Weaning Stress Perturbs Gut Microbiome and Its Metabolic Profile in Piglets

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Weaned piglets are vulnerable to nutritional, physiological, and psychological stressors, leading to abrupt taxonomic and functional shifts in the intestinal microbiome. In this study, an integrated approach combination of 16S rDNA gene sequencing and the mass spectrometry-based metabolomics techniques was used to investigate the effects of weaning stress on intestinal microbial composition and its metabolic profiles of piglets. Three litters of suckling piglets with same parity were chosen. The samples of colonic contents were collected from each selected piglets (weaned day, 3 days after weaned) for microbial and metabolomics analysis. The results showed that Lachnospiraceae, Negativicutes, Selenomonadales, Campylobacterales and other 15 species increased after weaning, while Porphyromonadaceae, Alloprevotella, Barnesiella and Oscillibacter decreased. Based on the function profiles prediction and metabolomic analysis, five key metabolic pathways including Phenylalanine metabolism, Citrate cycle (TCA cycle), Glycolysis or Gluconeogenesis, Propanoate metabolism, Nicotinate and nicotinamide metabolism might be the relevant pathways involved in weaning stress-induced gut microbiota dysbiosis. Taken together, these results indicated that weaning stress not only changed microbial composition and function but altered the microbial metabolic profiles in the intestine, which might provide a new insight in alleviating weaning stress and facilitating disease prevention during the period of weaning in piglets.

Enormous numbers and diverse microbiota population inhabit the gastrointestinal tract in human beings and animals, being responsible for gut maturation, pathogens resistance and immune modulation. Shaped by co-evolution more than millions of years, a symbiotic relationship has formed between gut microbiota and host. Gut microbiota play a critical role in nutrient metabolism and in return occupy the nutrient-rich environment1. Increasing evidence showed that the dysbiosis of gut microbiota was intimately associated with many diseases, including inflammatory bowel disease, cardiovascular diseases, allergies, diabetes and obesity2,3. Thus, improvement in host health requires a better understanding of intestinal environment especially the gut microbiota. Gut microbiota remains relatively stable once established. However, many factors such as host genetics, environment, diet, immunological pressures and antibiotics can cause dramatic changes in the microbial community4. Owing to the similarities to human beings in anatomy and nutritional physiology, pigs often served as a biomedical model of human5,6. Investigation of gut microbiota in pigs not only benefits to construct a healthy gut of the animal but also provide important evidence for human.

The pig gut microbiota shows dynamic composition and diversity which shifts over time and along the whole gastrointestinal tract7. Colonization was started at birth and shaped by consumption of the sow’s milk, building a milk-oriented microbiome8. Therefore, the suckling period offers a special window of gut microbiota modifications. Weaning is usually conducted at around 3–4 weeks in the modern swine industry, and piglets are fed with solid diets instead of liquid milk. It is a sudden, complex and highly stressful event in pig’s life. Weaning piglets are usually vulnerable to nutritional, physiological, and psychological stressors, leading to alterations of intestinal morphology, physiological function, and a shift in intestinal microbiome (e.g., increased potential pathogens and diarrhea)9–11. And the disruption of gut microbiota is regarded as one of the major factors leading to post-weaning diarrhea12. Previous study showed the temporal change of gut architecture and function after

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weaned could be divided two periods: an acute change (about 5 days) occurs immediately after weaned and an adaptive and maturity phase. Thus 1–5 days after weaned are considered to be a period in which piglets suffered the most severe weaning stress.

Recently, the association among gut microbiota, metabolites and host physiology has gained increasing attention. Microbial metabolites are indispensable to maintain the majority of the biological effects of gut microbiota. The extraction, synthesis and absorption of metabolites could be mediated by intestinal commensal bacteria. Microbiota could exploit ingested dietary components, such as carbohydrates, proteins, and lipids to produce metabolites. These metabolites are mainly the short-chained fatty acids (SCFAs), as well as branched-chain fatty acids. Besides, there are some other metabolites produced by microbiota, including choline metabolites, bile acid metabolites, indole and phenolic derivatives. These metabolites have been appreciated for their beneficial effects on intestinal barrier, immune regulation and inflammation. Therefore, it is of particular significance to probe the metabolic changes associated with weaning-perturbed gut microbiota. The present work was designed to investigate the effects of weaning stress on gut microbiome and its metabolic profiles in piglets via an integrated approach combining 16S rDNA gene sequencing and GC-TOF/MS (Gas Chromatography Tandem Time-of-Flight Mass Spectrometry) method.

