

Selective maternal seeding and rearing environment from birth to weaning shape the developing piglet gut microbiome

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Abstract

Background: The acquisition and development of the mammalian microbiome early in life are critical to establish a healthy host-microbiome symbiosis. The maternal and environmental microbial reservoirs are considered the main sources of microbial communities in newborn mammals. However, the timely relative contribution of various microbial sources to the colonization of the gut microbiota in newborn piglets remains unclear. The aim of this study was to investigate the influence of the sow and delivery environment microbes on nursing piglets from birth through weaning.

Results: We longitudinally sampled the microbiota of 20 sow-piglet pairs (three piglets per sow) from multiple body sites and the surrounding weaning environment from birth to 28 days postpartum (1,119 samples in total) reared under identical conditions. Source-tracking analysis revealed that the contribution of various microbial sources to the piglet gut microbiome gradually changed over time. The neonatal microbiota was initially sparsely populated and predominantly comprised taxa from the maternal vaginal microbiota that increased gradually from 69.0% at day 0 to 89.3% at day 3 and dropped to 0.28% at day 28. As the piglets aged, the major microbiota community patterns were most strongly associated with the sow feces and slatted floor, with contributions increasing from 0.52% and 9.6% at day 0 to 62.1% and 33.8% at day 28, respectively. The intestinal microbial diversity, composition and function significantly changed as the piglets aged, and 30 age-discriminatory bacterial taxa were identified with distinctive time-dependent shifts in their relative abundance, which likely reflected the effect of the maternal and environmental microbial sources on the selection and adaptation of the piglet gut microbiota.

Conclusions: Vaginal microbiota is the primary source of the gut microbiota in piglets within three days after birth and are gradually replaced by the sow fecal and slatted floor microbiota over time. These findings may offer novel strategies to promote the establishment of exogenous symbiotic microbes to improve piglet gut health.

Introduction

The mammalian gastrointestinal tract is a diverse and complex ecosystem of microbes that play fundamental roles in metabolic, developmental and physiological processes affecting host health [1–3]. It is now widely recognized that the first 2–3 years of life is a critical window for the acquisition and colonization of the host gut microbiome, and gut microbial colonization during infancy plays an instrumental role in the development of the host mammalian immune system [4–6]. In addition, initial microbial exposure is important in defining successional trajectories, leading to a more complex and stable adult microbiome [7, 8]. Aberrant neonatal gut microbiota has been reported to be linked to many diseases during childhood and later in life, including inflammatory bowel diseases (IBDs) [9, 10] and Crohn's disease (CD) [11]. While the importance of gut microbiota colonization early in life to host health is not in question, the main sources of infant gut microbiota and the relative contribution of various microbial sources remain largely unexplored.

The ecological succession of the gut microbiota early in life is the result of a dramatic and complex transition from a sterile state to extremely dense colonization ending with the establishment of a functional mature microbial community [12, 13]. The first major exposure of the neonate to microbes occurs during the birthing process and is highly dependent on delivery mode. The guts of vaginally delivered neonates are enriched with *Lactobacillus* and *Prevotella*, which resemble the maternal vaginal microbiota [14–16]. In contrast, the gut of cesarean-section-born infants are instead colonized by microbes from the maternal skin or the hospital environment, such as *Staphylococci*, *Streptococci* or *Propionibacteria* [17, 18]. In addition, *Bifidobacteria* communities are present in both mother and infant feces and in maternal milk, indicative of a vertical transmission route from the maternal gastrointestinal tract [19, 20]. Thus, intimate contact with the mother, breastfeeding and general environmental exposure play critical roles in the early gut microbial acquisition in the infant.

The pig is both a major source of meat for human consumption and a valuable biomedical model. It is evident that the gut microbiota plays fundamentally important roles in pig health, providing pigs with many functions, including improved energy harvesting capacity, the production of volatile fatty acids, the production of vitamin K, cellulose fermentation, and enhanced resistance against pathogenic bacteria [21, 22]. The acquisition and establishment of the gut microbiota early in life is crucial for piglets, since early gut colonizers are pivotal in the establishment of permanent microbial community structures affecting the health and growth performance of pigs later in life [23]. However, despite the emerging understanding of the gut microbiota of pigs, few studies characterize the development of the early gut microbiota of piglets, and no current studies have systematically characterized the main sources of the piglet gut microbiota early in life.

To this end, we conducted a large-scale study to investigate the multiple maternal and environmental sources of microbial transmission and how these sources contribute to the acquisition of the nursing piglet gut microbiome from birth through weaning up to 4 weeks of age. Additionally, we assessed the dynamic changes in the composition and potential metabolic function of the piglet gut microbiota longitudinally. In this study, we collected a total of 1,119 samples from sows (vagina, feces, milk, and breast skin), the environment of the nursing house (air, water, and slatted floor), and piglet feces. 16S rRNA gene sequencing was performed on collected samples to assess the relative contribution of various bacterial sources to the piglet gut microbiota. Overall, this study provides insight into the origin of gut bacteria and new information about the development of the gut microbiota in piglets.

Methods

Animal experiments

Samples were collected from April to May 2017. Throughout the study, all animals were housed under similar conditions on a commercial farm in Guangdong Province, China. The pens in the unit were furnished with a polypropylene plastic slatted floor with 3.91 m² space per sow. Thirty healthy Large-White/Landrace pregnant multiparous sows with similar expected delivery dates were selected and

intramuscularly injected with cloprostenol (0.2 mg per sow) on day 113 of gestation to ensure synchronous delivery. Candidate sows that differed in delivery time by more than 3 h were excluded. In total, 20 sows with litters of 10 or 11 piglets were used in this study. Upon delivery, 60 infant pigs (three piglets per litter per sow) were cohoused with sows by litter and ear notched for individual identification, following standard husbandry practices for swine. Sows were given *ad libitum* access to feed at 8:30 am and 2:30 pm and received water freely throughout the day, while sucking piglets had free access to water after 7 days. The infant piglets were allowed to nurse freely until weaning at 21 days of age without creep feed and did not consume sow feed. On weaning day, sows were removed from the piglets, and the piglets remained in the nursing pens for one week until the end of the experiment at day 28 to avoid the stress caused by environmental changes. None of the studied sows or piglets required antibiotics during the sampling period.

