Feeding a Low-protein Maternal Diet Affects Qinghai Bamei Piglet Jejunal Microbiome-metabolome Response

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Research Article

Keywords: Bamei suckling piglets, 16S rRNA, metabolic profiles, low-protein diet, Bioinformatics

Posted Date: June 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-591216/v1

Abstract

This experiment investigated the impacts of feeding a maternal low-CP concentration diet having iso-essential amino acids on new born suckling piglets intestinal microbial composition and metabolic profiles. The Bamei swine breed was selected due to high meat quality and flavor, but demonstrates slower growth rates which may be related to jejunal nutrient supply. Forty randomly selected purebred Bamei sows were divided into two groups and fed a low dietary CP (12%, LP) or a normal CP (14%, CON) diet, respectively, but formulated to contain similar (iso-) essential amino acid concentrations per current recommendations. At 21 days, 12 piglets were randomly selected from each treatment and euthanized with jejunum content samples collected. The 16S rRNA gene sequencing and mass spectrometry-based metabolomics profiling were combined as an integrated approach for evaluating the functional impact of maternal CP concentrations on piglet intestinal microbiome. Even though piglets demonstrated similar 0 to 21 d ADG among treatments, the jejunum relative weight, villus width, crypt depth and muscular thickness were increased ($P < 0.05$), while villus height, and villus height:crypt depth were reduced ($P < 0.05$) for the material LP compared to the maternal fed CON diet. Maternal CP concentrations can modify the intestinal microbial composition of Bamei suckling piglets. The relative abundances of the bacterial species *Escherichia-Shigella*, *Actinobacillus*, *Clostridium_sensu_stricto_1*, *Veillonella*, and *Turicibacter* were increased ($P < 0.05$) in the maternal LP fed diet compared with the maternal fed CON diet. Jejunal digesta metabolomics analysis indicated that several amino acids were metabolized (i.e. cys, met, tyr phe and trp), biosynthesized (arg phe, tyr, and trp), or degraded (lys) were enriched ($P < 0.05$) for the maternal fed LP compared with the maternal fed CON. Correlation analysis demonstrated that certain intestinal bacterial genera were highly related to the histomorphology and altered intestinal microbiota metabolites. In conclusion, maternal dietary CP concentrations in excess of protein and amino acid requirements not only altered suckling Bamei piglets histomorphology, microbial composition and function, but also modulated jejunum microbial metabolic profiles, which aids in understanding the beneficial effects when feeding a maternal LP diet on piglet intestinal health.

1. Introduction

The Bamei swine breed is a local swine breed in the Qinghai Province of the People's Republic of China. Bamei pigs are known for their meat quality and flavor, but are known to be slow growing (Jin, 2006; Yang and Gun, 2007). The Qinghai plateau has used both natural and artificial selection practices for developing Bamei pigs developing a strong adaptability to the plateau, fat deposition, and good meat quality characteristics. However, Bamei's slow growth rates combined with the plateau's low feed quality/digestibility are important constraints limiting the Qinghai's growth potential of the Bamei swine industry. This results in international implications for meat imports along with grains and protein sources that need to be addressed for food production. Bamei adipose growth rates increase dramatically after 35 kg, while muscle growth rates decrease dramatically after achieving 55 kg (Yang and Song, 1991). China's continuous improvement of people's living standards has resulted in Bamei pork becoming more popular.

The gastrointestinal tract's microbial ecosystem is dynamic and complex with the composition known to vary widely across healthy individuals (Huttenhower et al., 2012). In the human and animal gastrointestinal tract lives a large and diverse microbial community playing a vital role in host health (Kuang et al., 2019), muscosal immunological environment maturation (Pattaroni et al., 2018), precision medicine development (Kuntz and Gilbert, 2017), and assisting with intestinal barrier integrity (Martinez-Lopez et al., 2019). Over the last decade, numerous studies have reported that the intestinal microbiome composition plays an important role in regulating the metabolic health of both rodents and humans (Kreznar et al., 2017). Recent rodent work suggests the major dietary factors regulating intestinal microbiome taxonomic composition are protein and carbohydrate intake (Holmes et al., 2017).

The intestinal microbiome is a complex and dynamic ecosystem of bacterial species being in a continual state of flux and highly susceptible to numerous environmental factors, especially dietary nutrient supply. Reducing CP by 2 to 4 percentage units by adding crystalline amino acids (AA) to meet NRC (2012) nutrient recommendations has increased nitrogen utilization, reduced feed costs and nitrogen excretion, while promoting intestinal health and meat quality with similar growth performance (Wang et al., 2018). The high-quality protein source shortage is a global problem, but especially for China's large population. Since 2002, the world's largest soybean importer is China (Zhang and Reed, 2008). In 2016, China imported 8.391 billion tons soybeans, which is > 26% of global production. Decreasing dietary protein concentrations can effectively reduce pressure on protein source availability (Wang et al., 2018). Many studies demonstrate dietary CP concentrations versus CP source, have a greater impact on intestinal microbiota composition (Rist et al., 2013). Previous studies have focused on changes in large intestinal microbiota, while ignoring the bacteria's role for the small intestine (Dai et al., 2010). Moderate diet protein restriction may alter intestinal microbiota composition while improving adult pig ileal barrier function (Fan et al., 2017). Chen et al. (2018) reported that decreasing dietary CP concentration 3 % units reduced ileal Streptococcus spp., while increasing Lactobacillus spp. and Bifidobacterium spp. These ileal microbiota alterations improved intestinal stem cell proliferation and altered tight junction protein distribution resulting in similar intestinal barrier function. Therefore, feeding dietary LP concentrations has advanced while maintaining essential amino acid supply and has been applied to swine production. The application of 16S rRNA high-throughput sequencing technology provides methods for determining if maternal dietary CP concentration can alter intestinal microbial composition at different physiological stages and intestinal locations.