Results
Gut Microbiota Diversity and Structure of Nursing and Weaned Piglets. An average of 35595 high quality sequences per sample was obtained from 12 colonic content samples (Supplementary Table S1). The rarefaction Curves tended to be flat as shown in Fig. 1A,B, which demonstrated that almost all the bacterial species were detected in the colonic microbiota. Further, these sequences in the colonic contents of nursing and weaned piglets were assigned to 1,157 and 1,584 OTUs (operational taxonomic units) based on a 97% sequence similarity (Supplementary Table S2), respectively. Venn diagram reflected the number of OTUs that were common in groups as well as within the groups. This analysis showed there were 137 and 85 unique OTUs in weaned piglets and nursing piglets, respectively (Fig. 1C). The indexes of Chao1, observed species, PD-whole-tree, Shannon and Simpson were calculated to estimate alpha diversity. Shannon and Simpson indices, which reflect the species diversity, showed no significant difference in two groups. While, there were significant decrease in richness indices (Chao 1 and observed species) in weaned piglets compared to nursing piglets (Table 1). Unweighed Unifrac Anosim and unweighed Unifrac cluster tree based on UPGMA were used to analyze beta diversity (that is, diversity between individuals). Figure 1E showed a strong difference in the community composition of gut microbiota between two groups.

Four predominant phyla (Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria), which consisted of over 1% of total sequences on average, were found in colon as shown in Fig. 2A. There was no difference detected in dominant bacterial phyla between two groups (Supplementary Table S3). At family level, Prevotellaceae was predominant, followed by Ruminococcaceae, Lachnospiraceae, Porphyromonadaceae, and Bacteroidaceae (Fig. 2B and Supplementary Table S4). At genus level, Prevotella, Bacteroides, Lactobacillus and Alloprevotella were the predominant genera (Fig. 2C and Supplementary Table S5).

Differences in Microbiome between Nursing and Weaned Piglets. Figure 3 showed the microbiota with significant differences between nursing and weaned piglets. Lachnospiraceae, Negativicutes, Selenomonadaceales, Campylobacterales and other 15 species increased in weaned piglets, while Porphyromonadaceae, Alloprevotella, Barnesiella and Oscillibacter decreased.

Predicted Function of Gut Microbiota in Nursing and Weaned Piglets. PICRUSt analysis was performed to predict the potential functions of gut microbiota. In comparison two groups, nursing piglets had higher enrichment of the pathways in Lipid biosynthesis proteins, Energy metabolism, Transcription machinery, Nicotinate and nicotinamide metabolism, Carbon fixation pathways in prokaryote, Propanoate metabolism, naphthalene degradation, Streptomycin biosynthesis and peroxisome. While other pathways such as Photosynthesis proteins, Thiamine metabolism, Sulfur relay system were overrepresented in weaned piglets (Fig. 4).

Metabolic Differences of Gut Microbiota between Nursing and Weaned Piglets. Untargeted metabolomics analysis was performed to evaluate the metabolic differences of gut microbiota in two groups. An OPLS-DA method was performed to better understand the different metabolic patterns. Figure 5A showed significantly separated clusters between two groups. The values for $R^2$X and $Q^2$ and the results of permutation tests indicated that the samples were of reasonable quality (Fig. 5B). In total, relative levels of 433 biomarker metabolites differed significantly between nursing and weaned piglets. There was a clear distribution in metabolite between two groups (Supplementary Fig. 1). Figure 6 showed the variety of metabolic profiles in gut microbiome, with 239 increased and 194 decreased molecular features based on a VIP > 1 in 95% jack-knifed confidence intervals, respectively. And a total of 83 biomarker metabolites were filtered according to the standard with similarity > 600, VIP > 1 and p < 0.05. These metabolites, including lipids, amino acids, carbohydrates, benzene, organic acids, amines and others, involved in multiple biochemical processes in the colon (Table 2).

Key metabolic pathways analysis. The commercial databases including KEGG (http://www.genome.jp/kegg/) and MetaboAnalyst (http://www.metaboanalyst.ca/) was applied for search to the pathways of metabolites. The result showed that beta-Alanine metabolism, Arginine and proline metabolism, Alanine, aspartate and glutamate metabolism, Nitrogen metabolism and other pathways were altered (Table 3). Among them, five metabolic pathways including Phenylalanine metabolism, Citrate cycle (TCA cycle), Glycolysis or gluconeogenesis, Propanoate metabolism and Nicotinate and nicotinamide metabolism were also found in the functional prediction of gut microbiota as shown in Fig. 4.
Discussion

