobacillus* were enriched in the RA-treated group compared with the control (Fig. 4d).

Metabolism-related functions played a major role in the RA and control groups

Next, we investigated the total gene expression of faeces samples in two groups. Compared with the control group, the total gene expression level was changed dramatically under RA treated which affected metabolic pathway via gene expression regulation. Figure 5a displayed that in RA *versus (vs)* control

group, 5824 unigenes were up-regulated and 11467 were down-regulated in the differentially expressed genes.

The gene ontology (GO) analysis provided controlled vocabularies of defined terms representing gene product properties. Molecular functions and biological processes were clearly influenced by RA compared to the control groups (Fig. 5b). The results of kyoto encyclopedia of genes and genomes (KEGG) analysis (Fig. 5c) suggested that metabolism-related pathways took the lead, including global and overview maps, carbohydrate metabolism, and amino acid metabolism. So we focused on carbohydrate metabolism. Dysregulation of the microbiota caused alterations in carbohydrate-active enzymes (CAZymes), which interfere with carbohydrate metabolism [22]. The main role of CAZymes was to generate and break down complex carbohydrates and glycoconjugates, allowing them to exert a huge number of biological effects [23]. CAZymes are primarily studied through the CAZy database. Our experiments illustrated that the order of the proportion of the experimental group and the control group from high to low was glycoside hydrolase (GH) > glycosyltransferase (GT) > carbohydrate-binding module (CBM) > carbohydrate esterifying enzyme (CE) > polysaccharide lyase (PL) > auxiliary redox enzyme (AA) (Fig. 5d).

Metabolically related pathways were enriched in the RA and control groups

Since there was a study found that *Lactobacillus* could cause metabolic disorders [24], our results detected that *Lactobacillus* enriched in the RA-treated group compared with the control, and metabolism-related pathways were the most differentially expressed pathways between two groups by KEGG, we then analyzed the relevant pathways which manifested the changes in the digestive tracts in order to further investigate the relationship between microbiota and metabolism in both RA-induced and control pregnant mice. We integrated the function information related to each gene in GO and KEGG databases. In GO enrichment analysis, tricarboxylic acid (TCA) cycle, succinate dehydrogenase activity, porin activity, and anaerobic respiration enriched in both RA and control groups (Fig. 6a); In KEGG enrichment analysis, ribosome, metabolic pathways, lipopolysaccharide biosynthesis, and histidine metabolism enriched in both RA and control groups (Fig. 6b). Analysis of differential gene function (GO, CAZy, and KEGG pathway) and gene set enrichment analysis (GO and KEGG pathway) manifested significant changes in carbohydrate metabolism and energy metabolism in both the RA and control groups. The reason might be due to the number of *Lactobacillus* changes that could cause metabolic disorders, which might play a key role in the potential interaction effects between CP in fetal mice and gut microbiome in pregnant mice.

Discussion

At present, the effect of lipid metabolism on the formation of CP has been discovered. The unsaturated triglycerides increased in the RA-treated mice could play an important role in the formation of CP [25]. In the meantime, the gut microbiota was one of the causative factors affecting metabolic syndrome. It acted as a critical part in regulating dietary fat absorption and lipid metabolism by influencing bile acid

metabolism, producing short-chain fatty acids, and regulating the intestinal endocrine system [26]. Recent studies have proved that the maternal gut microbiome could promote healthy development by regulating metabolites entering the fetal brain [16], but the effect of maternal gut microbiome on the fetal cleft palate remains unclear.

In our research, since there was no significant difference in biodiversity between the two groups, we speculated that RA only affected the abundance of some species during cleft palate formation, but did not affect the appearance or disappearance of some species. Therefore, we shifted our focus on species abundance and function. Interestingly, we found that the expression of *Lactobacillus* was significantly increased in the RA group, including *Lactobacillus intestinalis*, *Lactobacillus sp. ASF360*, *Lactobacillus paragasseri*, and *Lactobacillus amylovorus*. As one of the most common probiotics, the antibacterial action of *Lactobacillus* reaps huge fruits. On one hand, *Lactobacillus* can colonize in the intestine and directly affect intestinal homeostasis. On the other hand, the antibacterial products of *Lactobacillus* reduce intestinal permeability by inhibiting pathogens [26]. *Lactobacillus* decreased the level of serum cholesterol and reduced inflammation and oxidant damage by regulating gut-derived metabolites [27]. It was previously found that gut microbiota also acted as a critical role in the reproductive system's development [28]. *Lactobacillus* made up 90–95% of the vaginal microbiota (VMB). *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii*, and *Lactobacillus gasseri* were the four most abundant species in VMB [29]. *Lactobacillus paragasseri* represents a novel sister taxon of *Lactobacillus gasseri*. Its gene encoding the oxalate catabolism could catalyze the transfer of Coenzyme A from formyl-CoA to oxalic acid, such as the formyl-CoA transferase encoded by the frc gene, indicating that it has potential probiotic properties [30, 31]. That is maybe the reason that the metabolically related pathways esp. CAZymes were enriched after RA treatment in our study. Moreover, *Lactobacillus intestinalis* reduced menopausal symptoms by modulating the gut microbiota, involved in increased fat mass, decreased bone mineral density, increased pain sensitivity, depression-like behavior, and cognitive impairment [32]. Crucially, these results inferred that we should pay attention to the role of *Lactobacillus intestinalis* as a probiotic drug in the treatment of menopausal symptoms.