Sample Collection

Fecal samples were collected rectally from each piglet using a sterile cotton swab (Hua Chen Yang, Shen Zhen, China) premoistened with sterile phosphate-buffered saline (PBS), and then the swab head was placed in a 5 mL sterile screw top collection tube (Corning, NY, USA). Sow feces were collected on days 3, 5, 7, 14 and 21 because they did not defecate on days 0 and 1 after delivery. Vaginal swabs were collected on days - 1 and - 2 (prepartum) and day 0 (before delivery). The vulva was cleaned with water and wiped with 75% ethyl alcohol to avoid dragging bacteria into the vagina. The vaginal introitus was swabbed in a circular motion 5 times, and then the swab head was placed in a 5 mL sterile screw top collection tube. Bacterial samples from the skin surface were collected by swabbing the anterior, middle and posterior parts of the sow's breast skin surface for approximately 30 s in a back-and-forth motion with swabs premoistened with sterile PBS, and then the swab head was placed in a 5 mL sterile screw top collection tube. After the breast skin swabs were collected, the nipple and surrounding area of the sow were cleaned with soap and sterile water and then cotton soaked with 75% ethyl alcohol to minimize contamination with skin bacteria. Milk samples (approximately 15 mL) were collected manually in a sterile tube after the first few drops (approximately 1 mL) were discarded.

Five water samples (approximately 1 L each) were collected from the water trough and placed into sterile containers at each sampling time. Indoor air samples were collected using an SKC Bio Sampler (SKC Inc., PA, USA), which was placed ~ 50 cm above the floor. Three air sampling replicates were collected at 10:00, 14:00 and 18:00 by drawing air through the impingers filled with sterile molecular-grade water for 1 h at a rate of 13 L per min. For each replicate, the air sample was collected five times simultaneously at five points indoors, and the resulting samples (10 mL each) were pooled (50 mL total volume). Slatted floor surface samples of each pen were collected from five sites. During this procedure, a swab premoistened with sterile PBS was rubbed back and forth several times at each sampling site, and then the swab head was placed in a 5 mL sterile screw top collection tube. Samples were immediately placed in liquid nitrogen after collection, transferred to the laboratory within 24 h and stored at -80 °C until DNA extraction. A total of 482 piglet fecal samples, 99 sow fecal samples, 140 milk samples, 56 vaginal swabs and 136 breast skin swabs from sows, 30 water samples, 27 indoor airborne samples and 168

slatted floor samples were collected at different sampling time points at days 0, 1, 3, 5, 7, 14, 21, 23 and 28 (Fig. 1a, Additional file 1: Table S1).

DNA Extraction, Library Preparation, And Sequencing

DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) or PowerWater DNA Isolation kit (MoBio, Hilden, Germany). For samples collected via swabbing (feces, skin, slatted floor, and vagina), the swab head was placed in a bead tube containing 0.7 mL of sterile PBS. The tube was centrifuged at $15,000 \times g$ for 10 min, and the pellets were suspended in 1.4 mL of ASL buffer from the DNA isolation kit. Zirconium glass beads (400 mg; diameter, 0.1 mm) (BioSpec, Bartlesville, OK, USA) were added to the suspension that was shaken twice vigorously using a FastPrep-24 Instrument (MP Bio, Santa Ana, CA, USA) at a speed of 6.0 m/s for 2×45 s. The mixture was then incubated at 95°C for 5 min to maximize bacterial DNA extraction. All remaining steps followed the manufacturer's protocol of the DNA stool mini kit. For the milk samples, a total of 2 mL of milk sample was thawed on ice and centrifuged at $15,000 \times g$ for 10 min to separate the fat and cells from the whey. Then, DNA was extracted from pellets as described above.

For DNA extraction with a PowerWater DNA isolation kit, the water samples and impinger liquid from air samples were vacuum filtered onto sterile $0.2 \mu\text{m}$ polycarbonate filters (Sigma, St. Louis, MO, USA) and transferred to 0.7 mm garnet bead tubes containing 1 mL PW1 solution. The mixture was shaken vigorously using a FastPrep-24 Instrument, and the remaining steps followed the manufacturer's protocol.

Extracted DNA was used as a template for PCR using barcoded primers to amplify the V4 region of the 16S rRNA gene. The V4 region of the 16S rRNA gene was amplified using universal primers, where PCRs were carried out in triplicate using $10 \mu\text{M}$ primer 515F and 806R, $1 \times$ GoTaq Green Master Mix (Promega), 1 mM MgCl_2 , and $3 \mu\text{L}$ of DNA template or nuclease-free water as a negative control. The amplification conditions included an initial denaturation of 3 min at 94°C , followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension for 10 min at 72°C . PCR products were pooled at equimolar concentrations and purified using a QIAquick PCR purification kit, and 250-bp read sequencing was performed on the Illumina HiSeq platform. Raw sequencing data in this study have been deposited in the European Nucleotide Archive database with the accession number PRJEB 28241.

Metagenomic And Statistical Analysis

The raw reads of 16S rRNA gene sequencing were demultiplexed and quality-filtered using the Quantitative Insights Into Microbial Ecology (QIIME) program (v 1.9.1). Reads were trimmed and removed based on quality scores < 25 and lengths > 225 bp, respectively [24]. Chimeras and error sequences in the optimized data were removed using QIIME software (v1.9.1) by clustering the data into operational taxonomic units (OTUs) for species classification with 97% similarity [25]. Taxonomy was assigned using a metagenomics workflow based on an Illumina-curated version of the Greengenes database.

The relative abundances of taxa were compared using Metastats analyses, and Venn diagrams were generated using Venny [26]. The Chao1 and Shannon indices were calculated in the R (v3.0.3) “fossil” and “vegan” packages, respectively. The alpha diversity was assessed using one-way analysis of variance (ANOVA), and the sequencing depth was evaluated using rarefaction curves with metagenomics rapid annotation using subsystems technology (RAST). To represent the distance between samples, the nonmetric multidimensional scaling (NMDS) based on Bray – Curtis dissimilarity was calculated using PAST3, in which nonparametric multivariate analysis of variance (PERMANOVA) was used to test differences among groups. A similarity percentage (SIMPER) analysis was conducted in PAST3 to measure the differences in bacterial communities between groups and to identify which taxa were primarily responsible for the differences. An unweighted pair group method with arithmetic averages (UPGMA) clustering analysis was performed at the genus level using PAST3 to compare microbiota compositions [27]. Cooccurrence patterns of genera were constructed in the network interface by Spearman’s rank correlations based on bacterial abundance. A valid cooccurrence event was based on strong (Spearman’s $r_s < -0.7$ or $r_s > 0.7$) and significant ($P < 0.01$) correlations between genera. PICRUSt was used to predict metagenome function by 16S rRNA marker gene sequences and referencing published complete genome sequences [28]. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify microbiota and functional genes showing differential abundance between groups ($\alpha = 0.05$ and with an LDA score > 3.0).