Weaning is a sudden, complex and highly stressful event in piglets’ life, accumulating evidence indicated that piglets have an abrupt taxonomic and functional shift in the intestinal microbiota after weaned\(^8,42\). However, the effects of weaning stress on microbial metabolites in piglets are still an unsolved issue. In this study, piglets at weaned day (nursing group) and 3 days after weaned (weaned group) were chose to investigate the effects of weaning stress on the composition of gut microbiota and its metabolites in the colon. The results showed that the diversity of gut microbiota in weaned piglets was similar with nursing piglets, while the richness decreased. These results were analogous with previous study, which found a continuously decreased alpha diversity until 11 days after weaned\(^40\). However, other studies presented opposite phenomenon. Those showed a continuous increase in
weaning. What's more, the results showed that several bacterial species had a remarkable difference between nursed and weaned piglets, suggesting that these bacterial species may not adapt to the intestinal tract environment after weaning. What's more, the results showed that several bacterial species had a remarkable difference between nursing and weaned piglets. And Candidatus Saccharibacteria, Planctomycetes, Verrucomicrobia and Lentisphaerae even cannot be detected in the gut of weaned piglets, suggesting that these bacterial species may not adapt to the intestinal tract environment after weaning. What's more, the results showed that several bacterial species had a remarkable difference between nursing and weaned piglets. For example, Alloprevotella and Oscillospira were decreased, while Campylobacterales, Campylobacteraceae and Campylobacter were increased in weaned piglets. Alloprevotella mainly produce succinate and acetate, which could improve the gut barrier and exhibit anti-inflammatory function. Oscillospira species are butyrate producers and can use host glycans as a source of energy. Study found that the presence of Oscillospira is reduced in diseases that involve inflammation. Campylobacterales was one of opportunistic pathogens, causing a life-threatening gastrointestinal disease. And Campylobacteraceae and Campylobacter were also increased after weaning. The increase of this bacterial species in weaned piglets may be one of the major reasons of post-weaning diarrhea. These results indicated that unhealthy alterations in the composition of gut microbiota triggered by weaning stress.

Gut microbiota participate in many functional metabolic pathways through which nutrient digestion and absorption, lipid metabolism, and hormone biosynthesis are influenced. To investigate how the functional capacity of the gut microbiota changed after weaning in piglets, Functional metagenomics prediction approach was used to analyze the KEGG pathways compositions in microbial populations. The results showed that many metabolic pathways such as lipid biosynthesis proteins, energy metabolism and phenylalanine metabolism were changed during the period of weaning. Gut microbiota could also directly alter its metabolic capacity through microbial products, affecting intestinal function. Butyrate, a kind of metabolite, produced by gut microbiome could regulate energy metabolism and autophagy in the mammalian colon. So GC-TOF/MS method was applied in the current study to quantitatively measure small molecular metabolites in biological samples and identify relevant Pathways. Based on PICRUSt and metabolomics analysis, we found that five metabolic pathways including Phenylalanine metabolism, Citrate cycle (TCA cycle), Glycolysis or Gluconeogenesis, Propanoate metabolism and Nicotinate and nicotinamide metabolism were the key relevant metabolic pathways involved in weaning stress-induced gut microbiota dysbiosis.

During a short period after weaning, small intestine length, microvilli height, crypt depth, and barrier function of piglets were changed. What's more, digestive enzyme activity and absorptive capacity of small intestine were reduced, resulting in a low absorption capacity of amino acids and carbohydrates. In the present study, decreased essential amino acids such as valine and some carbohydrate in weaned piglets supported this view. TCA cycle is responsible for the oxidative degradation of sugars, fats, and amino acids and could link different types of nutrients together. Pyruvic acid, as an important intermediate involved in the Citrate cycle (TCA cycle), was decreased in the weaned piglets. The reduction in energy metabolism may be part of the gut adaptation process. Microorganisms may engage energy for adaptation mechanisms to ensure necessary physiological functions.

Table 1. Richness and diversity of indices of colonic microbiota in nursing and weaned groups. The coverage percentage, richness estimators (Chao1, Observed species), and diversity indices (PD_whole_tree, Shannon and Simpson) were calculated using the QIIME program. PD_whole_tree: (phylogenetic distance) whole tree.
in Propanoate metabolism were more abundant in nursing piglets. And the bacteria *Oscillospira*, known to be as a butyrate producer, decreased in the gut microbiota of weaning piglets, which might indicate the decreased SCFA production after weaning. SCFAs are major anions in the gut and could be absorbed rapidly by colonic epithelial cells. Growing evidence proved that these small molecules could protect the host against colonic diseases, improve the gut barrier function and exhibit anti-inflammatory effects\(^33,34\). In addition, SCFA-mediated beneficial metabolic effects on health can be mediated by induction of intestinal gluconeogenesis\(^35\). A recent study showed that SCFAs could also improve the feed efficiency of swine\(^36\). Thus the reduction in Propanoate metabolism of weaned piglets in the current study might be related to a low growth performance and unhealthy gut environment after weaning.