The benefits of *Lactobacillus* to our health and the protection of disease have been fully verified, but an excessive increase in *Lactobacillus* can also be harmful, which could cause increased lactic acid. *Lactobacillus*-derived lactic acid as an essential metabolite triggering NADPH oxidase complex (NOX)-dependent reactive oxygen species (ROS) production and ISCs proliferation, which lead to premature aging [33]. 16S rRNA gene sequencing demonstrated that *Lactobacillus* significantly increased in osteoporosis and type 2 diabetes (T2D) patients compared with controls [34, 35].In addition, the mother suffered from lactic acidosis, while her child presented with skull abnormalities such as larger frontal sinuses and thicker frontal bones [36].

In our study, we found the expression of *Lactobacillus* was significantly increased in the faeces of RA-induced pregnant mice, and the results of stereo microscope and hematoxylin-eosin (H&E) staining also discovered CP in RA-induced fetal mice. Current research has manifested that RA is one of the crucial trace elements in embryonic development, which plays an essential role in the regulation of morphology,

cell proliferation and differentiation, and the production of extracellular matrix [37]. The proliferation of palatal mesenchymal cells was inhibited by RA at E10.5, resulting in cleft palate and no apoptosis of palatal epithelial cells [38]. In Alzheimer's disease, RA affects the intestinal flora by modulating immune cells, thereby affecting the function of neurons [39]. These results indicated that the formation of fetal cleft palate was associated with the excess increase of *Lactobacillus* in the intestinal flora of RA-treated pregnant mice. And whether *Lactobacillus* is one of the causes of CP or the feedback of the pregnant mice to RA to prevent the formation of cleft palate in fetal mice needs further investigation.

Up to now, most studies only observed the changes of gut microbiota and metabolic phenotype between pregnant women and fetus [17, 40], but the concrete mechanism of cleft palate between them is not clear. Metagenomics is an effective way to clarify the relationship between gut microbiome and pathogenesis. In our work, to further explore the mechanisms involved in CP formation between pregnant mice and fetuses, the GO and KEGG enrichment analysis implied that metabolic-related pathways were significantly enriched in gut microbiome of pregnant mice, including metabolic pathways, TCA cycle, anaerobic, and so on. An earlier study on chicken and mouse embryos confirmed that energy metabolism was tightly regulated during development [41]. Mouse early preimplantation embryos did not rely on glucose as their primary energy source, but participated in the TCA cycle and produce ATP using pyruvate and lactate [42]. An important metabolic shift occurs during embryo implantation, resulting in increased glucose uptake and enhanced glycolysis activity. At this point, most of the glycolysis activity co-exists with an active TCA cycle and oxidative phosphorylation, causing the production of lactic acid. However, with the formation of organs, the intense glycolysis activity of embryos declined, and respiration became the main way of energy generation [43, 44]. In addition, maternal gut microbiota was associated with offspring metabolic phenotype. During pregnancy, the SCFAGPR41 and SCFA-GPR43 axes could pass on the mother's gut microbiota to offspring to make them resistant to obesity. GPR41 and GPR43 in the sympathetic nerve, intestinal tract, and pancreas of the embryo could sense SCFAs in the maternal gut microbiota, thereby affecting prenatal development of the metabolic and neural system [13].

CAZy analysis demonstrated that the percentage of GH and GT family enzymes in the control group and experimental group were higher than other enzymes. GT and GH play important roles in the formation of glycosylation [23]. Many of the proteins produced by cells are attached to sugar molecules, and these additives were called glycosylation. The process of glycosylation facilitated the transport of proteins to the parts of the cell where the protein was needed [45]. In some genetic disorders, individuals had abnormalities in glycosylation caused by genetic mutations that could lead to a variety of symptoms, including epilepsy, cleft palate, and heart defects [46]. A report showed that in the process of mammalian organ formation, golgin subfamily B member 1 (Golgb1) as a large coiled-coil protein located at the cytoplasmic surface of the golgin apparatus, could involve in the early steps of regulating O-glycosylation [47]. Golgb1 mutant embryos caused cleft palate in mice, which was due to reduce hyaluronan accumulation and impair protein glycosylation in the palatal mesenchyme [48]. All of these results indicated that the formation of CP was related to metabolism, and maternal environment also affected the development of the fetal palate.

