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The Changes of Fecal Steroid Hormones and Bacterial Composition in Different Gestation Stages of Meishan Sows

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Abstract

Currently, the importance of gut microbiota to the health of their host has been well discussed, yet the knowledge about the host hormonal effect on the gut microbiota is limited. In this study, a combination of the high-throughput 16S rRNA gene-based pyrosequencing and the Mass Spectrometry (MS)-based metabolomics techniques were used to investigate the stool microbial composition and microbial metabolites at different gestation day in Meishan sows. The results showed that, the stool steroid hormones including estradiol (E_2), progesterone (P_4) and cortisol (CORT) were increased at gestation day 90 (P<0.05), when compared to gestation day 30. In coincide with the changes of steroid hormones, the relative abundance of Burkholderiales and Selenomonadales of the stool microbiota at d90 of pregnancy was significantly increased than that of at d30 of pregnancy at the order level (P<0.05). However, no significant difference of the richness estimators (ACE and Chao), and the diversity indices (Shannon and Simpson) were found between the d30 and d90 of gestation. Further, the metabolomics profile revealed 13 metabolites changed greatly from the gestation day 30 to 90, including amino acid metabolic pathway, lipid and carbohydrate metabolic pathway (P<0.001). The results suggest that the interaction of the steroid hormones and the gut bacteria may account for the metabolic changes throughout the length of pregnancy.

Keywords: Stool microbiota; Metabolite; Meishan sow; Pregnancy

Introduction

There is growing evidence that the gut microbiota and its bacterial genome play causal regulation in the host physiology, metabolism and immunity [1]. For instance, germ free wide mice transplanted fecal microbiota from ob/ob mice gained more fat than recipients with the lean donors gut microbiota [2]. Streptomycin treatment decreased the number of fecal bacteria and disrupted the intestinal homeostasis by affecting the intestinal metabolites, including bile acid, eicosanoid, and steroid hormone synthesis [3]. Thus, changing gut microbiota by means of fecal transplant comes to be the alternative method for healthy improvement. However, the interaction between the host and its gut-microbial is dynamic and highly susceptible to numerous factors, and the composition of intestinal microbiota are changing with the host's diet alteration [4], age [5], and even differing at different pregnant stage [6]. As we know, the pregnant animal is faced to numbers of variation, such as the development of fetus, changes of hormones, metabolism and immunity. Yet, despite the clear importance of the intestinal microbiome, our understanding on the causal changes or the relationship of gut microbiome, metabolism and immunity of host throughout the gestation is to date very limited.

Meishan pig, a Chinese indigenous breed and well known for its large litter size of 15-16 piglets, is traditionally raised on diet containing high level of crude fiber. Usually in the practical pig farm management, the diet composition of the first two pregnant trimesters of sows is keeping consistence, it is a good model for understanding the causal effects on gut flora diversity throughout the length of gestation by excluding dietary influence. Therefore, the purpose of this study was to investigate the periodically changes of the microbial composition and metabolites, steroid hormones in the feces of the pregnant Meishan sows during the first 90 days of gestation, and to reveal the interaction of host steroid hormones and the gut microbes.

Materials and methods

Animals and sampling

All animal care and experimental protocols were in accordance with the Guidelines of the Institutional Animal Care and Use Committee of the Institute of Animal Husbandry and Veterinary Sciences, Shanghai Academy of Agricultural Sciences, and were in compliance with standard international regulations.

A total of 6 pregnant Meishan sows (2^{nd} ~ 3^{th} parity) were used in the study and raised in the National conservation farm of Meishan pigs (Jiading, Shanghai). The animals were fed on a standard diet (2.38 kg/day) composed of corn, soybean, alfalfa meal and soybean oil (60.8% corn, 9.2% full-fat soybean, 20.4% alfalfa meal, 5.6% soybean oil and balanced with vitamin and mineral supplements). All sows were fed individually twice daily (7:00 and 14:00), ad libitum access to water, and normal epidemic prevention. The experiment was carried out from the previous end of suckle to the 90^{th} day of present pregnancy.

The fresh stools of sows were individually collected in the early morning on d30 and d90 of pregnancy, and immediately mixed over ice and separated into 3 parts. One part of faecal samples was stored at -20°C for P_4, E_2 and cortisol (CORT) assay. The second part (0.3 g of stool) was kept in tubes that contained 0.9 mL ethanol, gently mixed with ethanol

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and then stored at -20°C until DNA extraction [7]. The third part was immediately kept frozen at -80°C until metabolomics analysis.