Analysis of the sources of bacterial taxa in piglet guts using SourceTracker

The relative contribution of maternal and environmental substrates to the assembly of bacteria in the piglet gut was analyzed with SourceTracker (V.1.0) in QIIME. OTUs present in less than 1% of samples were first filtered, and the resultant OTU table was imputed with default parameters [29], with the piglet feces at different ages as the “sink” and the samples from sows (feces, milk, breast skin, and vagina) and surrounding delivery environment (air, water, and slatted floor) identified as the “source” regardless of sampling time. The results were aggregated into three categories, vagina, milk and other (breast skin, sow feces, water, air, slatted floor and unknown), and visualized as ternary plots with the R package ggtern.

Modeling the maturation process of gut microbiota using the Random Forest algorithm

Random Forest models were used to regress relative abundances of OTUs in the time-series profile of the microbiota of piglets against their chronologic age, using default parameters of the R implementation of the algorithm (R package “randomForest”, ntree = 10,000, using default mtry of $p/3$ where p is the number input 97%-identity OTUs (features)) [30]. The Random Forest machine-learning algorithm was used to determine a ranked list of all bacterial taxa in the order of age-discriminatory importance. The “rfcv” function was applied over 100 iterations to estimate the minimal number of top-ranking age-discriminatory taxa required for prediction. A sparse model with 30 predictors (variance explained) was selected on the basis of 10-fold cross-validation. A smoothing spline function was fit between microbiota age and chronologic age of the piglet (at the time of fecal sample collection) in the validation sets to which the sparse model was applied.

Results

Characteristics of the study samples

In our study, 1,119 high-quality microbial samples were collected and used for downstream analysis including piglet feces ($n = 482$), sow feces ($n = 86$), milk ($n = 139$), vaginal ($n = 56$), breast skin ($n = 136$), water ($n = 25$), air ($n = 27$) and floors ($n = 168$) (Fig. 1a, Additional file 1: Table S1). The microbiome of all samples was analyzed by 16S rRNA gene sequencing, yielding 74,032,942 high-quality sequences after quality control, with an average of $66,160 \pm 391$ sequences per sample (ranging from 7,746 to 92,011). The overall number of OTUs detected by the analysis reached 40,533 based on $\geq 97\%$ nt identity. Rarefaction curves based on the Chao1 and Shannon indexes of all samples nearly reached a plateau, indicating that the sampling depth was sufficient to characterize the bacterial communities (Additional file 2: Figure S1).

Similarity Of The Microbial Community Structure Between Sample Types

Alpha diversity analysis revealed that the species richness and diversity of the microbial communities were distinct at different sources. The Chao1 index of the piglet microbiome was significantly lower than all environmental and maternal microbiomes except for the vaginal microbiome, and the Shannon diversity index of the sow fecal microbiome was significantly higher than that of other samples (Fig. 1b). In addition, we observed higher alpha diversity indexes in the piglet fecal microbiome at the first time point (day 0), and then they decreased shortly (to the bottom in day 5 for Chao 1 and in day 1 for Shannon diversity index) and rebounded over time.

The NMDS ordination based on Bray-Curtis dissimilarity showed distinct clusters between the sample types, and the early piglet microbiome did not consistently resemble one specific sow or environmental sample (Fig. 2a). For example, the early piglet fecal samples (at days 0 and 1) clustered with the sow vaginal samples, while they gradually shifted towards sow fecal samples as the piglets aged. Permutation Multivariate Analysis of Variance (PERMANOVA) showed that sample types significant effect on the bacterial community structure (stress = 0.16, $P = 0.001$). The fecal microbiomes in the piglets were relatively divergent from each other and had high intersubject variability, particularly on days 0, 1 and 3, compared with those of the sows and the environment (Fig. 2a, Additional file 3: Figure S2). Similarity percentage (SIMPER) analysis on the microbial community dissimilarity further confirm the result in the NMDS (Additional files 4: Figure S3). SIMPER analysis of the sow and environmental microbiota compared with the piglet fecal microbiota indicated that the piglet fecal microbiota on day 0 was more similar to the vaginal (index = 13.3), milk (index = 11.0) and breast skin microbiota (index = 12.6) than to the other microbiota groups. This high similarity between the piglet feces (day 0) and vagina and the sow milk and breast skin were attributed to the dominance of Proteobacteria (30.3%-41.1%, Additional files 5–7: Table S2-4). The similarity between the piglet fecal and sow vaginal microbiota increased within the first three days after birth and then gradually decreased, but the similarity between the piglet fecal and the sow fecal microbiota gradually increased as the piglets aged. At day 28, the piglet fecal microbiota was more similar to the sow fecal microbiota (index = 9.3) than to other

samples, which was attributed to the dominance of Firmicutes (36.3%, Additional files 8: Table S5). The bacterial profiles at the genus level were analyzed using the UPGMA method to measure the similarity of bacterial community compositions between different sample types. Consistent with the NMDS analysis, the UPGMA clustering analysis revealed that the microbiota of piglet feces on days 0 and 1 clustered with sow vaginal samples and appeared to be more similar to feces on day 28 (Fig. 2c).

Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the four most abundant phyla in all samples except sow feces and accounted for 93.6% – 95.9% of the different sample types (Fig. 2b, Additional files 9: Table S6). Firmicutes, Bacteroidetes, Tenericutes and Spirochaetes were the most abundant phyla and accounted for 94.0% of the sow fecal microbiota. At the genus level, 23, 20, 25, 21, 22, 26, 22 and 26 predominant bacterial taxa (average relative abundance of > 1%) were identified in piglet feces (65.6% of the total sequences), sow feces (78.9%), milk (52.5%), breast skin (53.7%), vagina (55.4%), air (50.9%), slatted floor (55.9%) and water (55.6%) samples, respectively (Fig. 2c).

SourceTracker analysis highlights the contribution of sow and environmental sources to the piglets

SourceTracker, a Bayesian probability tool [29], was used to predict the relative contributions of the sow and delivery environment microbiome to the piglet fecal microbiota. The results revealed that the vaginal microbiota contributed the most to the meconium (day 0) microorganisms compared with other sources, followed by the slatted floor (9.6%), milk (9.4%) and air (8.5%) (Fig. 3a). The relative contribution of the vaginal microbiota to the piglet fecal microbiome increased in the first three days from 69.0–89.3% and then gradually decreased to 0.28% on day 28. Interestingly, the relative contribution from sow feces gradually increased after day 5 and finally reached the highest on day 28 (62.1%). However, the relative contribution of bacteria from sow milk was increased only on days 0 (9.4%) and 21 (15.0%). Apart from the vertical transmission of the sow microbiota, the neonatal piglets were also exposed to a wide variety of environmental microbiota. The environment (water, air and slatted floor) contributed 18.1% of the bacterial communities on day 0, rapidly decreased in contribution to 4.0% on day 3, and gradually increased in contribution to 34.1% on day 28, indicating that the slatted floor was the primary environmental source of bacterial communities in piglets, especially five days after birth, while air and water contributed less to the colonization of piglet bacterial communities than the slatted floor.