Administration of niacin (nicotinic acid and nicotinamide) has been shown to beneficially effect the host-microbiome interaction in a mouse model\(^37\). Nicotinamide adenine dinucleotide (NAD\(^+\)) is the central cofactor of metabolism, mediating ATP generation, reactive oxygen species (ROS) detoxification, biosynthetic processes, DNA repair, and nutritionally sensitive gene regulation\(^38\). In the present study, decreased nicotinic acid was found in weaning piglets, which indicated an adverse influence on piglets triggered by stress.

**Figure 2.** Gut microbial community structure in nursing and weaned groups. (A) Microbial community bar plot by phylum. (B) Microbial community bar plot by family. (C) Microbial community bar plot by genus. Each bar represents the average relative abundance of each bacterial taxon within a group. The top 20 abundant taxa are shown.

**Figure 3.** Cladogram. Bacterial taxa significantly differentiated between Nursing and Weaned group identified by linear discriminant analysis coupled with effect size (LEfSe) using the default parameters.
Conclusion
In conclusion, our current work demonstrated that weaning stress not only disturbed the gut microbiota but also altered the metabolome profiles in the colon. After weaning, some benefit bacterial species such as *Alloprevotella* and *Oscillibacter* decreased in weaned piglets, while some opportunistic pathogens such as *Campylobacterales*, *Campylobacteraceae* and *Campylobacter* increased. In addition, based on PICRUSt and metabolomics analysis, five key metabolic pathways including Phenylalanine metabolism, Citrate cycle (TCA cycle), Glycolysis or Gluconeogenesis, Propanoate metabolism, Nicotinate and nicotinamide metabolism were identified as the relevant pathways associate with weaning stress-induced gut microbiota dysbiosis. These results may provide a new insight in alleviating weaning stress of piglets and facilitating disease prevention during weaning by manipulating host-microbiome metabolites interactions. And future study might focus on the potential functions of neurotransmitters in gut microbiome, neurogenesis, behavior and immune of piglets suffering weaning stress.

Materials and Methods

Animals and Sample Collection. All methods in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Zhejiang University, and animals used in this experiment were approved by the principles of the Zhejiang University Animal Care and Use Committee (NO. 2012-0178). Three litters of suckling piglets (21d, Duroc × Landrace × Yorkshire) with same parity were chosen from a commercial pig farm located in Ningbo.

Figure 4. Predicted microbial function in the colon lumen of nursing and weaned groups. The third level of KEGG pathway was showed in the extended error bar. Blue and red represent nursing group and weaned group, respectively.
city, Zhejiang province, China. Two barrows from each litter were randomly selected and humanely euthanized. The samples of colonic content were collected and transferred into sterile conical tubes. Then remaining piglets in the same trial were separated from sows and transferred into the same pen. These weaned piglets were fed a corn/soybean-based diet and had free access to water and feed. All nutrients reached or exceeded NRC (2012) recommendations for piglets. Three days after weaned, two barrows from each litter were selected and humanely euthanized. The samples of colonic content were collected into sterile conical tubes. All collected colonic samples were immediately frozen in liquid nitrogen and stored at −80°C.

**DNA extraction and 16S rDNA gene amplicon sequencing.** Total Microbial genomic DNA extraction was performed from colonic lumen using E.Z.N.A.® Stool DNA Kits (Omega Bio-tek, Norcross, US) according to the instructions of the manufacturer. The method of microbial community analysis was performed according to previous study39. Bacterial universal primers (341 F: ACTCCTACGGGAGGCAGCAG, 806 R: GGACTACHVGGGTWTCTAAT) were used for the amplification of the V3–V4 region of the bacterial 16S rDNA gene and subsequent pyrosequencing of the PCR products. Amplicons were confirmed by electrophoresis on a 2% agarose gel, purified with the AxyPrep DNA kit (AXYGEN, Tewksbury, MA, US), quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, US) to pool into even concentration. Amplicon libraries were sequenced on Illumina Miseq PE250 platform (Illumina, San Diego, US).

**Figure 5.** PCA 3D OPLS-DA score plots and corresponding validation plots of OPLS-DA derived from the GC-TOF/MS metabolite profiles in the colon lumen of nursing and weaned piglets. (A) OPLS-DA score plots showed significantly separated clusters in nursing and weaned group. (B) Permutation test of OPLS-DA model of nursing and weaned group. Blue and red represent nursing group and weaned group, respectively.