Fecal steroid hormones measurement

The fecal samples were extracted following the protocol described previously [8]. In brief, samples were lyophilized for 72 h, pulverized, and 0.10 g of the fecal powder was extracted in 3 mL of 80% aqueous methanol by vortex for 15 min. Following extraction, we centrifuged the suspension, and recovered the supernatant for hormones measurement. The measurement of fecal P_4, E_2 and CORT content were performed using the commercial EIA kits (Cayman, P_4, Item No. 582601, Intra-assay CV<7.3%, Inter-assay CV<16.4%; E_2, Item No. 582251, Intra-assay CV<12.3%, Inter-assay CV<5.5%; Cortisol, Item No. 500360, Intra-assay CV <5.1%, Inter-assay CV<6.7%). All samples were run in duplicates, and all final hormone concentrations were expressed as micro gram or nano gram per gram fecal dry weight.

Pyrosequencing analysis of fecal microbiota diversity

The total Genomic DNA was extracted from the stool sample using the commercially available DNA isolation kit according to the manufacturer's instructions (ultra clean fecal DNA isolation kit, Solarbio Co., Ltd., CHN). The concentration of the extracted DNA was determined using a Nano-Drop 1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA). Amplification of fecal DNA was performed using a barcode-tagged primer set for pyrosequencing of the bacterial 16S rRNA gene. This primer set targeted the V4 and V5 hypervariable regions of the 16S rRNA genes using the 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') primer set. The PCR was performed using 4 μL 5×FastPfu Buffer, 2 μL dNTPs (2.5 mM), 0.4 μL FastPfu Polymerase, 0.8 μL of each primer (5 μM), and 10 ng of fecal DNA as the template. The following PCR cycles were used: initial denaturation at 95℃ for 5 min, 27 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 45 s, and a final extension at 72℃ for 10 min. The amplification products from each sample were evaluated by electrophoresis in 2% agarose gel and purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The products were quantified using QuantiFluor™-ST fluorescent quantitation system (Promega, Madison, WI, USA), and were then mixed in equivalent proportions. Sequencing was performed using Illumina Miseq PE250 according to the manufacturer's instructions.

A quality control of sequences was conducted and only high-quality sequences were used for subsequent analyses. The Operational Taxonomic Units (OTU) were clustered with a 97% similarity cut off was compiled with Qiime using default parameters. Taxonomic classification was performed based on the OTU database. The richness estimators (Abundance-Based Coverage Estimator (ACE) and the bias-corrected Chao), and the diversity indices (Shannon and Simpson diversity) were calculated using the MOTHUR program (http://www.mothur.org).

Sample preparation for LC-MS Analysis

Fecal metabolites were extracted with methanol using an extraction method that has been shown to effectively isolate a range of metabolites, including water-soluble and lipophilic compounds [9]. 800 μL of cold methanol and 10 μL of internal standard (2.9 mg/mL, DL-o-Chlorophenylalanine) were added to 50 mg of fecal sample. And all samples were grinded to fine powder using Grinding Mill at 65 Hz for 90 s. The samples after grinding were vortexed for 30 s, and centrifuged at 12000 rpm and 4°C for 15 min. 200 μL of supernatant was transferred to vial for LC-MS analysis by using an Acquity chromatograph (Waters, USA). Fecal metabolites were eluted using an Acquity UPLC HSS T3 column 2.1 × 100 mm, 1.8 μm (Waters, USA). Gradient elution was performed using water 0.1% formic acid as aqueous (A) mobile and acetonitrile 0.1% formic acid as organic mobile phase (B): 0 min, 5% B; 2 min, 5% B; 12 min, 95% B; 15 min, 95% B; 17 min, 5% B; 20 min, 5% B. Flow rate was set at 0.3 mL/min and the injection volume was 4 μL.

Metabolites contributing to the discrimination between d30 of pregnancy samples and d90 of pregnancy samples were identified through a multiple-step procedure. First, feature detection, peak alignment and retention time correction were performed using Masslynx4.1 package. The preprocessed data obtained by Masslynx in negative and positive ionization were separately exported to SIMCA-P13.0 software (Umetrics) to perform multivariate analysis. Principal component analysis (PCA) was used to check the quality of the data. Then, the supervised model partial least square discriminate analysis (PLS-DA) was used to analyze fecal metabolites differences between d30 of pregnancy and d90 of pregnancy. The quality of the model was evaluated using the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2). Metabolites that were discriminating between the d30 of pregnancy samples and d90 of pregnancy samples (VIP>1.0 and P<0.01) were identified comparing the exact mass with the METLIN Metabolite.