Ternary plot was used to more intuitively reflect the contribution of various bacterial sources to each fecal microbiome of piglets on different days. As shown in the plot, the piglet fecal samples were more closely related to the sow vaginal sample on the first 7 days (Fig. 3b). The piglet fecal samples diverged in their distributions among the vertices in the ternary plot at days 14 and 21, indicating that the bacterial sources during these times were more complex. At day 24, almost all fecal samples were uniformly distributed between the slatted floor and sow fecal samples, and most of the piglet fecal samples were close to the sow fecal samples in the plot at day 28. Analysis of the OTU cooccurrence patterns showed a hierarchy among the sample sources that were shared with piglet feces (Fig. 3c). This result indicates that more OTUs of piglet feces were shared in the milk, breast skin and slatted floor samples than in the water and air samples. The disparity between the similarity and the proportion of the OTUs shared

between the piglet and sow fecal microbiota might be related to reduced diversity and therefore competition in the piglet gut microbiota [31]. Overall, the relative contribution of various sources of bacteria to the microbial composition of the piglet gut gradually changed as the piglets aged, and the main source of microbes in the fecal microbiota of the piglets was the vagina of the sow within 3 days after birth, which was gradually replaced by the sow feces and the slatted floor.

Maturation Of The Piglet Fecal Microbiota

Tracking individual OTUs within different phyla revealed distinct temporal dynamics within Firmicutes, Bacteroidetes and Proteobacteria. Many of the Firmicutes OTUs displayed dynamic volatility, with 16.4% disappearing between days 0 and 1; 77.8% of those that disappeared eventually reappeared at later times (Fig. 4a, left panel). A smaller proportion of the Bacteroidetes and Proteobacteria OTUs also showed dynamic changes. The greatest number of Bacteroidetes OTUs disappeared at days 0 and 1 (49.3%) but reappeared at later time points (Fig. 4a, middle panel). The greatest number of Proteobacteria OTUs disappeared from days 3 to 5 (35.1%) but reappeared at later time points (Fig. 4a, right panel). We used BugBase to further predict phenotypes in the piglet fecal microbiomes [32] BugBase predicted the fecal microbiome of the piglets to have a higher proportion of facultative aerobic bacteria than aerobic and anaerobic bacteria on days 0 and 1 (Additional files 10: Figure S4). The proportion of anaerobic and facultative anaerobic bacteria showed a contrasting trend, in which the proportion of anaerobic bacteria gradually increased from 22.3–68.0% during the first four weeks postpartum, while the proportion of facultative anaerobic bacteria gradually decreased from 55.75 to 11.8%. The proportion of aerobic bacteria in the piglet fecal microbiomes was only 15.2% at day 0, and this proportion decreased over the first five days postpartum before recovering over time (Additional files 10: Figure S4).

The relative abundances of OTUs were regressed against the chronologic age of each piglet using the Random Forest machine-learning algorithm to probe the age-dependent development of the piglet fecal microbiota. The regression explained 98.4% of the variance related to chronologic age. The top-ranking age-discriminatory taxa were selected according to their variable importance measures using 10-fold cross-validation. Thus, the top 30 age-discriminatory taxa were identified and used for the subsequent construction of the microbiota-based model for discriminating the degree of microbiota maturity, as inclusion of any taxa beyond these top taxa produced only minimal improvement in model performance (Fig. 4b). This model consisted of 21 genera that distinguished the maturity of the gut microbiota during the 28 days of the experiment. Although the natural development of the gut microbiota exhibited a smooth curve that gradually increased, the curve did not reach a plateau until day 28 (Additional files 11: Figure S5), indicating that the gut microbiota had not reached maturity by the end of this study. These age-discriminatory taxa were primarily affiliated with Lachnospiraceae and Erysipelotrichaceae, and the relative abundance of these age-discriminatory taxa significantly changed across the sampling times (Fig. 4b).

To explore bacterial interactions within piglet feces and environment samples, we used network analysis based on strong (Spearman's $r_s < -0.7$ or $r_s > 0.7$) and significant ($P < 0.01$) correlations of genera. In this

network, it was assumed that cooccurring genera interacted with each other in either a positive or negative manner. The piglet feces network consisted of 53 nodes (genera) and 211 edges (relations) with an average degree (the mean number of connections per node) of 3.98 (Fig. 4c). According to the modularity algorithm, the piglet feces genera were partitioned into five modularity structures, where major age-discriminatory taxa such as *Actinomyces* and *Bacteroides* were part of the same subcommunity and had positive correlations. In addition, *Actinobacillus*, *Epulopiscium* and *Pasteurella* were also major age-discriminatory taxa, which were part of the same subcommunity and positively correlated. Most of the piglet fecal age-discriminatory taxa were also identified in the network of other sow and environmental samples (Additional files 12: Figure S6).

Diversification Of The Microbial Community Function

We next sought to examine how the microbial metabolic and functional pathways of the early piglet fecal metagenome changed over time. The majority of functional genes of the piglet fecal microbiota were associated with transporters (6.90%), ABC transporters (3.58%) and DNA repair and recombination proteins (2.66%) (Fig. 5a). The relative abundance of transporters was also the highest in the other samples. Principal coordinates analysis (PCoA) showed that the functional profiles of piglet fecal microbiota clustered more closely to the vaginal microbiota of sows at days 0 and 1, while they were more similar to the sow fecal microbiota at days 24 and 28 (Fig. 5b). The LEfSe analysis revealed that 63 differentially abundant bacterial functions were present across the piglet sampling times (Additional files 13: Figure S7).