The Bamei pig was selected due to its popularity with local consumers, but it is a slower growing swine breed, which needs to be addressed. He et al. (2016) reported that IGF-1, insulin, leptin and amino acids may be associated with slow growth. The hypothesis was that altering maternal dietary CP concentrations would alter the intestinal microbiota and metabolites for the suckling piglets. The 16S rRNA gene sequencing method was integrated with LC-MS metabolomics to analyze maternal dietary LP concentration on piglet intestinal microbiome and metabolite profiles. The relationships between metabolites and microbiota were explored as well.

2. Material And Methods

2.1 Ethics Statement

The use of animals was kept to an absolute minimum required to achieve statistical significance for validation purposes; a total of 40 animals were used for the work described in this paper. All procedures involving the Huzhu Bamei sows were conducted at the Qinghai Province Huzhu County Bamei Pig Seed Breeding Farm (Huzhu, China). All procedures were conducted in accordance with China animal welfare law Act 2011, approved by institutional ethical review committees (the State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University Animal Ethics Committee)and conducted under the authority of the Project Licence (IACUC permit number: 2016080301). All procedures involving the Huzhu Bamei sows were conducted at the Qinghai Province Huzhu County Bamei Pig Seed Breeding Farm (Huzhu, China) for Scientific Purposes. These Huzhu Bamei sows were experimental animals, and animal feeding followed the recommendations in the ARRIVE guidelines, animals slaughtering followed the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020) and was approved by the National Administration of Swine Slaughtering and Quarantine regulations (Qinghai, China).

2.2 Animals and Diets

Forty (40) purebred Huzhu Bamei well body condition (score 4) sows were sourced through the Qinghai Province Huzhu County Bamei Pig Seed Breeding Farm (Huzhu, China) having similar body weight (BW), health status, and 3 to 4 years of age being randomly assigned to one of two treatments (20/treatment). The LP treatment diet (12% CP) was balanced for the five EAA Lys, Met, Thr, Trp, and Val for their standardized ileal digestibility (SID) concentrations and then decreased CP by 2% compared to a control (CON; 14% CP) diet balanced for the same SID EAA according to Chinese feeding standards for a 90 kg heavy body conditioned sow. The complete diet composition is given in **Table 1**. After 5 d of facility and diet acclimation, the sows were fed the assigned treatment diet while skipping one estrous cycle (21 days) during natural estrus and then mated. The newborn piglets were maintained with their mothers prior to weaning with litter size, live birth %, birth weights, and diarrhea rates being published previously (Jin et al., 2019). All sows at all time had ad libitum access to feed and fresh water.

2.3 Sample Collection

Randomly, 12 piglets were selected from each treatment group, fasted for 12-hour, weighed, and euthanized with 50 mg/kg sodium pentobarbital on day 21 of age. The small intestine was ligated at the pylorus, duodenum, jejunum, and ileum and dissected. The ligated jejunum was weighed. The jejunal contents were sampled at approximately the half-way point of the jejunal length, placed into 1.5 mL sterile polypropylene tubes, and stored in liquid nitrogen until analyses were conducted for intestinal microbiome and metabolome. An approximate 1.5 cm jejunal tissue sample was collected, washed, and placed in 4% paraformaldehyde for histomorphometric analysis at the same time.

2.4 Histomorphometric analysis

Jejunal tissue samples fixed in 4% paraformaldehyde were embedded in paraffin (5 μm) and stained with HE (hematoxylin-eosin). In each jejunal section, 12 intact villi were randomly selected from each piglet. The jejunum villus height, villus width, crypt depth, and muscular layer thickness were measured using an image analysis system (Caseviewer 2.0 software, 3DHISTECH, Hungary).

2.5 gDNA Extraction, 16S rRNA Gene Sequencing and Microbial Function Prediction

The jejunal content samples were extracted to harvest total bacterial DNA using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA samples were stored at -80℃ until outsourced for analyzing the 16s rRNA gene by BIOMARKER (Beijing, China). The 16S rRNA gene sequence (Illumina HiSeq 2500) was used to measure microbial diversity and bacterial community composition. The extracted DNA was used as a template and PCR was performed using barcode primers located on both sides of the V3-V4 hypervariable region of the bacterial 16S rRNA gene. The primer sequences used were: 338F: 5'-ACTCCTACGGGAGGCAGCA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3'. Amplification was performed for 30 cycles using a DNA thermal Cycler (Bio-Rad, Hercules, CA, USA). The first cycle was at 98°C for 2 min followed by 30 subsequent cycles of 98°C × 30 s, 50°C × 30 s, then 72°C × 1 min, and the last cycle at 72°C for 7 min.

The original DNA fragments from the raw sequencing reads were merged using FLASH v1.2.7 (http://ccb.jhu.edu/software/FLASH/). The reads were assigned to each sample according to the unique barcodes. The selected high-quality reads were used for bioinformatic analysis. Each sample's unique read was clustered into operational taxonomic units (OTU) based on a 97% sequence similarity determined accordingly by UCHIME v4.2 (http://drive5.com/usearch/manual/uchime_algo.html).

2.6 Sample Processing for Metabolomics

The samples of jejunal contents were thawed at 4℃. Then, 60 mg were mixed with 200 μL ultrapure water to assist in homogenization, followed by adding 800 μL of methanol/acetonitrile (1:1, v/v). Then samples were vortexed followed by sonication on ice. The samples were incubated for 1 hour at -20℃ to remove protein followed by 15 min centrifugation (13,000 × g at 4°C), The supernatants were collected followed by vacuum drying followed by storage at -80°C until analyzed using ultra-high-performance liquid chromatography equipped with quadrupole time-of flight mass spectrometry (UPLC-Q-TOF/MS). The quality control (QC) samples were prepared following the same procedures as previously described. For the UPLC-Q-TOF/MS analysis, the samples were re-dissolved in 100 μL acetonitrile/water (1:1, v/v). Instrument stability and repeatability was monitored using QC samples prepared by pooling 10 μL of each sample and analyzed after every 10 experimental samples.