**Figure 6.** Volcano plot of nursing and weaned group. Each point represents a kind of metabolite, the scatter color represents the final screening result. Significant up-regulated metabolites are indicated in red, significantly down-regulated metabolites are indicated in blue, and non-significant differences in metabolites are grey.
| Metabolic         | Similarity | RT† (min) | MEAN - Weaned | MEAN - Nursing | VIP       | t-testa |
|-------------------|------------|-----------|---------------|----------------|-----------|---------|
| **Lipids**        |            |           |               |                |           |         |
| heptadecanoic acid | 828        | 20.337    | 5.57818E-11   | 0.000117       | 1.09225   | *       |
| Sphingosine       | 904        | 23.217    | 0.0003573     | 0.004645       | 1.50233   | ***     |
| phytosphingosine  | 872        | 24.471    | 0.000206      | 4.89435E-11    | 1.31316   | **       |
| alpha-Tocopherol  | 861        | 28.163    | 8.809993E-05  | 0.000391779    | 1.50347   | ***     |
| Cholesterol       | 899        | 28.352    | 0.002760734   | 4.89435E-11    | 1.31090   | **       |
| Cholestan-3beta-ol| 778        | 28.426    | 0.00120339    | 0.01103108     | 1.53157   | ***     |
| Coprostan-3-one   | 767        | 28.529    | 5.57818E-11   | 8.6931E-05     | 1.54168   | ***     |
| **Carbohydrates** |            |           |               |                |           |         |
| Lyxose            | 828        | 15.414    | 0.001186306   | 9.8097E-05     | 1.51917   | ***     |
| xylose            | 953        | 15.589    | 0.031706877   | 0.00242436     | 1.51325   | ***     |
| 6-deoxy-D-glucose | 807        | 16.220    | 0.000764127   | 0.00445482     | 1.34719   | **       |
| fructose          | 900        | 17.856    | 0.000335583   | 0.000993779    | 1.49148   | ***     |
| Sedoheptulose     | 628        | 18.461    | 0.000268555   | 4.89435E-11    | 1.09879   | *       |
| D-galacturonic acid| 864        | 18.579    | 0.000413546   | 4.89435E-11    | 1.31565   | **       |
| lactose           | 708        | 25.021    | 0.00031404    | 1.14153E-05    | 1.01325   | ***     |
| trehalose         | 839        | 25.328    | 0.000393928   | 4.89435E-11    | 1.54237   | ***     |
| maltose           | 775        | 25.296    | 0.000274583   | 0.000162243    | 1.03989   | *       |
| **Organic acids** |            |           |               |                |           |         |
| Pyruvic acid      | 921        | 7.430     | 6.32753E-05   | 0.000116271    | 1.25376   | **       |
| glycolic acid     | 930        | 7.829     | 0.007297916   | 0.000592752    | 1.45508   | **       |
| 3-Hydroxypropionic acid | 843  | 8.826     | 0.00325326    | 8.7607E-05     | 1.39673   | ***     |
| glutaric acid     | 708        | 12.458    | 0.001529942   | 0.000342019    | 1.28988   | **       |
| oxoproline        | 950        | 13.968    | 0.04241735    | 0.082504831    | 1.38890   | ***     |
| **Amino acids**   |            |           |               |                |           |         |
| valine            | 810        | 8.041     | 0.001631261   | 0.000197131    | 1.46254   | **       |
| Maleimide         | 927        | 8.098     | 0.00013127    | 9.90848E-05    | 1.04632   | **       |
| sarcosine         | 733        | 8.732     | 5.97859E-05   | 0.000118821    | 1.08313   | *       |
| serine            | 907        | 10.480    | 5.57818E-11   | 0.000131568    | 1.54214   | ***     |
| isoleucine        | 841        | 10.923    | 0.000249148   | 8.0456E-06     | 1.35077   | **       |
| proline           | 960        | 11.050    | 0.002575346   | 0.012998171    | 1.50201   | ***     |
| glycine           | 957        | 11.163    | 0.002625406   | 0.016532029    | 1.47505   | **       |
| beta-Alanine      | 945        | 12.773    | 0.001489759   | 9.94769E-05    | 1.43201   | **       |
| aspartic acid     | 956        | 13.927    | 0.00684775    | 0.015713239    | 1.25782   | ***     |
| 4-aminobutyric acid| 844        | 14.067    | 0.007020642   | 0.000668468    | 1.51846   | ***     |
| D-alanyl-D-alanine| 755        | 14.881    | 0.000863753   | 0.00017924     | 1.33635   | **       |
| asparagine        | 865        | 15.681    | 5.57818E-11   | 0.000313104    | 1.30777   | **       |
| alpha-Aminoadipic acid | 806  | 16.208    | 0.000124128   | 4.9774E-06     | 1.33593   | ***     |
| glutamine         | 692        | 16.834    | 5.57818E-11   | 0.00042769     | 1.30790   | **       |
| ornithine         | 940        | 17.323    | 0.001909938   | 0.00740552     | 1.48481   | **       |
| citrulline        | 806        | 17.389    | 0.000271653   | 0.000529457    | 1.19390   | *       |
| histidine         | 833        | 18.369    | 5.57818E-11   | 0.00052798     | 1.09632   | *       |
| tyrosine          | 933        | 18.574    | 0.004176509   | 0.014777744    | 1.50781   | ***     |
| **Benzene**       |            |           |               |                |           |         |
| Continued         |            |           |               |                |           |         |
Sequencing data analysis. The paired-end reads were obtained 2 × 250 bp using MiSeq platform. In order to obtain high quality sequences, tags with length of <220 nt, average quality score of <20, and tags containing >3 ambiguous bases were removed by PANDAseq. Data was de-chimeric and clustered using Usearch software, a standard clustering of 97% similarity was applied to obtain OTU (Operational Taxonomic Units). And the taxonomy-based analysis to the OTUs was performed by RDP algorithm using GreenGene database (http://greengenes.lbl.gov). QIIME was used for analyzing alpha diversity, which included calculation of the observed species, Chao 1, Shannon, and Simpson indices. And principal coordinate analysis (PCoA), Anosim Analysis, and unweighted pair group method with arithmetic mean (UPGMA) were performed using QIIME for beta-diversity analysis. Linear discriminant analysis coupled with effect size (LEfSe) was applied to identify the bacterial taxa differentially represented between groups at genus or higher taxonomy levels.