To sum up, our results suggested that changes in maternal gut microbiome could affect fetal palatal development, which might be related to changes in *Lactobacillus* and metabolic disorders. These results comparing the gut microbiome between normal and CP mice could lead to the discovery of new therapeutic approaches. Undeniably speaking, the limited samples may also be one of the reasons for the indifference of biodiversity which we will consider to enlarge the samples size in our subsequent experiments.

Conclusion

The present study showed that the changes of gut microbiome in pregnant mice may affect the palatal development of fetal mice, which is related to the expression of *Lactobacillus* and metabolic disorders. Our works implied a new direction for the pathogenesis of CP, providing a potential relationship between the gut-brain axis and CP.

Methods

Animals and sample collection

C57BL/6J mice were purchased from the Sibeifu Company (Beijing, China). Female C57BL/6J mice (age, 9–10 weeks; weight, 20–25 g) and mature male mice (age, 9–10 weeks; weight, 20-25g) mated at 9 pm. Female mice were detected for vaginal plugs after 10 h and designated as E0.5. Ten pregnant mice were equally divided into control group and RA group. Female mice at E10.5 were administered RA (100 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) dissolved in corn oil by oral gavage [21]. Control mice were given an equal amount of corn oil. RA-treated and untreated pregnant mice were sacrificed via cervical dislocation at E16.5. All mouse experiments were approved by the Animal Care and Use Committee of the School of Stomatology, Capital Medical University (Beijing, China, permit number: KQYY-20080312), and all experiments met the relevant regulatory standards.

All eligible faeces samples were sent to the laboratory immediately after self-sampling of faeces samples [49], each sample was divided into 3 parts, loaded into 3 cryopreservation tubes, and stored at − 80°C after overnight freezing of liquid nitrogen.

Stereomicroscope observing and H&E staining

Embryos from RA-treated mice and control mice were isolated at E16.5. Palate tissues of half of the embryos were detached by ophthalmic shears and then observed under stereomicroscope (Olympus, Japan). Other embryos were fixed in 4% paraformaldehyde at room temperature for 24 h. All fixed samples were dehydrated by the ethanol gradient, and after 4 h of preservation of n-butanol, they were embedded with paraffin wax and sliced at 5 μm intervals to make tissue sections. After dewaxing, the structure was observed by H&E staining.

Metagenomics sequencing

5 RA samples and 5 control samples were used to perform metagenomics analysis by LC-BIO TECHNOLOGIES (HANGZHOU) CO., LTD., Hang Zhou, Zhejiang Province, China. The specifically sequencing methods were described by Wang et al [27]. The lowest common ancestor taxonomy of unigenes was obtained by aligning them against the NCBI NR database by DIAMOND v 0.9.14. Similarly, the functional annotation (GO, KEGG, and CAZy) of unigenes were obtained. The differential analysis was carried out at each taxonomic or functional or gene-wise level by Kruskal-Wallis test.

Statistical analyses

The Wilcoxon rank-sum test was used to exam the result of alpha diversity (observed species, Shannon, Simpson, and Chao1) and LEfSe analysis. The beta diversity was performed by ANOSIM test. The Kruskal-Wallis test was used to bacteria abundance analysis. $p < 0.05$ were considered statistically significant.

Abbreviations

CP
Cleft palate
RA
retinoic acid
GI
gastrointestinal
E
embryo day
H&E
hematoxylin and eosin
LEfSe
Linear discriminant analysis effect size
LDA
loglinear discriminant analysis
GO
gene ontology
KEGG
kyoto encyclopedia of genes and genomes
CAZymes
carbohydrate-active enzymes
GH
glycoside hydrolase
GT
glycosyltransferase
CBM

carbohydrate-binding module
CE
carbohydrate esterifying enzyme
PL
polysaccharide lyase
AA
auxiliary redox enzyme
TCA
tricarboxylic acid
VMB
vaginal microbiota.

Declarations

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Author Contributions

Juan Du designed the experimental studies, Jing Chen, Xiaotong Wang, Xia Peng, Tianli Li, and Ying Liu conducted the research, and Yijia Wang wrote the manuscript. All authors contributed to interpretation of the results and manuscript drafting. All authors read and approved the final manuscript.

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Availability of data and materials

The clean data of Metagenomics are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive: http://www.ncbi.nlm.nih.gov/sra under accession number PRJNA811741. The URL is: https://dataview.ncbi.nlm.nih.gov/object/PRJNA811741?reviewer=1a5na3o6kglcjkfvjkipb9gfg8.