2.6 UPLC-Q-TOF/MS Analysis

Jejunal content samples metabolic profiles were measured using an Agilent 1290 Infinity LC system (Agilent Technologies, Santa-Clara, California, USA) coupled with an AB SCIEX Triple TOF 6600 System (AB SCIEX, Framingham, MA, USA). An ACQUITY UPLC BEH Amide 1.7 μm (2.1 × 100 mm) column for

both positive and negative models was used for chromatographic separation. The A mobile phase was 25 mM ammonium acetate and 25 mM ammonium hydroxide in water and the B mobile phase was acetonitrile. The solvent gradient was 85% B mobile phase for 1 min followed by linearly reducing to 65% by 11 min followed by further reduction to 40% in 0.1 min. This mobile phase concentration was maintained for 4 min followed by increasing to 85% in 0.1 min increments with a 5 min re-equilibration period.

The ESI source parameters were: Ion Source Gas1 = 60, Ion Source Gas2 = 60, curtain gas = 30, source temperature = 600℃, and IonSpray Voltage Floating ± 5500 V. In the MS acquisition only mode, the instrument was set to acquire data covering the m/z range of 60-1000 Da. The TOF MS scan accumulation time was set at 0.20 s/spectra and product ion scan accumulation time was 0.05 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire data covering the 25-1000 Da m/z range, and the product ion scan accumulation time was set at 0.05 s/spectra. The product ion scan was acquired using information dependent acquisition by selecting the high sensitivity mode. The parameters were set as follows: collision energy fixed at 35 V with ± 15 eV; declustering potential at 60 V (+) and − 60 V (−); isotopes were excluded within 4 Da and candidate ions were monitored at 10 per cycle.

2.7 Statistical Analyses

All data were checked for outliers before any statistical analyses were conducted. Data were either plotted or the box and whisker plots and the Shapiro Wilk Test were used to verify that the data were normally distributed ($P > 0.15$). All data were subjected to least squares analysis of variance (ANOVA) for a completely random design (CRD; Steel and Torrie, 1980) having 2 treatments using SPSS 21 software (SPSS Inc., Chicago, IL, USA). The statistical linear additive model was:

$Y_i = \mu + T + e_i$

Where Y_i = depended variable, μ - overall mean, T = treatment of LP or CON and e_i = residual random error. Least squares means were separated using the Least Significant Difference (LSD) and significant was declared at $P < 0.05$.

Microbial Data Analysis. The OTU were rarified based on several metrics for alpha diversity analysis including OTU rank curves, rarefaction, and Shannon, along with Shannon, Chao1, Simpson, and ACE calculated indices. Principal Coordinates Analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) were performed using QIIME based weighted unifrac distance for beta diversity analysis (Jin et al., 2019). Finally, PICRUSt (Parks et al., 2014) was used to predict microbial function. Bacterial domains, phyla, and genera were compared using Wilcoxon rank-sum test, with the FDR adjusted P value < 0.05 being considered as significantly different. Finally, Spearman's rank correlations among jejunal microbiome changes, histomorphometric, and shifted metabolome were calculated to examine functional impacts of material LP diet concentrations on the small intestinal microbiome.

Metabolomics Data Analysis. UPLC-Q-TOF/MS raw data were converted to mzXML files using Proteo Wizard MSconventer tool and then processed using XCMS online software (https://bioconductor.org/packages/release/bioc/html/xcms.html). The XCMS parameters were: feature detection centwave settings (Δm/z = 25 ppm, peakwidth = c (10, 60)); retention time correction obiwarp settings (profStep = 1); and minfrac parameters = 0.5, bw = 5 and mzwid = 0.025 for chromatogram alignment. After being normalized and integrated using support vector regression, the processed data were uploaded into MetaboAnalyst 4.0 software for further evaluation (www.metaboanalyst.ca). Orthogonal partial least square discriminant analysis (OPLS-DA) and 3D-Principal Component Analysis (3D-PCA) for both positive and negative models were performed after log transformation and pareto scaling. For each variable, the variable importance projection (VIP) value in the OPLS-DA model was calculated to determine the classification contribution. Metabolites having VIP values > 1 were further evaluated using Student's t-test at univariate level for each metabolite with $P < 0.05$ considered as statistically significant. Changes in microbial community metabolite profile can reflect microbial community dynamic alterations. Therefore, defining relationships between metabolic function and microbial community structure via microbial and metabolomics data using correlation analysis may provide insight for a comprehensive understanding of microbial composition and community function.

3. Results

3.1 Piglet Performance

Piglet birth BW (day 0) was greater for sows fed LP compared with piglet birth BW for sows fed CON, while 21 d piglet BW tended ($P < 0.07$) to be greater for piglets from sows fed LP compared with sows fed CON (**Table 2**). However, these initial and final piglet BW differences did not affect piglet ADG, which was similar ($P > 0.36$) among both treatments.

3.2 Jejunal Morphology

Intestinal HE staining demonstrated that piglets nursing sows fed a maternal LP diet demonstrated reduced ($P <0.05$) villus height and ratio of villus height to crypt depth, while jejunum relative weight, villus width, crypt depth, and muscle thickness were increased ($P < 0.05$) compared with piglets from sows fed the maternal CON diet (**Table 3**).