Statistical analysis

Data were analyzed by SPSS 17.0 and the effects of different pregnant stages on microbial metabolites in the stool of sows were tested for significance using Student's t-test. Significant differences were declared when P<0.05.

Results

The changes of steroid hormones

The steroid hormones showed a periodic change with the development of pregnancy. In the feces of Meishan sows at d90 of pregnancy, the concentrations of P_4, E_2 and CORT were all significantly up-regulated (P<0.05), when compared with d30 of pregnancy (Figure 1).

Prosequencing analysis of stool bacteria community

Pyrosequencing profiled a total of 1,557,991 valid reads that were assigned to 1,241 OTUs after screening them with strict criteria. As indicated in Table 1, no significant difference of the richness estimators (ACE and Chao), and the diversity indices (Shannon and Simpson) were found between d30 and d90 of gestation.

At the phylum level (Figure 2A), we observed that the majority proportion of sequences (>80%) were attributed to Firmicutes (51.00%) and Bacteroidetes (33.37%), and followed by Spirochaetes (8.15%), Tenericutes (3.13%) and Proteobacteria (1.70%). No significant changes of the fecal bacteria composition were found between gestation day 30 and day 90 at the phylum level and the class level. However, at the order level, we observed that the relative abundance of Burkholderiales and Selenomonadales were significantly increased at gestation day 90, as compared with the gestation day 30 (P=0.041, 0.023, Figure 2B).

Metabolomics profiles

The PLS-DA models indicated 2 principal components in the positive mode, R^2X=0.465, R^2Y=0.959, Q^2=0.739, and 2 principal

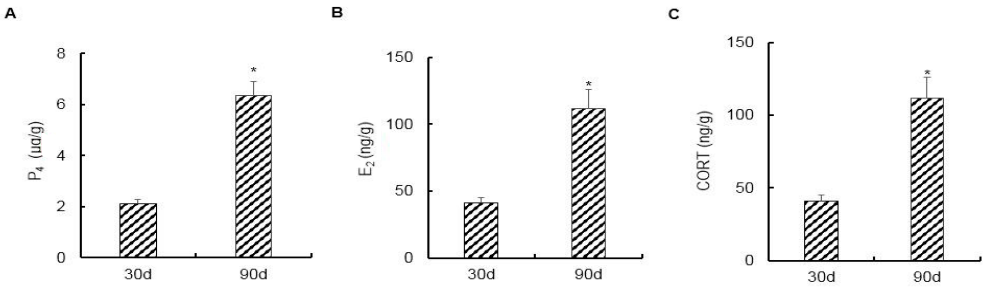

Figure 1: The changes of the fecal steroid hormones at gestation day 30 (d 30) and gestation day 90 (d 90). A) Progesterone (P_4); B) Estradiol (E_2); C) Cortisol (CORT). The data were represented as mean ± SEM (N=6). *P<0.05 means significant difference between gestation day 30 and 90.

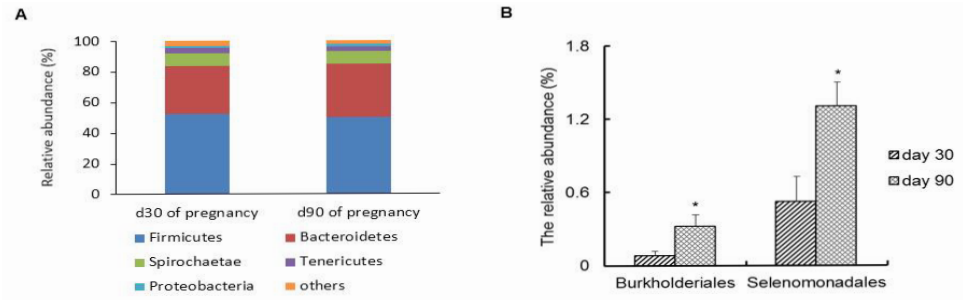

Figure 2: The relative abundance of fecal bacteria at the phyla level (A) and order level (B). The data were represented as mean ± SEM (N=6). *P<0.05 means significant difference between gestation day 30 and day 90.