The piglet fecal microbiota at day 0 was enriched for several microbial pathways, including secretion systems, pore ion channels, bacterial secretion systems, fatty acid metabolism, tryptophan metabolism and butanoate metabolism. In comparison, the piglet fecal microbiota at day 28 was significantly enriched for pathways related to sporulation, metabolism and biosynthesis, including starch and sucrose metabolism, methane metabolism, lysine biosynthesis and terpenoid backbone biosynthesis (Fig. 5c). There were no significant differences in the metabolic pathways of functional genes in the microbiota among piglet feces at day 0, sow vaginal samples, and piglet and sow feces at day 28 according to the LEfSe analysis. However, 62 differentially abundant bacterial functions were observed between the piglet and sow feces at day 0 (Fig. 5d). Metabolic functions, including fatty acids, tryptophan, glutathione and butanoate and valine, leucine, isoleucine, lysine, geraniol and caprolactam degradation, were overrepresented in the piglet fecal microbiota. In contrast, ribosome, methane metabolism, transcription machinery, DNA replication proteins and amino acid-related enzymes were underrepresented in the sow feces. The relative abundance of hypertrophic cardiomyopathy (HCM), the renin angiotensin system and the ubiquitin system were overrepresented in the vaginal microbiota of sows compared to the piglet fecal microbiota at day 28 (Fig. 5e).

Discussion

The gut microbiota of mammals rapidly develops after parturition through microbial exposure. In this study, we investigated the early acquisition and development of the piglet gut microbiota and in particular, the role of different sow and delivery environment sources in this process by means of a longitudinal multiple site metagenomic sequencing approach. We found that the maternal vaginal microbiota was the primary source of the piglet gut microbiota during the first three days after birth, but the main source was gradually replaced by the sow fecal and slatted floor microbiota, followed by the concomitant coalescence of predicted functions and changes in the biomarkers of taxa. These processes reflected the powerful selection forces of the host or adaptations of the different sources of the microbiota.

The high alpha diversity in the piglet gut microbiota at birth reflects the rapid influx of the pioneering microbiome, primarily from the sow's vagina and other environmental sources after birth, and is consistent with previous reports [18, 33, 34]. Vaginally delivered infants are first exposed to the maternal vaginal microbiota, which results in neonatal gut colonization by vaginal microbes [14, 16]. In our study, the relative contribution of vaginal microbiota rapidly decreased after three days and was gradually replaced by the sow fecal and environmental microbiome (Fig. 3a), which was consistent with the decrease in the initial alpha diversity and intersubject diversity of the gut microbial diversity in the piglets. The mammal is subjected to largely anaerobic conditions in utero, but the infant gut is mostly facultatively aerobic at birth and gradually forms a strictly anaerobic state [35, 36]. These results suggest that the gut microbial colonization of piglets is a process of niche selection, which was confirmed by the initial decrease in the diversity of microbiota (Fig. 1b) and the increased proportion of facultative aerobic bacteria (Additional files: Figure S4), which were subsequently replaced by strict anaerobes consistent with the biochemical changes in the gut environment.

The decrease in the relative contribution of vaginal microbiota indicated that these pioneering microbes were probably poorly adapted or unsuited to colonize the piglet lower gastrointestinal tract and, as such, were easily lost or replaced. Although the facultative aerobic bacteria as pioneering microbes are present only transiently in the piglet gut, these pioneering microbes mediate the shift from aerobic to anaerobic conditions typically associated with the adult state [17, 36]. Most likely due to the shift in the gut environment to an anaerobic state, the relative abundance of strict anaerobic bacteria increased over time. The relative contribution of the sow fecal microbiota gradually increased and became the main source of the piglet gut microbiota after day 21, suggesting that vertically transmitted microbes from the sow vagina to the piglet were more ecologically adaptable in the piglet gut compared with other sow-derived microbes. This finding is in disagreement with a study of human infants in which the contribution of the maternal fecal microbiota to the anal microbiota of vaginally delivered infants gradually decreased within 30 days after delivery [18]. The reason for the difference might be that the piglets were raised with their mothers for an extended period of time and had more frequent exposure to the sow fecal microbiota. In addition to vertical transmission of the vaginal and gut microbiota, neonatal human babies are exposed to a myriad of other microorganisms from different body sites and other maternal sources [18, 37, 38]. The relative contribution of the milk and breast skin microbiota to the piglets was lower than that of other sow sources of transmission, which was inconsistent with previous reports on the sources of gut

microbiota in breastfed infants [39]. This finding may be due to the previous study sequencing only communities from milk and areolar skin while ignoring other maternal or environmental sources, which may have influenced the results of the source-tracking estimates [29]. In our study, more than 97% of piglet gut bacteria were successfully predicted and exceeded previous studies on gut microbial sources with human infants [18, 39], which may be due to the large sample size and the piglets and sows being co-raised under a relatively stable environment throughout the trial. Overall, our results reinforce the importance of this vertical sow-to-piglet microbial transmission from multiple sources, and further studies are needed to elucidate the mechanism of microbial vertical transmission.

In addition to maternal sources, the environment surrounding the newborn is also a natural source of microbes that can colonize different body sites by frequent contact [40, 41]. Our results showed that the slatted floor was the most important source of environmental microorganisms for the colonization of the piglet gut microbiota, and similar results were also observed in the studies of the human infant gut microbiota [37, 41]. The frequent activities of piglets and sows created a unique microbial environment in the slatted floor that differs from sows and environmental microbiome (Fig. 2a), indicating that the slatted floor is not a simple carrier for the vertical transmission of sow fecal microbes. In swine production, biological additives are commonly used to spray confinement swine buildings to reduce the emission of odor, dust and bioaerosol and may also colonize slatted floors [42, 43]. Our results indicated that these biological additives need to be carefully selected before application, as this might affect the colonization of the piglet gut microbiota. Previous studies using both culture-dependent and culture-independent methods showed that there are complex microbial communities in the air [44, 45]. However, the contribution of air bacteria to the colonization of the piglet gut was very low in our study. The reason may be that the aerobic bacteria in the air did not adapt well to the anaerobic environment of the piglet gut.

Few studies have examined the microbiomes of nursing pigs and followed the animals from birth past weaning longitudinally. The dramatic changes in the gut microbial communities and the function of such changes in piglets early in life were observed in our study, and the results were in agreement with those of a previous study [46]. As the piglets grew, the bacterial community became more complex, as reflected by the increase in richness and evenness. Our study is the first to explore the gut microbiota maturity of newborn piglets using a Random Forest regression model, although the results indicate that the intestinal microbiota of piglets did not reach maturation at day 28, indicating that more time is needed to reach a steady state. Previous studies have shown that a number of factors can influence the maturity of gut microbiota, including probiotic and antibiotic feeding [30], health status [47] and age of solid food introduction [38]. However, the potential factors affecting the gut microbiota maturity of newborn piglets still require further study. Consistent with a previous study based on the gut microbiome in human infants, the results of this study showed an enhancement in carbohydrate digestion and absorption capacity, especially starch and sucrose metabolism and other glycan degradation [48]. Furthermore, the significantly increased relative abundances in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways belonged to amino acid metabolism, especially lysine biosynthesis, further supporting the enhanced capacity of protein digestion and absorption as the piglets aged. Considering that gut microbes

utilize host nutrients for survival, it is possible that the enhancement in the gut bacterial digestive system in carbohydrates and proteins is the result of the increased intake of solid feed composed of more complex carbohydrates and proteins than those in sow milk.