3.3 The Diversity and Composition of Jejunal Microbiota

The 16S RNA jejunal microbiota samples after data filtering, quality control, and low-confidence singletons removal resulted in an average of 42,718 V_3-V_4 16S rRNA gene sequence reads being obtained for the 21 d samples (two piglet litters were not yet weaned due to late farrowing). The sequence lengths ranged from 415 up to 429 bp. The rarefaction curves resulted in new OTU diminishing identification rates with increasing number of reads per sample. This implies that the jejunum bacterial community has adequate sampling depth for identifying dominant members. Similarly, the Good's coverages exceeded 99% demonstrating excellent sequence accuracy and reproducibility (**Table 4**). Of the 482 total OTU numbers, 452 OTU were detected in both groups. Based on the Shannon ($P < 0.001$), and Simpson ($P =$

0.001) indices piglets from the maternal fed LP diet demonstrated more diversity and greater evenness compared with piglets from the material fed CON diet (**Table 4**). The Chaol (P = 0.519) and Ace (P = 0.435) indices were similar for piglets from the maternal fed LP compared with the maternal fed CON. Taxonomic analysis revealed the predominant phyla Firmicutes and Proteobacteria being 67.21% and 24.97%, respectively of total reads identifying 16 bacterial phyla. (**Figure 1A**). At the genus level, 232 genera were identified in the jejunal samples. The predominant genera were Lactobacillus (51.11%), Escherichia-Shigella (9.00%), Actinobacillus (7.41%), Clostridium_sensu_stricto_1 (5.60%), Romboutsia (4.35%), and Buchnera (3.54%), respectively (**Figure 1B**).

Furthermore, using a PCoA plot illustrated microbial community dissimilarity and revealed distinct structures between piglets from the maternal fed LP compared with maternal fed CON (**Figure 1C**). The PCoA plot uses a weighted method for unifrac similarity, which revealed PC1 and PC2 explained 55.61% and 13.98% of sample variation, respectively. Similarly, the jackknifed beta diversity and hierarchical clustering analysis via the Unweighted Pair-group Method with Arithmetic Mean (UPGMA) demonstrated that different piglets fed different maternal CP diets were clustered in their individual groups (**Figure 1D**). In addition, piglets from maternal fed CON diets in the PCoA plot were clustered into two subgroups (**Figure 1C**) and UPGMA hierarchical clustering analysis (**Figure 1D**), which was attributed to individual variations of jejunum microbiome profiles.

3.3 Differences in Jejunal Bacterial Community Composition

Relative phylum abundances of Firmicutes, Proteobacteria, Bacteroidetes, and unknown were > 1% for both treatments (**Table 5**). Firmicutes relative abundance was decreased (P = 0.002) and Proteobacteria (P = 0.001) was increased for piglets from the maternal LP treatment compared with piglets from the sows fed maternal CON. Thirty-two (32) specific genera demonstrated relative abundances > 0.1%. The relative bacterial community abundances of Escherichia-Shigella (P = 0.050), Actinobacillus (P = 0.050), Clostridium_sensu_stricto_1 (P = 0.003), Veillonella (P = 0.015), and Turicibacter (P = 0.011) were higher and Lactobacillus was lower (P < 0.001) for piglets from the maternal fed LP treatment compared with piglts from the maternal fed CON treatment (genus level; **Table 6**).

The receiver operating characteristic curve (ROC) predicted different microorganisms for piglets from maternal fed LP compared to maternal fed CON piglets for inducing jejunal development. The area under the curve (AUC) judged via diagnosis test (Xia et al., 2013) that Lactobacillus is the most likely biomarker (0.9 < AUC < 1.0) for piglets from both treatments, while Clostridium_sensu_stricto_1 and Turicibacter are more likely biomarkers (0.8 < AUC < 0.9) for piglets from maternal fed LP sows.

3.5 Predicted Function of Jejunal Microbiota

The PICRUSt analyzed pathway compositions for evaluating jejunal bacterial community functional capacity is a functional-gene-count matrix. Second level KEGG (levels) metabolism pathway analysis via global and overview maps demonstrated that biosynthesis of other secondary metabolites were enriching amino acid, cofactors, and vitamins metabolism (P < 0.05), while lipid and nucleotide metabolism were

decreased (P < 0.05) for piglets when maternal sows were fed LP diet compared with piglets from the maternal fed CON (**Figure 2**).

3.6 Correlations between Intestinal Microbial Species and Jejunum Morphological Traits

Numerous correlations via Spearman's correlation analyses (correlation coefficient | > or < 0.4, P < 0.05, **Figure 3**) were investigated between the different genera (n=6) relative abundances and morphological parameters (n = 7). Clostridium_sensu_stricto_1 was positively correlated with villus width, crypt depth, and muscular thickness, while being negatively correlated with villus height, and ratio of villus height: crypt depth. Escherichia-Shigella was positively correlated with muscular thickness and negatively correlated with villus height. Turicibacter was positively correlated with crypt depth and muscular thickness, while Veillonella was positively correlated with villus width. Lactobacillus was positively correlated with villus height, and villus height: crypt depth, and negatively correlated with jejunum weight, villus width, crypt depth, and muscular thickness.