Ethics approval and consent to participate

All mouse experiments were approved by the Animal Care and Use Committee of the School of Stomatology, Capital Medical University (Beijing, China, permit number: KQYY-20080312). Precise details of the animal handling protocols and experimental procedures involving the animals are given in the ARRIVE Essential 10: Compliance Questionnaire, in the methods sections and Additional file 4.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Figures

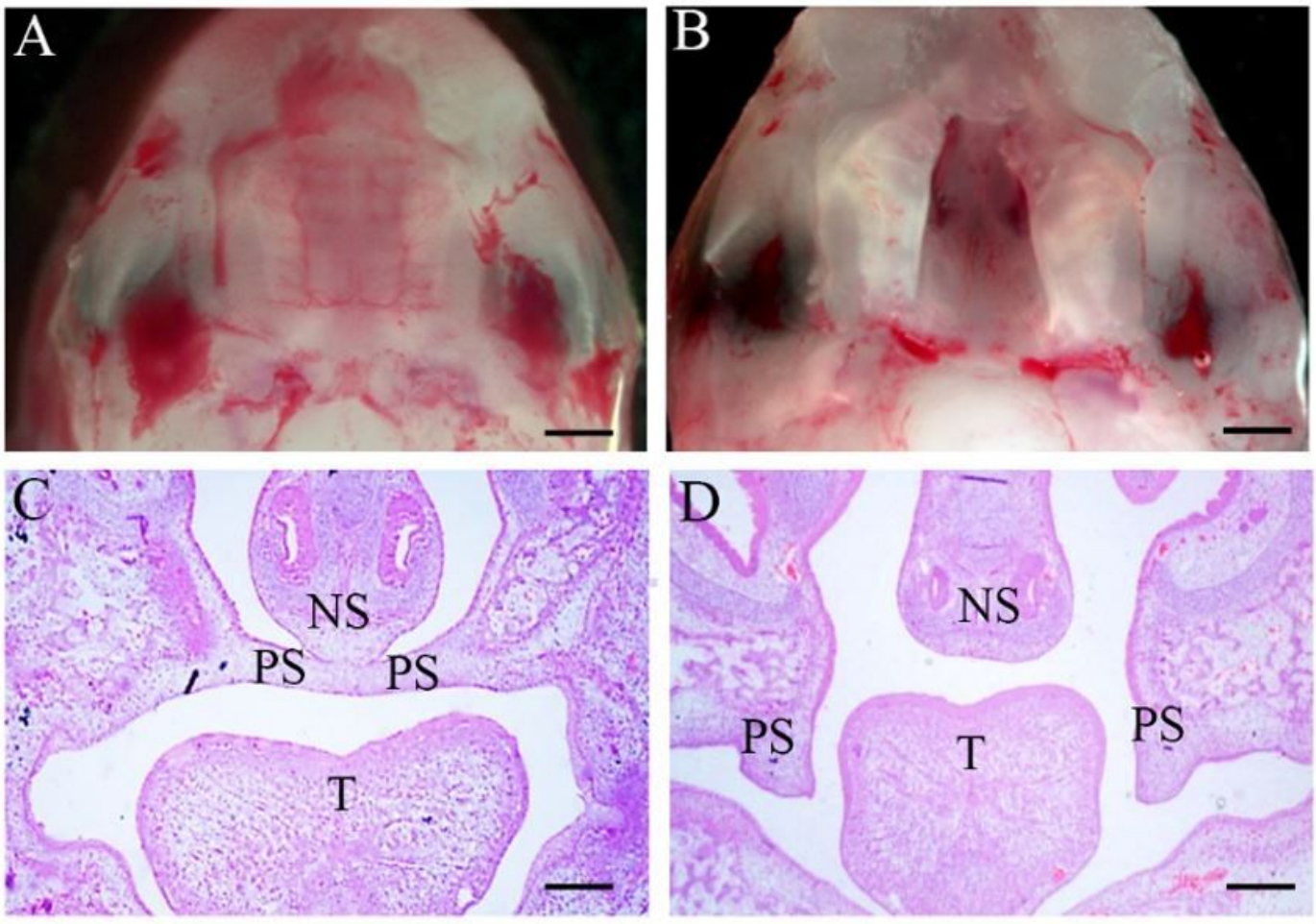

Figure 1

Morphology and H&E of palate shelf tissues at E16.5.(a, c) The palatal shelf contacted the midline and fused through the formation of the midline epithelial seam (MES) in the mid-anterior region of a control embryo. (b, d) Unfused, separated palatal shelf from an embryo of a RA-treated mouse. (a, b) Morphological specimens (×10); (c, d), H&E staining results (×4). PS, palatal shelf; T, tongue; NS, nasal septum; H&E, hematoxylin and eosin.