Conclusions

For the first time, we comprehensively analyzed the relative contribution of sow and environmental microbial sources to the colonization of gut microbiota in piglets. Ordination and cluster analyses revealed that the gut microbiota of piglets is closely related to the vaginal microbiota at days 0 and 1, while they gradually shifted towards the sow fecal samples over time. In addition, the proportion of gut anaerobic bacteria gradually increased, while that of facultative anaerobic bacteria gradually decreased. More importantly, the initial colonizers in piglets, especially within the first three days of life, largely originated from the sow vaginal microbiota and were gradually replaced with the sow fecal and slatted floor microorganisms as piglets aged according to SourceTracker analysis. These results indicate that the gut microbial succession of piglets is a process of niche selection. Furthermore, gut microbiota maturity revealed that the intestinal microbiota of piglets did not reach maturation at day 28, and more time is needed to reach a steady state. These findings underscore the importance of sows and the rearing environment in the development of the piglet gut microbiome.

Declarations

Ethics approval

This animal study was approved by the Animal Experimental Committee of South China Agricultural University.

Consent for publication

Not applicable.

Availability of data and material

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request; sequence data will be submitted to the European Nucleotide Archive database.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CW, JM, NL, JG, JC conducted the sample collections. JM, CW and YJ conducted bioinformatics analyses. CW and NL conducted the nucleic acid extractions. CW, JM and YJ were major contributors to the manuscript. All co-authors provided comments for the manuscript. JM, TL and XL directed the overall research project. All authors read and approved the final manuscript.

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Figures

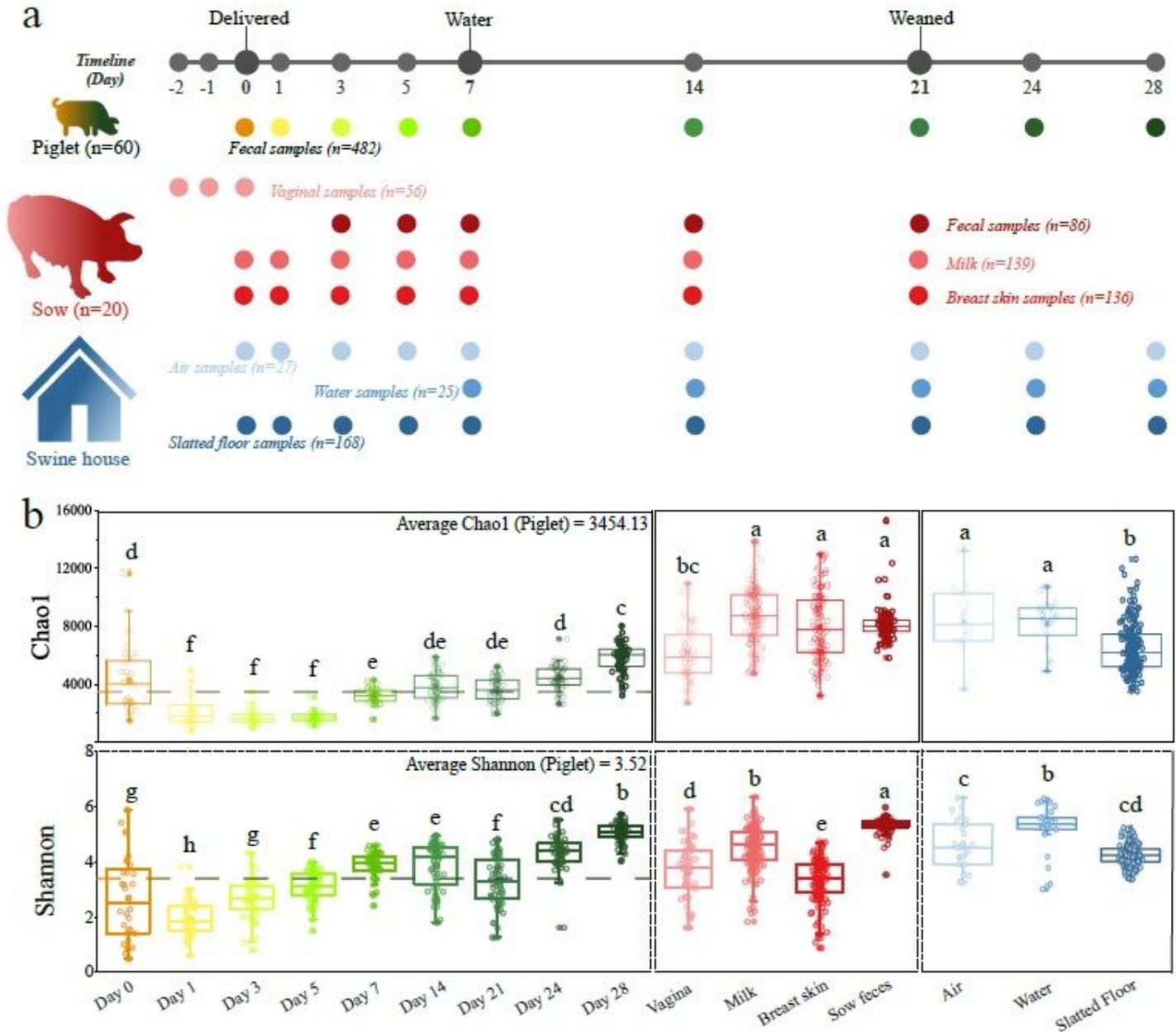


Figure 1

Longitudinal exploration of microbiome between both sow-piglet pairs and housing environment. a, Overall workflow of samples collection from piglets, environment and sows at each sampling time point. All sows (n=20) and their piglets (n=60, three piglets per litter) were co-raised in the same environment. b, Microbial alpha-diversity in different sample types. Different letters indicate significant differences (one-way ANOVA, $P < 0.05$) in the mean values.