3.7 Jejunum Metabolites and Metabolic Pathways

The 3D-PCA and OPLS-DA multivariate statistical analysis models were applied to evaluate the different group classifications via Score plots (**Figure 4**). The 3D-PCA Score plots were derived from the LC-TOF/MS jejunal metabolic profiles demonstrated separation between the LP and CON fed diets. A clear separation and discrimination were observed between the two groups, which indicated that the OPLS-DA model could be used to identify piglet differences between maternal fed LP and CON diets. In addition, the volcano plots highlight the 44 metabolites being altered (VIP > 1.0 and P < 0.05) for piglets from the maternal fed LP fed treatment (**Table 7**). Thirty-four (34) metabolites were increased and 10 metabolites were decreased in piglets from the maternal fed LP diet compared with piglets from the maternal fed CON (**Table 7**). These amino acids, nucleotides, lipids, organic acids, and numerous metabolites are involved in multiple jejunum biochemical processes of the Bamei piglet. The hierarchical clustering analysis (HCA) with a heat map was performed to visualize the Bamei piglet jejunum metabolome differences associated with two maternal CP concentrations. The positive ionization data (**Figure 5**) and negative ionization data (**Figure 6**) clearly demonstrate similar clustering patterns of molecular features within each treatment. The maternal CP concentration demonstrated an impact (P < 0.05) on jejunum metabolome, while cluster differences were clearly observable in the HCA generated heatmap plot. The AUC value for each metabolite was calculated, such that metabolites with an AUC > or equal to 0.85 were selected as potential signatures. Six metabolites determine via the AUC elimination step (**Figure 7A**) were: L-Methionine (AUC = 0.875), Taurochenodeoxycholate (AUC = 0.882), Taurodeoxycholic acid (AUC = 0.875), Tauroursodeoxycholic acid (AUC = 0.896), 4-Androsten-17 beta-ol-3-one glucosiduronate (AUC = 0.931), and Cholic acid (AUC = 0.882). Furthermore, the metabolic pathway enrichment analysis (**Figure 7B**) demonstrated that feeding a maternal LP diet altered (P < 0.05; rich factor > 0.10) Arg, Cys, His, Met, Phe, Try, Tyr, and linoleic acid metabolism, biosynthesis of Phe, Tyr, and Try, and Lys degradation. Consistent with the PICRUSt function prediction pathway, amino acid metabolism was enriched for

piglets from sows fed a maternal LP concentration diet compared with piglets from sows fed a maternal CON.

3.8 Correlations between Differential Genera and Metabolites

The functional correlation between intestinal microbiome changes and metabolite perturbations (VIP > 2, P < 0.05) was evaluated using a correlation matrix generated by calculating the Spearman's correlation coefficient. Clear identifiable correlations between perturbed intestinal microbiome and altered metabolite profiles were found (r > 0.5 or < -0.5, P < 0.05). Clostridium_sensu_stricto_1 was negatively correlated with L-His; Lactobacillus was positively correlated with chenodeoxycholate, cholic acid, glycocholic acid, L-His, L-Leu, L-Met, L-Try, L-Val, taurochenodeoxycholate, taurodeoxycholic acid, and tauroursodeoxycholic acid, but was negatively correlated with hypoxanthine, linoleic acid, palmitoyl ethanolamide, PC (16:0/16:0), and uracil; Turicibacter was negatively correlated with the D-Pro; Veillonella was positively correlated with uracil (Figure 8). In summary, dietary maternal CP concentrations induced a piglet intestinal microbiome taxonomic perturbation, which in turn substantially alters the intestinal metabolomic profile as observed due to changes in the diverse intestinal microbiota-related metabolites.

4. Discussion

In this study, reducing maternal dietary protein concentrations by 2% units resulted in similar 21 d ADG. The main nutrient digestion and absorption site is the jejunum (Low, 1976). Maternal suckled milk enters the piglet's gastrointestinal tract, thereby promoting crypt cell proliferation and proliferation. Suckling piglet jejunal development directly affects post-weaning growth performance (Buddington and Sangild, 2011). The small intestinal growth rate before and after birth of the piglet is greater than the whole body (Cheng and Leblond, 1974). The small intestine relative weight 24 h after birth is 50% greater than at birth (Adeola and King, 2006). Intestinal crypt depth increases 40% and villus height increases 35% within 3 d (Godlewski et al., 2005). These crypt stem cells divide and differentiate to form intestinal epithelial cells that gradually migrate to the villi tip (Godlewski et al., 2005) for nutrient absorption.

The small intestine has an important role in defense against health challenges in addition to nutrient digestion and absorption. These new intestinal epithelial cells replace intestinal villi epithelial cells loss caused by apoptosis, while allowing the piglet to adapt to intestinal adversity. Through this process, the digestive and absorption functions of intestinal epithelial cells are gradually improved (Wang et al., 2016; Papadopoulos et al., 2017). To some extent, the crypt depth of small intestinal villi reflects the production efficiency of intestinal epithelial cells. The villus height / crypt depth reflects the overall physiological function of the small intestine. AS muscle thickness increases, the intestinal villi muscle fibers and rhythmic contractions increase, which improves mechanical digestion efficiency and nutrient absorption.

The combined data using Bamei piglets demonstrated that maternal dietary LP concentrations resulted in significant changes in intestinal microbiome composition compared with CON piglets. The altered intestinal microbial compositions were strongly associated with numerous changes of intestinal microbiota-related metabolites. These data demonstrate that a 2% reduction in dietary CP with similar

SID AA concentrations not only alters intestinal bacteria abundance levels, but alters intestinal microbiome metabolic profile, which makes the homeostasis of host metabolites rebuilt. These findings may provide useful insights into a mechanism of feeding a maternal diet for altering the piglet's intestinal microbiome as an alternative mechanism of using dietary intervention for disease treatment.