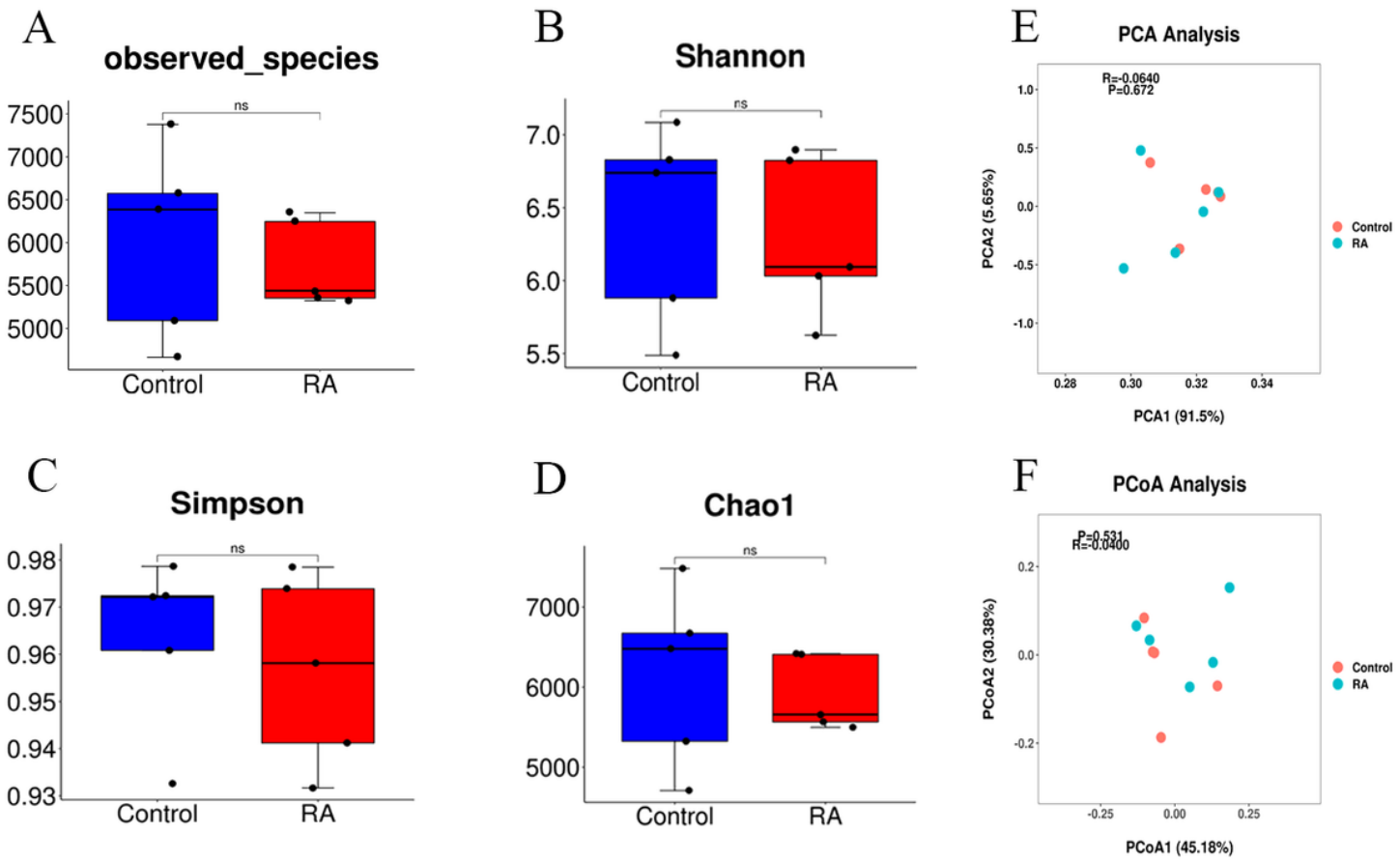

Figure 2

Gut microbiome diversity and structure analysis. (a-d) Alpha diversity differences between the RA and control groups were estimated by the observed species(a), Shannon(b), Simpson(c), and Chao1(d) indices. NS, not significant. (e, f) Beta diversity differences between the RA and control groups were estimated by the PCA (R=0.0640, P=0.672) (e) and PCoA (R=0.532, P=0.04) (f) of the microbiota.