Metagenome prediction based on 16S rDNA gene data. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), which makes up for the shortage of 16S rDNA studies, was used for predicting the gene family abundances of bacterial populations based on the precalculated GreenGenes (v13.5) database. In brief, the analysis process is divided into two steps: gene content inference and metagenome inference. The software STAMP was conducted to detect the differentially abundant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in two groups with false discovery rate correction. The rank sum test was used to identify species with significant differences between groups. Two-sided Welch's t-test and Benjamini-Hochberg FDR correction were performed in two-group functional prediction analysis. P < 0.05 was considered statistically significant. And the prediction accuracy of PICRUSt was evaluated by the Nearest Sequenced Taxon Index (NSTI), with lower value indicating a higher accuracy of prediction.

Table 2. Identified metabolites for discriminating between nursing and weaned groups based on the untargeted metabolomics study. aRetention time. bStudent t-test of the relative levels of compounds detected in weaned and nursing piglets (n = 6). *p < 0.05, **p < 0.01 and ***p < 0.001, respectively.
Sample preparation for GC-TOF/MS analysis. Briefly, approximately 1.0 g colonic contents were mixed with 3 ml 4 °C H2O, followed by centrifugation at 3,200 × g for 15 minutes. 50 μl of the supernatant was taken and added with 200 μl of methanol, which contains 12.5 μg/ml of [13C2] myristic-acid. The mixture was maintained at 4°C for 1 h and subsequently centrifuged at 20,000 g for 10 minutes. Then the supernatant fraction was transferred to a glass vial and dried in a vacuum concentrator. And added methoxyamine salt reagent (methoxyamine hydrochloride, dissolved in pyridine 20 mg/mL), incubated in an oven at 80 °C for 30 min, then derivatized using N, O Bis (trimethylsilyl) trifluoroacetamide (BSTFA), incubated for 1 hour at 70 °C. The final mixture was strongly vortexed for 1 min and then subjected to detection.