After the piglet's birth, there are 2 sources of gut microbes with one being the maternal microbes, which are vertically passed, while the 2nd source is environmental, which are horizontally passed. In agreement with previous pig studies (Fan et al., 2017; Yan et al., 2017; Chen et al., 2018; He et al., 2019; Wang et al., 2019), the Bamei piglet's dominant jejunum core microbiome was the phyla Firmicutes, Proteobacteria, and Bacteroidetes. The dominant genus level Bamei suckling piglet jejunum bacteria were: Lactobacillus, Escherichia-Shigella, Actinobacillus, Buchnera, Romboutsia, and Clostridium_sensu_stricto_1. The bacterial community diversity and richness are known to be influenced by dietary intervention (Smits et al., 2016; Johnson et al., 2019a). Alpha diversity metrics (Shannon and Simpson index) demonstrated a higher piglet bacterial diversity from sows fed lower maternal dietary CP concentrations compared with piglets from sows fed the CON CP concentrations, suggesting that altering CP concentration has a direct impact on jejunal microbial composition of Bamei suckling piglets.

Lactobacillus are beneficial bacterial members of the small intestinal microbiota that were reduced for piglets from sows fed the LP diet. The intestinal bacterial environment can protect the intestine from toxic dietary ingredients (Di Rienzi et al., 2018). The reduction of Lactobacillus spp. abundance may result from decreased oligosaccharide ingestion (less soybean meal inclusion), which reduces nutrient availability, which relates to reduced piglet weight (Drissi et al., 2017). These results indicate that maternal dietary LP concentration alters Bamei piglets intestinal microbiota through altering the beneficial bacterial colony structure (Bian et al., 2016). Therefore, it is reasonable to hypothesize that intestinal microbiota differences are the result of early dietary intervention, host–microbe interactions, and/or host physiological state. The most important host-microbe interaction may occur on or at the intestinal barrier.

The metabolomics data revealed that maternal dietary CP concentration alters jejunal metabolite concentration indicating that jejunal metabolism may be linked with jejunal microbiota activity. Maternal LP concentration altered numerous metabolite concentrations associated with piglet protein digestion and absorption, as well as, amino acid biosynthesis. Amino acids are key precursors for protein and polypeptide synthesis and regulators of some metabolic pathways mainly derived from jejunal microbiota and host enzymatic degradation of dietary proteins and microproteins. The piglet L-Met concentration from sows fed the LP diet was higher compared with piglets from sows fed the CON diet. L-Met is a precursor to other sulfur-containing AA, including homocysteine, which are important for protein synthesis, polyamine formation, and synthesis of many metabolites. Additionally, L-Met plays important roles in intestinal bacteria cell protein synthesis and regulating mucosal antigen responses (Mariz et al., 2018). Bamei piglet jejunal linoleic acid metabolism was altered by dietary CP concentrations, which speculating could be contributing to improving intestinal barrier function by enhancing the GPR40-MEK-ERK pathway contributing to improved intestinal barrier integrity (Miyamoto et al., 2015; Di Rienzi et al.,

2018). Further studies are required to identify the AA source from either microbiota or host metabolism (diet and endogenous), while examining the effects on in vivo intestinal function and immune development.

This study demonstrates that dietary intervention can alter intestinal microbiota composition, function, diversity and activity to reduce protein supplementation and feed cost for maintaining host health by altering metabolite availability (such as amino acids; Li et al., 2018; Johnson et al., 2019b; Kovatcheva-Datchary et al., 2019). The Spearman's correlation analysis suggested changes in jejunal microbial abundance induced by feeding lower maternal dietary protein concentrations resulted in a shift in the microbial metabolome.

The correlation analysis between intestinal bacteria (Clostridium_sensu_stricto_1, Lactobacillus, and Turicibacter) and metabolites demonstrated that feeding a maternal LP diet can induce shifting abundance changes in the piglet's intestinal microbiome. Equally important, dietary interventions may not always alter the piglet's bacterial species and abundance but may alter the metabolites produced by these bacterial species thru influencing their metabolism and physiology. Therefore, changes in the metabolic profile of the gut microbiome may not completely depend on altering the microbial profile that was revealed by 16S rRNA gene sequencing. In addition to influencing bacterial types, intestinal numbers, metabolism and physiology, this metabolic change might be achievable through other mechanisms, such as regulating bacteria gene and protein expression. Lin et al. (Lin et al., 2019) demonstrated that acetate and butyrate production by the intestinal microbiome mediated the regulation of growth-related genes in the rumen epithelium which regulated many epithelial physiological processes. So, future research should focus on multi-omics methods to provide more information sources for the intestinal health of piglets when feeding maternal LP diets.

These data demonstrated that dietary CP concentrations altered the intestinal microbiome composition and associated metabolic profiles in Bamei piglets. This is the first key step to understand the impacts of feeding low protein diets influencing intestinal microbiome and function. This could be an exciting research field with the potential to solve many important problems. For example, does altering maternal dietary protein concentration influence intestinal microbiota and related metabolites for newborn piglets to improve growth performance and feed efficiency remains to be determined. The maternal intestinal microbiota largely determines infant intestinal microbiota, which Maqsood et al. (2019) demonstrated that 63% of the infant's bacterial flora originated via the maternal microbiota. Although dietary protein was reduced by 2% units for lactating Bamei sows, their dietary protein requirements during the gestation period are lower (NRC, 2012). Therefore, it may be necessary to determine CP concentration available for the intestinal microbiome at different pregnancy stages having similar SID EAA concentrations. What may be critically important is the impact of protein on piglet intestinal microbiome, due to bacterial flora during pregnancy affecting fetal development (McDonald and McCoy, 2019), and it's impact on the bacterial flora development of infants (Baumann-Dudenhoeffer et al., 2018; Parnanen et al., 2018).