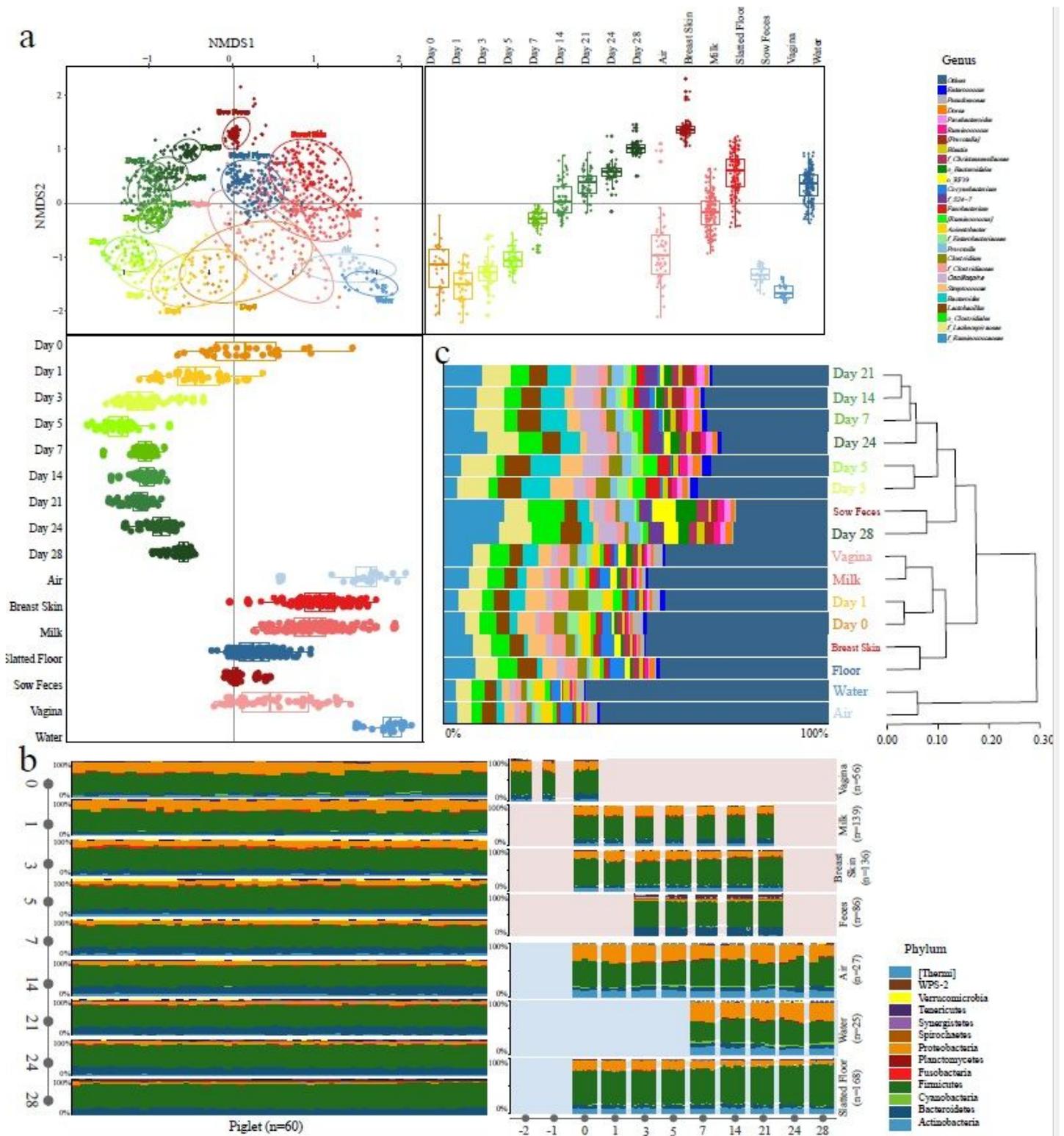


Figure 2

Characteristics of the microbiota in all types of sample. a, NMDS plot of Bray-Curtis dissimilarity between different types of samples. Bray-Curtis dissimilarity was calculated using the abundance of OTUs. Groups from different types were significantly different by a PERMANOVA analysis on Bray-Curtis dissimilarity ($p = 0.001$, stress = 0.16). Box-and-whisker plots shown along each NMDS axis represents the median and interquartile range with whiskers determined by Tukey's method, indicating the

distribution of samples along the given axis. b, Relative abundance of the phylum in different types sample. c, Relative abundance of the major genus in different types sample ($\geq 1.0\%$ of the total sequences) and hierarchical clustering using the UPGMA method performed on a Bray-Curtis dissimilarity matrix at the genus level. Reads representing $< 1.0\%$ of total were pooled and labelled “others”.