### Table 3. Pathway analysis for nursing group and weaned group.

| Pathway                          | Total | Hits | Raw p | -ln(p) | Holm adjust | FDR | Impact |
|----------------------------------|-------|------|-------|--------|-------------|-----|--------|
| Beta-Alanine metabolism          | 17    | 6    | 0.0812| 6.7056 | 0.0992      | 0.0992| 0.6667 |
| Arginine and proline metabolism  | 44    | 9    | 0.0055| 5.2020 | 0.4404      | 0.1825| 0.3289 |
| Alanine, aspartate and glutamate metabolism | 23 | 6    | 0.0068| 4.9967 | 0.5340      | 0.1825| 0.4633 |
| Nitrogen metabolism              | 9     | 3    | 0.0277| 3.5847 | 1           | 0.5618| 0.0 |
| Valine, leucine and isoleucine biosynthesis | 11 | 3    | 0.0486| 3.0246 | 1           | 0.6558| 0.6667 |
| D-Glutamine and D-glutamate metabolism | 5  | 2    | 0.0520| 2.9559 | 1           | 0.6558| 0.0 |
| Aminoacyl-tRNA biosynthesis      | 64    | 9    | 0.0567| 2.8704 | 1           | 0.6558| 0.0 |
| Glycine, serine and threonine metabolism | 32 | 5    | 0.0991| 2.3117 | 1           | 0.9605| 0.3528 |
| Panthothenate and CoA biosynthesis | 15   | 3    | 0.1067| 2.2375 | 1           | 0.9605| 0.0408 |
| Phenylalanine metabolism         | 9     | 2    | 0.1526| 1.8796 | 1           | 1     | 0.2222 |
| Butanoate metabolism             | 20    | 3    | 0.2024| 1.5974 | 1           | 1     | 0.0290 |
| Ubiquinone and other terpenoid-quinoine biosynthesis | 3  | 1    | 0.2173| 1.5265 | 1           | 1     | 0.0 |
| Tyrosine metabolism              | 42    | 5    | 0.2280| 1.4783 | 1           | 1     | 0.0532 |
| Histidine metabolism             | 14    | 2    | 0.3012| 1.1999 | 1           | 1     | 0.2662 |
| Pyrimidine metabolism            | 37    | 4    | 0.3286| 1.1129 | 1           | 1     | 0.0634 |
| Pentose and glucorionate interconversions | 15 | 2    | 0.3311| 1.1052 | 1           | 1     | 0.0 |
| Glutathione metabolism           | 26    | 3    | 0.3336| 1.0978 | 1           | 1     | 0.0057 |
| Cyanosaminoc acid metabolism     | 6     | 1    | 0.3877| 0.9475 | 1           | 1     | 0.0 |
| Biosynthesis of unsaturated fatty acids | 42  | 4    | 0.4210| 0.8651 | 1           | 1     | 0.0 |
| Pentose phosphate pathway        | 19    | 2    | 0.4461| 0.8072 | 1           | 1     | 0.1209 |
| Propanoate metabolism            | 20    | 2    | 0.4732| 0.7483 | 1           | 1     | 0.0 |
| Citrate cycle (TCA cycle)        | 20    | 2    | 0.4732| 0.7483 | 1           | 1     | 0.1398 |
| Sphingolipid metabolism          | 21    | 2    | 0.4995| 0.6942 | 1           | 1     | 0.0526 |
| Methane metabolism               | 9     | 1    | 0.5213| 0.6515 | 1           | 1     | 0.0 |
| Vitamin B6 metabolism            | 9     | 1    | 0.5213| 0.6515 | 1           | 1     | 0.0 |
| Valine, leucine and isoleucine degradation | 38 | 3    | 0.5838| 0.5383 | 1           | 1     | 0.0 |
| Riboflavin metabolism            | 11    | 1    | 0.5938| 0.5212 | 1           | 1     | 0.0 |
| Steroid hormone biosynthesis     | 67    | 5    | 0.6158| 0.4849 | 1           | 1     | 0.0541 |
| Nicotinate and nicotinamide metabolism | 13 | 1    | 0.6555| 0.4223 | 1           | 1     | 0.0 |
| Primary bile acid biosynthesis   | 46    | 3    | 0.7143| 0.3365 | 1           | 1     | 0.0667 |
| Purine metabolism                | 68    | 4    | 0.7967| 0.2274 | 1           | 1     | 0.0269 |
| Lysine degradation               | 20    | 1    | 0.8068| 0.2147 | 1           | 1     | 0.0 |
| Pyruvate metabolism              | 22    | 1    | 0.8363| 0.1788 | 1           | 1     | 0.1875 |
| Starch and sucrose metabolism    | 23    | 1    | 0.8493| 0.1633 | 1           | 1     | 0.0 |
| Porphyrin and chlorophyll metabolism | 25 | 1    | 0.8724| 0.1365 | 1           | 1     | 0.0 |
| Galactose metabolism             | 26    | 1    | 0.8826| 0.1250 | 1           | 1     | 0.0161 |
| Glycolysis or Gluconeogenesis     | 26    | 1    | 0.8826| 0.1250 | 1           | 1     | 0.0989 |
| Cysteine and methionine metabolism | 28 | 1    | 0.9006| 0.1047 | 1           | 1     | 0.0210 |
| Glycero phospholipid metabolism  | 29    | 1    | 0.9085| 0.0960 | 1           | 1     | 0.0 |
| Steroid biosynthesis             | 35    | 1    | 0.9446| 0.0570 | 1           | 1     | 0.0539 |
| Amino sugar and nucleotide sugar metabolism | 37 | 1    | 0.9532| 0.0480 | 1           | 1     | 0.0 |
| Fatty acid metabolism            | 39    | 1    | 0.9604| 0.0404 | 1           | 1     | 0.0 |