5. Conclusions

These data clearly demonstrates that maternal diet CP concentration alters the piglet's intestinal microbiome composition for altered metabolite concentrations used in various metabolic pathways when diet CP was reduced 2% units with similar EAA concentrations. Reducing maternal dietary CP demonstrated altered piglet histomorphology, microbiota composition and function, while modulating jejunum microbiota metabolic profiles that are associated with specific intestinal bacteria genera. These alterations aid in understanding the beneficial impacts of feeding maternal LP diets without affecting SID EAA concentrations on piglet intestinal health. Furthermore, the regulated intestinal microbiome-related metabolites may be potential biomarkers to be used in the future to explore functional impacts of maternal dietary interventions on the piglet's intestinal microbiome.

Declarations

Acknowledgments

The authors are grateful to all the participants who took part in this study.

This research was funded by the Key of laboratory of Plateau Ecology and Agriculture, Qinghai University (2019-zz-02) and National Natural Science Foundation of China (31660662).

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Tables

Table 1

Ingredient and nutrient composition of maternal diets (DM basis) containing 12% (LP) or 14% crude protein (CON).

Items	% of DM	
	LP	CON
Ingredient composition		
Corn	50.60	44.90
Soybean meal	4.50	9.80
Rapeseed meal	2.50	2.70
Wheat bran	37.78	38.14
Lys	0.34	0.20
Met	0.07	0.05
Thr	0.15	0.10
Trp	0.02	0.01
Val	0.04	0.10
4% premixb	4.00	4.00
Nutrient concentrations, calculated via formulation.		
DE (MJ/kg) a	11.72	11.72
CPb	12.04	12.04
Lys	0.81	0.81
Met+Cys	0.33	0.33
Thr	0.35	0.35
Trp	0.08	0.08
Val	0.26	0.26
Total Ca	0.62	0.62
Total P	0.51	0.51
Salt	3.20	3.20

Note: [a] DE=digestible energy; [b] CP=crude protein;

[b] The premix during pregnancy provided the following per kilogram of diets: vitamin A: 3.52 kIU; vitamin E: 20 kIU; vitamin D3: 0.76 kIU; vitamin K3: 2.6 mg; vitamin B2: 9.52 mg; vitamin B3: 24 mg; vitamin B5: 45 mg; Cu: 4 mg; Fe: 10 mg; Zn: 40 mg; Mn: 16 mg; Ca: 15 %; Total P: 1.8 %; NaCl: 8 %; Water: 10 %.

Table 2

Piglet body weight (BW) and average daily gain (ADG) when feeding maternal diets containing 12% (LP) or 14% crude protein (CON).

Items	LP	CON	SDM	P-value
Piglet BW, kg				
Day 0	0.90	0.88	0.02	0.020
Day 21	3.85	3.78	0.09	0.067
ADG, 0 – 21, g/d	135.8	134.0	1.38	< 0.37

Table 3

Jejunum weight and tissue morphology by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON).

Items	LP	CON	SDM	P-value
Jejunum weight, g	123.22	109.95	17.12	0.074
Jejunum relative weight, %	3.42	3.17	0.30	0.048
Villus height, μm	318.58	385.44	17.99	<0.001
Villus width, μm	96.44	83.43	3.62	<0.001
Crypt depth, μm	150.15	99.01	6.58	<0.001
Villus height: Crypt depth	2.13	4.62	0.19	<0.001
Muscular thickness, μm	65.17	60.75	2.24	<0.001

Table 4

Alpha diversity measures of bacterial communities by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON).

Items	LP	CON	SDM	P-value
Chao1	218.08	208.89	33.48	0.519
Ace	216.58	205.47	33.66	0.435
Shannon	2.72	1.67	0.68	< 0.001
Simpson	0.16	0.45	0.13	0.001
Coverage	0.9996	0.9996	<0.001	0.898

Table 5

Phylum-level taxonomic composition of the jejunal bacterial communities by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON).

Phylum	LP	CON	SDM	P-value
Firmicutes	0.51169	0.83253	0.17449	0.002
Proteobacteria	0.39987	0.09948	0.15060	0.001
Bacteroidetes	0.02626	0.02173	0.03188	0.299
Chlamydiae	0.00004	0.00804	0.01304	0.686
Epsilonbacteraeota	0.01906	0.00739	0.02340	0.166
Cyanobacteria	0.00210	0.00414	0.00565	0.773
Fusobacteria	0.00397	0.00372	0.00485	0.525
Actinobacteria	0.00452	0.00332	0.00593	0.356
Patescibacteria	0.00176	0.00111	0.00204	0.817
Acidobacteria	0.00110	0.00032	0.00140	0.840
Tenericutes	0.00070	0.00014	0.00112	0.544
Cloacimonetes	0.00009	0.00010	0.00035	0.544
Chloroflexi	0.00048	0.00007	0.00072	0.312
Verrucomicrobia	0.00008	0.00005	0.00020	0.356
Planctomycetes	0.00024	0.00002	0.00037	0.908
Gemmatimonadetes	0.00022	0.00002	0.00056	0.470
Unknown	0.02785	0.01781	0.02738	0.156

Table 6

Genus-level taxonomic composition of the jejunal bacterial communities by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON).