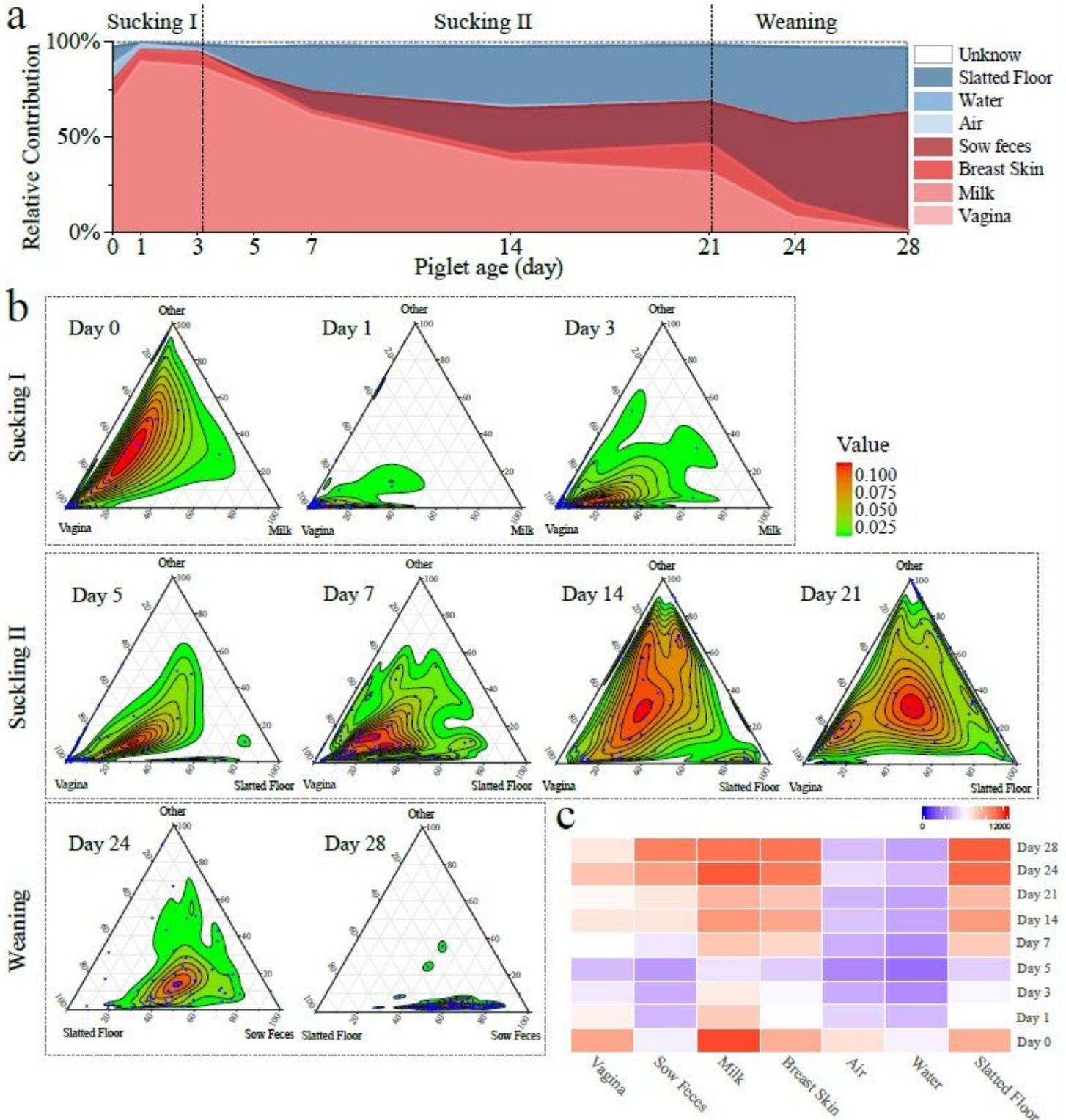


Figure 3

Source of piglet fecal microbiota at nine sampling times. a, Proportion of microbiota from piglet feces at different days that estimated the origin from different sow sources (milk, feces, breast skin, vagina) and environmental source (air, water, slatted floor). b, Three-axis ternary plots indicating the proportion of OTUs within a piglet's feces sample (each point) that is predicted to originate from sow's or environmental sample (indicated by the triangle vertices). Each blue point represents a piglet fecal sample, and its position indicates the predicated relative contribution from the sow's vagina, milk or other sow's sample (breast skin, sow feces) or environmental sample (air, water, slatted floor or unknow source). The two most important microbial sources for each sampling time were selected as vertices, and the rest sources were labeled as "others". Points closer to the vertices indicate that a greater proportion of the sample's OTUs are predicated to originate from the microbiota of the indicated sow's sample or environmental sample. c, Shared OTUs heatmap for piglet feces and others group samples after consolidation of samples taken from the same type across temporal sampling series.

discriminatory bacterial taxa were identified by applying Random Forests regression of their relative abundances in fecal samples against chronologic ages in 60 piglets. Shown are 97%-identify OTUs ranked in order of their importance to the accuracy of the model. The insert shows tenfold cross-validation error as a function of the number of input 97%-identify OTUs regressed against the age of piglets in the training set. Heatmap of mean relative abundances of the 30 age-predictive bacterial taxa plotted against the chronologic age of piglet used to train the Random Forest model. c, Network of co-occurring genera within piglet feces. The nodes represent the genera, and the size of each node is proportional to the degree (the numbers of connections). The edges stand for strong and significantly positive (red) or negative (green) correlations between genera. The nodes are colored based on modularity structure.

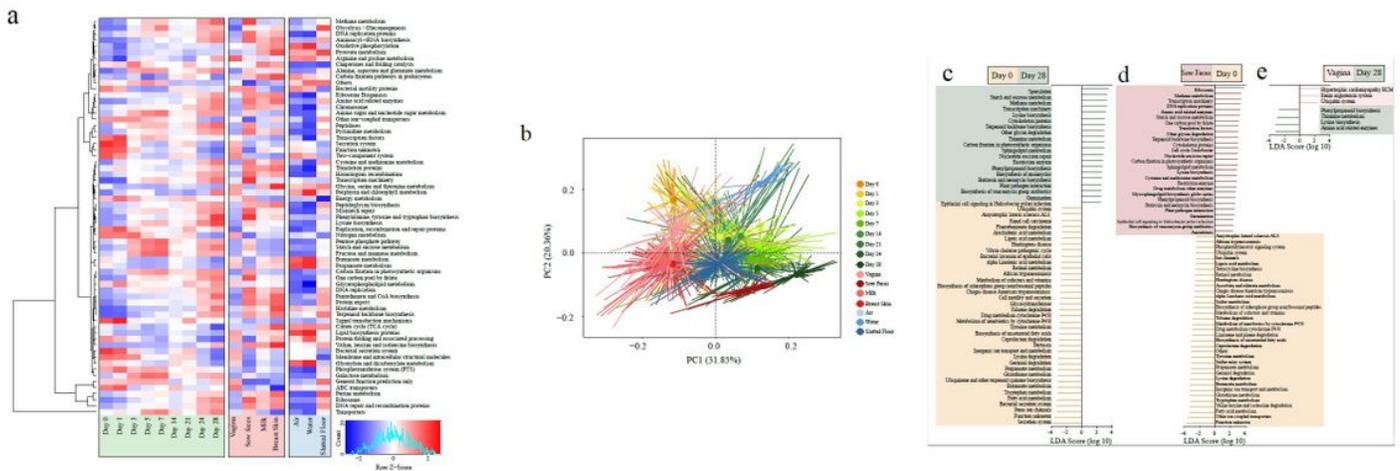


Figure 5

Metagenomic functional predictions for microbiota in different samples. a, Heatmap showing distinct microbial gene (according to KEGG pathway analysis at the third level; > 0.5% of the total sequences) profiles in the samples from piglet feces, sow (stool, vagina, breast skin, and milk) and environment (air, water and slatted floor). b, Principal coordinate analysis (PCA) of microbial functional diversity across the time points using the relative abundances of functional pathways. $P < 0.001$ by permutational analysis of variation (PERMANOVA). c, Results from LefSe analysis based on the PICRUST data set (third level), which was conducted to identify pathways that differentiated functional pathways between piglet feces at the 0 day and sow feces (left), the piglet feces at the 28 days and vaginal samples (middle), the piglet feces at different time points (right). Modules with a linear discriminant analysis (LDA) score > 3.0 are plotted.

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