Table 3. Pathway analysis for nursing group and weaned group. *The number of metabolites in the pathway. The number of differential metabolites that hit the pathway. The P value of Metabolic pathway enrichment analysis. The P value of Multiple Hypothesis Test Corrected by Holm-Bonferroni Method. The P values of Multiple Hypothesis Tests for Corrected via the false discovery rate (FDR) Method. The Impact value of metabolic pathway topology analysis.
GC-TOF/MS analysis. The derivatized samples were analyzed using an Agilent 7890 GC system equipped with a Pegasus 4D TOFMS (LECO, St. Joseph, MI) with a DB-5MS capillary column (30 m × 250 μm inner diameter, 0.25 μm film thickness coated with 5% dimethylpolysiloxane cross-linked with 5% diphenyl under the following conditions: initial temperature was kept at 80 °C for 0.2 min, increased to 180 °C at a rate of 10 °C/min, to 240 °C at a rate of 5 °C/min, and further to 290 °C at a rate of 20 °C/min; the column was then maintained for 11 min. One μL of sample solution was injected with helium as the carrier gas at a flow rate of 1 mL/minute. The temperatures of transfer line, and ion source were 245 °C, and 220 °C, respectively. The MS data were acquired with a mass-to-charge ratio (m/z) range of 20 – 600 in a full-scan mode.

GC-TOF/MS data acquisition and processing. The raw peaks extraction, data baselines filtering and calibration, peak alignment, deconvolution analysis, peak identification, and integration of the peak area were operated using the Chroma TOF4.3X software (LECO) and LECO-Fiehn Rtx5 database. The peak identification was tested using the R (retention time index) method. The missing values of the original data were filled by half of the minimum value via a numerical simulation method. Noise removal was performed based on an interquartile range to filter data, then, data were normalized by area normalization methods. The SIMCA14.1 software package (Umetrics, Umea, Sweden) was used for multivariate variable pattern recognition analysis: principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA). PCA was used to show the internal structure of the data and display the similarity and difference. OPLS-DA was applied to obtain a higher level of group separation and better explain the variables. To evaluate the predictive ability and fitting level of the model, the parameters R2Y and Q2 were employed using the Chroma TOF4.3X software (LECO) and LECO-Fiehn Rtx5 database. The peak identification, calibration, peak alignment, deconvolution analysis, peak identification, and integration of the peak area were operated using the Chroma TOF4.3X software (LECO) and LECO-Fiehn Rtx5 database. The peak identification was tested using the R (retention time index) method. The missing values of the original data were filled by half of the minimum value via a numerical simulation method. Noise removal was performed based on an interquartile range to filter data, then, data were normalized by area normalization methods. The SIMCA14.1 software package (Umetrics, Umea, Sweden) was used for multivariate variable pattern recognition analysis: principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA). PCA was used to show the internal structure of the data and display the similarity and difference. OPLS-DA was applied to obtain a higher level of group separation and better explain the variables. To evaluate the predictive ability and fitting level of the model, the parameters R2Y and Q2 were applied. The metabolites responsible for differentiating two groups were filtered with the following requirements: variable importance in the projection (VIP) > 1 and P-values of 0.05 (threshold) with 95% Hotelling’s T-squared ellipse.

Data was analyzed by SPSS 20.0 using Student’s t-test to profile metabolite differences between two groups. The commercial databases including KEGG (http://www.genome.jp/kegg/) and MetaboAnalyst (http://www.metaboanalyst.ca/) was utilized to search for the pathways of metabolites. We can further screen pathways and find the key pathways with the highest correlation with metabolites through comprehensive analysis of the pathways where the differential metabolites are located (including enrichment analysis and topological analysis).

Data Availability
All data generated or analysed during this study are included in this article and its Supplementary Information files.

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Author Contributions
X.H. conceived the experiment(s), Y.L., S.C. and S.G. conducted the experiment(s), Y.L., X.J. and X.M. analysed the results. All authors reviewed the manuscript.

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