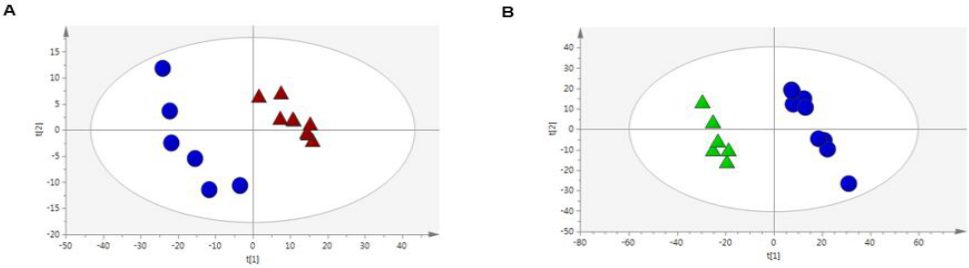

Figure 3: Partial least squares discriminant analysis (PLS-DA) of fecal metabolites. The PLS-DA models indicated 2 principal components in the positive mode (A), R^2X=0.465, R^2Y=0.959, Q^2=0.739, and 2 principal components in the negative mode (B), R^2X=0.279, R^2Y=0.996, Q^2=0.756. The resultant PLS-DA models showed a good discrimination between d30 of pregnancy and d90 of pregnancy.

components in the negative mode, R^2X=0.279, R^2Y=0.996, Q^2=0.756. The resultant PLS-DA models showed a good discrimination between the d30 of pregnancy and d90 of pregnancy (Figure 3). To identify which metabolites were responsible for this difference between the two gestation stage, the parameters of VIP>1, P<0.01, FC more than 10 or less than 0.1 were used as criteria. Accordingly, 11 metabolites in the stool were enriched (Table 2), while 3 metabolites (THF-L-glutamate, Phosphatidylcholine, and Diacylglycerol) were significantly increased (P<0.01) and the remaining (dodecanoic acid, triacylglycerol, LysoPE, LysoPC, TDP-4-oxo-6-deoxyglucose, GDP-4-oxo-6-deoxymannose, glucuronide and phylloquinone) were obviously decreased at the gestation day 90 (P<0.01), when compare with the gestation day 30.

Discussion

Present trail of fecal steroid hormones P_4, E_2 and CORT on gestation day 90 were obviously higher than that on gestation day 30. This is similar to the variation pattern of plasma steroid hormone in pregnant women that the level of P_4 and E_2 were significant increased through gestation week 12, 25 and 33 [10], and peak level was found in the third trimester [11,12]. And it also agrees with the tendency of saliva CORT level in gestation women, which was increased across the gestation period [13]. This confirms that the variation curve of fecal steroid hormones concentration can stand for the changes of steroid hormone secretion especially for periodic investigation by the advantage of non-invasive sampling [14].

Table 1: The index of stool bacterial diversity of Meishan pigs at different gestation day.

Items	d30 of pregnancy	d90 of pregnancy	*P*-value
OTUs	749.83 ± 39.94	810.67 ± 19.72	0.20
Ace	844.83 ± 40.28	922.50 ± 20.49	0.12
Chao	854.50 ± 40.23	938.83 ± 17.14	0.08
Coverage	1.00 ± 0.00	0.99 ± 0.00	0.24
Shannon	4.87 ± 0.13	5.09 ± 0.04	0.16
Simpson	0.02 ± 0.00	0.01 ± 0.00	0.16

The data were expressed as mean ± SEM, N=6

Table 2: Differential metabolites in stool at different pregnant stages.

Metabolite	VIP[a]	M/Z	FC[b]	*P*-Value
Amino acid				
THF-L-glutamate	1.30	575.2457	14.17	0.008
Lipid				
phosphatidylcholine	2.22	450.3005	22.75	<0.001
dodecanoic acid	1.81	199.1493	0.74	0.004
Diacylglycerol	1.31	511.4454	193.82	0.002
Triacylglycerol	1.35	1162.0779	0.01	0.005
LysoPE	1.29	552.2907	0.08	0.009
LysoPC	1.52	592.3288	0.00	<0.001
Carbohydrate				
TDP-4-oxo-6-deoxyglucose	1.30	547.135	0.10	0.008
GDP-4-oxo-6-deoxymannose	1.30	592.1588	0.08	0.008
glucuronide	1.36	477.3136	0.00	0.005

[a]VIP, variable importance in the projection; [b]FC, fold change, mean value of peak area obtained from gestation day 90/ mean value of peak area obtained from gestation day 30. FC value less than 1 mean that metabolites are less in d90 of gestation than in d30 of gestation. FC more than 10 or less than 0.1 was presented in this table.