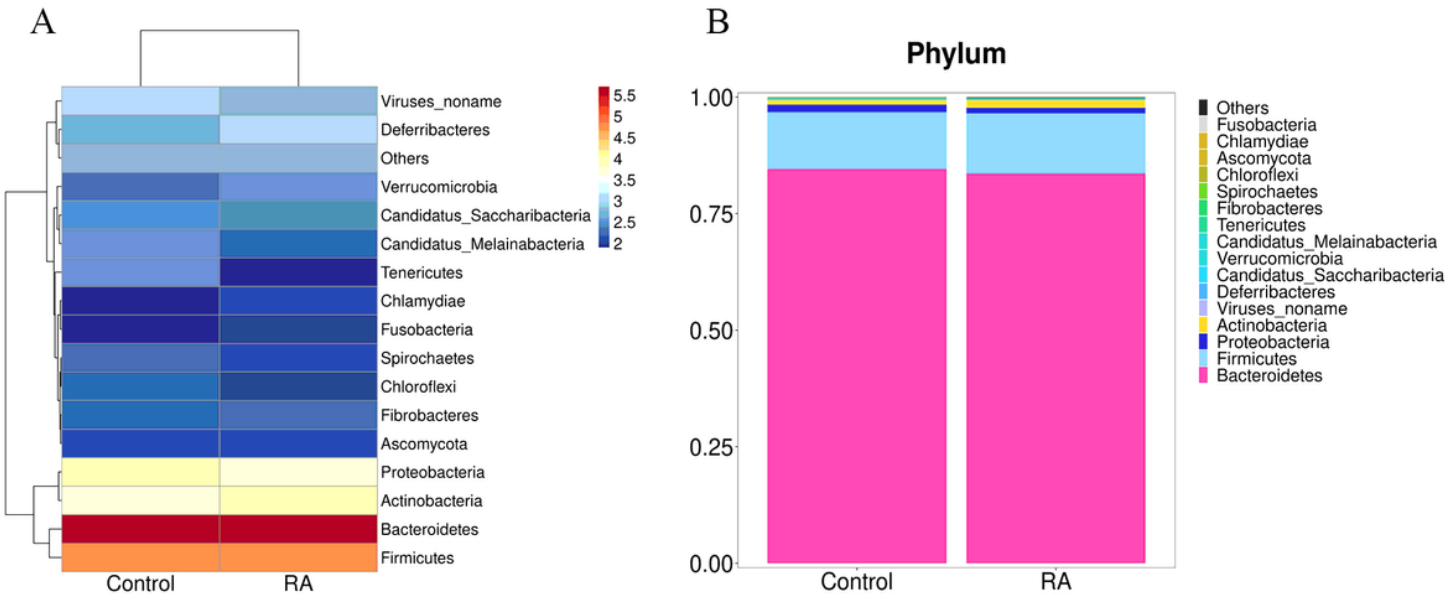

Figure 3

Gut microbiome structure analysis. Component proportions of bacterial phylum in the RA and control groups by heatmap(a) and stacked bar(b); n = 5 for the RA group and n = 5 for the control group.

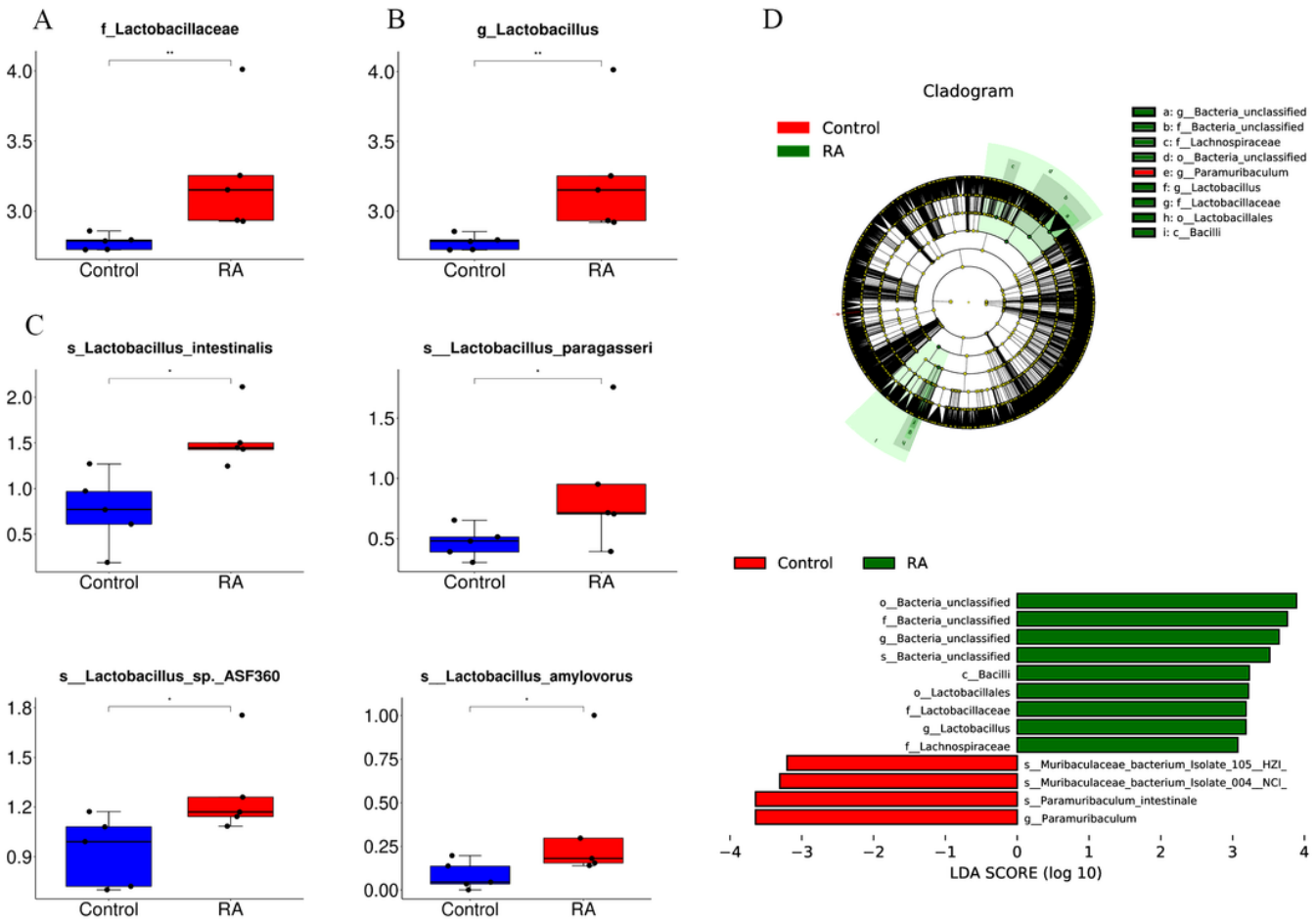

Figure 4

The relative abundance of microbial taxa at the family, genus, and species levels. (a, b) The relative abundance of *Lactobacillales* and *Lactobacillaceae* enriched in RA *vs* control at different levels. a, at family level; b, at genus level. (c) The relative abundance of *Lactobacillus intestinalis*, *Lactobacillus paragasseri*, *Lactobacillus sp. ASF360* and *Lactobacillus amylovorus* enriched in RA *vs*. control. The box represents the interquartile ranges, inner line denotes the median. (d) The differences in abundance between the RA and control group by LEfSe analysis. o, order; f, family; g, genus; s, specie. *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control group.