In coincide with the high level of steroid hormones in later stage of pregnancy, the relative abundance of Burkholderiales and Selenomonadales at order level of Meishan pig's fecal bacterium had a significant increase. The Burkholderiale is an order of Proteobacteria Phylum, Gram-negative. However, the phylum Proteobacteria encompass multiple pathogens and are often associated with inflammatory conditions [15]. Koren et al. [6] observed that the abundance of Proteobacteria in stool were increased at the third trimester pregnancy, and companied with high levels of inflammation markers (cytokines IFNγ, IL2, IL6, and TNFα) when contrasted with the first trimester. Transferring the trimester pregnant stool microbiota into female Germ free wildtype Swiss-Webster mice induced metabolic syndrome, high level of blood insulin, glucose, and more adiposity gain [6]. Therefore, the increased ratio of order Burkholderiales in stool bacterium may indicate the health status of Meishan sows during later pregnant period. Further, the Selenomonadales order belongs to the Firmicutes Phylum. Research in mice observed that the abundance of Firmicutes is proportionate to the obesity levels, high efficiency of fatty acid absorption and fat deposits [16,17]. In later pregnancy, the mother develops a physiological insulin resistance that to store an adequate nutrient supply for fetus growth and lactation energy demand, therefore, excessive adiposity is usual for later pregnancy [18]. In addition, the high level of E_2 and CORT in later period of pregnancy also indicate the metabolic changes, E_2 has the activity to stimulate energy expenditure, CORT can stimulate food intake, reduce food passage rate [19]. Thus, high composition of Burkholderiales and Selenomonadales order in stool microbiota may relate to the metabolic variation in later pregnancy of Meishan sows.

Consistent with the changes of fecal bacterial composition, the stool metabolites were differed greatly between the different gestation age, including amino acid metabolic pathway (THF-L-glutamate), lipid metabolic pathway (phosphatidylcholine, dodecanoic acid, Diacylglycerol, Triacylglycerol, LysoPE, LysoPC), and carbohydrate metabolic pathway (TDP-4-oxo-6-deoxyglucose, GDP-4-oxo-6-deoxymannose, glucuronide and phylloquinone). All these further confirm that Meishan pigs have different metabolic characters at different pregnant stage, and suggest the diet should be formulated different to match the nutrient requirement for different stage. Usually in practical, pregnant sows at the later stage are given more feeds or feeds contain high concentration of protein and energy as considering the fetus development and preparing for farrowing and lactation.

Researches about the interaction of hormone and bacterium illustrated that, a single-dose of 5 or 10 mg/kg dexamethasone injection increased the number of ileal aerobes and lactobacilli bacteria in Wistar albino rats, while 0.1 mg/kg dexamethasone increased the coliform bacteria [20]. Researches indicated that CORT increased the proliferation of Salmonella in primary porcine alveolar macrophages [21]. Hosoda et al. [22] revealed that estradiol and progesterone inhibited the growth of Helicobacter pylori in vitro culture [22]. All these suggest that the steroid hormones may influence the replication of bacteria. However, whether the increasing of steroid hormones throughout the pregnancy account to the change of the fecal bacteria composition in Meishan sows need to be elucidate in the future. However, present sequencing profile did not find obvious difference in the richness estimators (ACE and Chao), and the diversity indices (Shannon and Simpson) of fecal bacterial community between the different gestation time in Meishan pigs, this did not agree with the studies in men and postmenopausal women, in which, the urinary estrogens concentration were changed strongly and directly associated with all measures of fecal microbiome diversity and composition [23]. Numbers of studies have proved that, the dietary composition can modify the gut microbial profiles [24]. Flores et al. [23] had excluded the dietary patterns effect by dietary restrictions (vegan or vegetarian, gluten, lactose, peanuts, pork or shellfish) [23], but it is difficult to make all volunteers refine their diet on one recipe throughout the investigation. Whereas, the research on pigs has the advantage of fixing feed composition through the experimental period.

Conclusion

In conclusions, present study observed that, consistent with the development of pregnancy the level of fecal steroid hormones and the proportion of Burkholderiales and Selenomonadales were increased, and the fecal metabolites changed. The interaction of steroid hormones and gut bacteria may account for the metabolic character changes throughout the length of pregnancy. However further studies are necessary to distinguish the individual action and combination action of steroid hormones on the gut bacterial community.

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