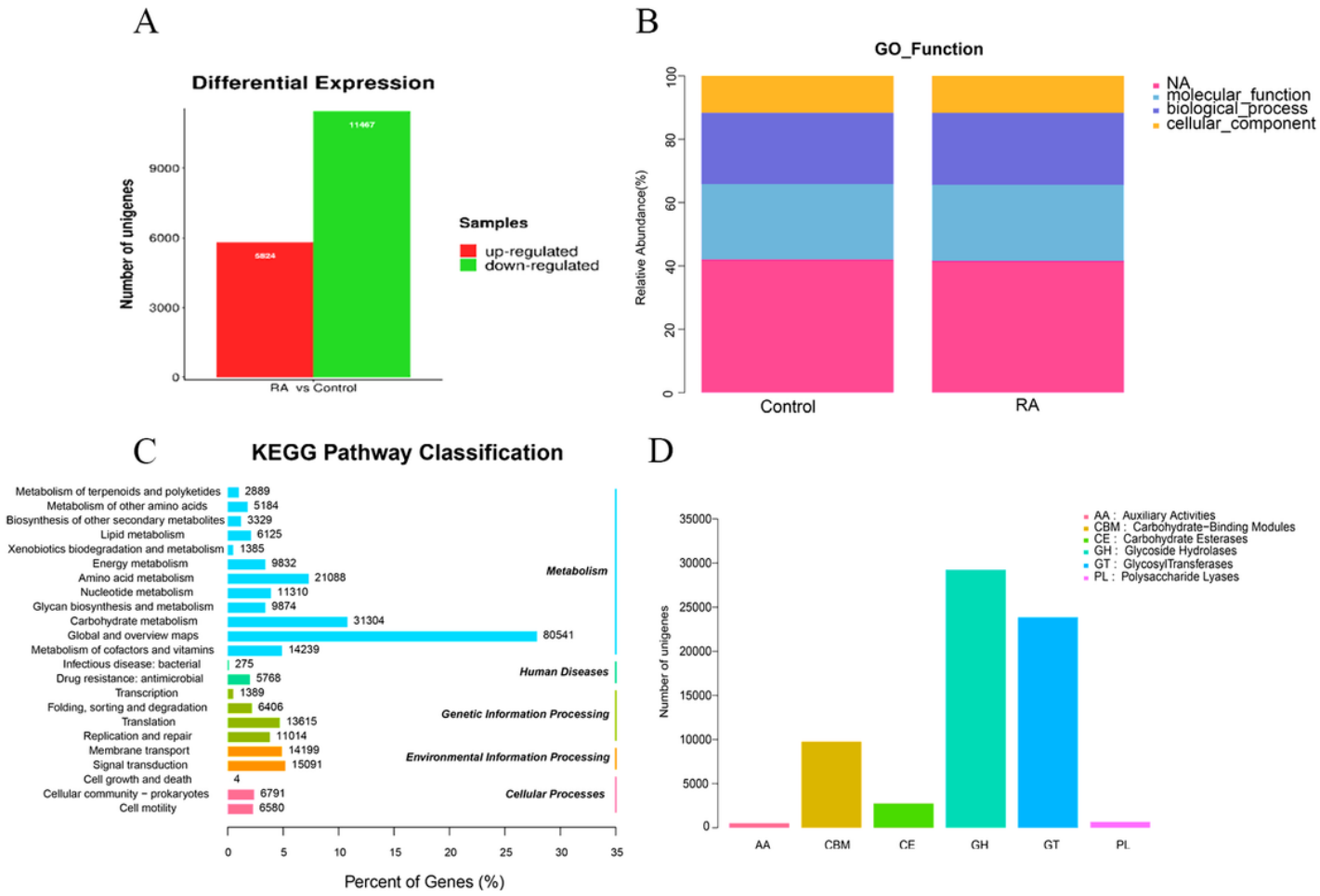

Figure 5

The differential gene expression and gene function in RA and control group. (a) Differential expressed unigenes between RA and control groups. (b) Relative abundance of GO function. (c) KEGG pathway classification. (d) CAZy category.

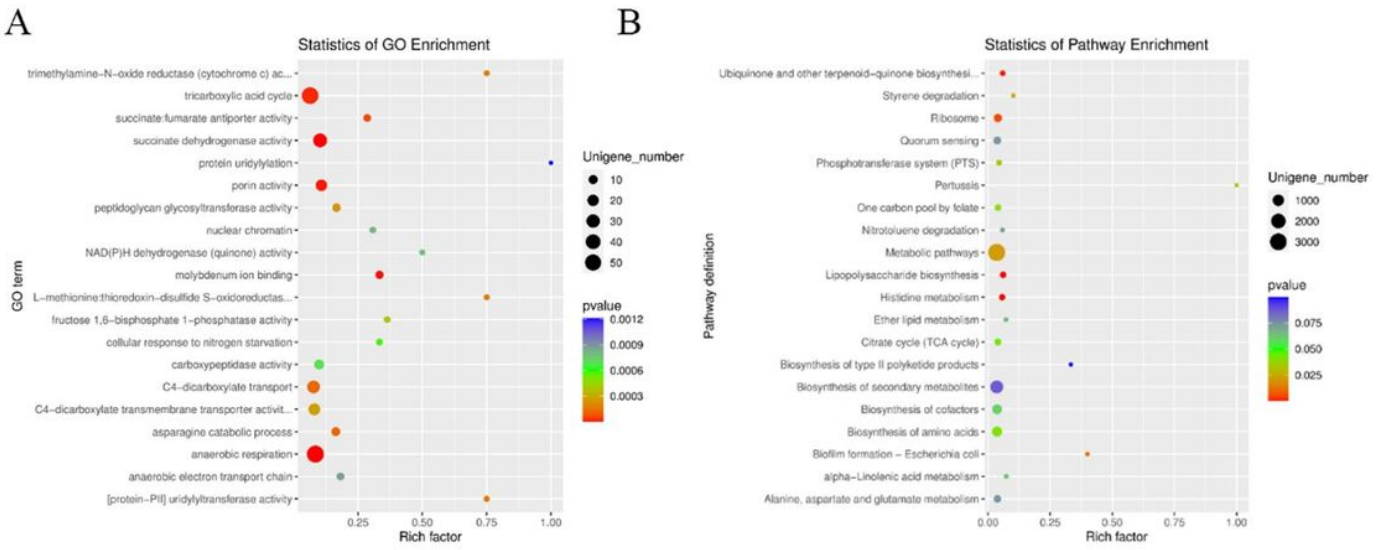

Figure 6

The enrichment analysis in RA and control group. (a) GO enrichment analysis of differentially expressed unigenes between RA and control groups. (b) Pathway classification based on KEGG enrichment analysis of differentially expressed unigenes between RA and control groups. Rich factor, the ratio of the number of differentially expressed genes (DEGs) to the number of total genes in this pathway.

Supplementary Files

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