Genus	LP	CON	SDM	P-value
Lactobacillus	0.25881	0.76331	0.13670	< 0.001
Escherichia-Shigella	0.15483	0.02514	0.12003	0.050
Actinobacillus	0.12509	0.02318	0.07921	0.050
Buchnera	0.05169	0.01920	0.05861	0.488
Romboutsia	0.06841	0.01856	0.06543	0.166
Clostridium_sensu_stricto_1	0.09503	0.01698	0.07304	0.003
Acinetobacter	0.01295	0.00957	0.01571	0.248
Prevotella_7	0.00384	0.01020	0.02064	0.436
Chlamydia	0.00004	0.00804	0.01298	0.686
Helicobacter	0.01813	0.00691	0.02292	0.094
Veillonella	0.02581	0.00659	0.01388	0.015
Turicibacter	0.00703	0.00440	0.01058	0.011
Rickettsia	0.01763	0.00407	0.01963	0.686
uncultured_bacterium_f_Muribaculaceae	0.00853	0.00352	0.00993	0.326
Fusobacterium	0.00326	0.00329	0.00419	0.644
Pseudomonas	0.00922	0.00300	0.01422	0.106
Terrisporobacter	0.01388	0.00331	0.01267	0.299
Bacteroides	0.00514	0.00264	0.00618	0.184
Enterobacter	0.00117	0.00237	0.00358	0.603
Megasphaera	0.01073	0.00276	0.01537	0.386
Streptococcus	0.00261	0.00183	0.00164	0.149
Pasteurella	0.00642	0.00150	0.00635	0.194
uncultured_bacterium_f_Lachnospiraceae	0.00161	0.00105	0.00270	0.795
Epulopiscium	0.00100	0.00116	0.00153	0.225
Citrobacter	0.00164	0.00093	0.00206	0.453
Prevotellaceae_UCG-001	0.00160	0.00064	0.00226	0.149
Lachnoclostridium	0.00174	0.00070	0.00185	0.100
uncultured_bacterium_f_Clostridiales_vadinBB60_group	0.00295	0.00067	0.00352	0.260

Wolbachia	0.00205	0.00058	0.00233	0.624
Acidaminococcus	0.00419	0.00065	0.00800	0.386
Sutterella	0.00240	0.00023	0.00299	0.356
Others	0.05272	0.03520	0.01200	0.150
Unknown	0.02785	0.01781	0.02738	0.156

Table 7

Detailed information of significantly different metabolites at variable importance in projection (VIP) > 1 and P < 0.05 (t-test) by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON).

Metabolite name	adduct	m/z	RT(s)	VIP	FC (LP/Con)	P-value
Cytosine	(M+H) +	112.0496	190.3420	5.0629	1.8512	0.0202
Uracil	(M+H) +	113.0341	91.9875	3.0508	0.8447	0.0143
D-Proline	(M+H) +	116.0704	298.3865	8.4162	1.2016	0.0166
L-Valine	(M+H) +	118.0854	287.4770	2.7252	1.2772	0.0099
Methylmalonic acid	(M+H) +	119.0336	168.9700	1.3445	0.8350	0.0033
Nicotinate	(M+H) +	124.0382	203.9075	2.8918	1.9715	0.0351
5-Methylcytosine	(M+H) +	126.0646	188.8230	1.3633	1.6714	0.0277
L-Leucine	(M+H) +	132.1010	252.5760	5.5735	1.2929	0.0046
Dopamine	(M+H-H2O) +	136.0744	289.8395	1.8309	1.2324	0.0313
Hypoxanthine	(M+H) +	137.0451	168.9640	11.3354	0.8197	0.0041
DL-O-tyrosine	(M+H-2H2O) +	146.0581	249.2860	1.0860	1.3591	0.0089
DL-3-Phenyllactic acid	(M+H-H2O) +	149.0577	246.7525	1.1128	1.2927	0.0057
L-Methionine	(M+H) +	150.0565	274.8420	2.4280	1.2986	0.0027
3-Methyl-L-histidine	(M+H-H2O) +	152.0801	46.0890	3.9454	0.3010	0.0357
trans-2-Hydroxycinnamic acid	(M+H) +	165.0532	289.8605	1.9537	1.2321	0.0359
L-Phenylalanine	(M+H) +	166.0851	246.5560	5.2926	1.2407	0.0113
L-Citrulline	(M+H) +	176.1007	378.7415	1.2356	1.3942	0.0480
L-Tyrosine	(M+H) +	182.0796	289.7825	2.5042	1.2456	0.0250
DL-Indole-3-lactic acid	(M+H-H2O) +	188.0691	249.5480	3.9166	1.3756	0.0078
Val-Ala	(M+H) +	189.1215	46.9895	2.0810	1.8284	0.0104
L-Tryptophan	(M+H) +	205.0947	249.2050	2.4176	1.4057	0.0247
Pantothenate	(M+H) +	220.1164	257.4480	2.2720	0.8360	0.0199
Val-Leu	(M+H) +	231.1675	185.4245	1.2683	1.7742	0.0160
Linoleic acid	(M+NH4) +	298.2712	42.8735	2.1834	0.7078	0.0268

Palmitoyl ethanolamide	(M+H) +	300.2855	37.4730	2.1904	0.5074	0.0372
Decanoyl-L-carnitine	M +	316.2439	178.3130	1.5638	2.3734	0.0289
(2E,6E)-Farnesol	(M-2H+3K) +	337.2477	195.5165	1.0404	3.6265	0.0051
Chenodeoxycholate	(M+NH4) +	410.3206	144.7795	3.5345	3.4210	0.0292
Cholic acid	(M+NH4) +	426.3154	210.5310	5.4764	8.8444	0.0092
Glycocholic acid	(M+H) +	466.3105	227.7410	15.0858	3.9528	0.0033
Tauroursodeoxycholic acid	(M+H-H2O) +	482.2866	131.6380	5.7124	5.4911	0.0040
Taurochenodeoxycholate	(M+H) +	500.2976	130.9580	11.9799	4.7479	0.0001
Taurodeoxycholic acid	(M+NH4) +	517.3238	131.8080	17.7858	5.0351	0.0004
PC (16:0/16:0)	(M+Na) +	756.5424	132.4325	4.4768	0.4513	0.0018
Thioetheramide-PC	(M+Na) +	758.5586	133.0820	10.8346	0.4940	0.0034
Taurocholate	(2M-H) -	1029.5826	174.7330	2.0868	14.2093	0.0107
Uracil	(M-H) -	111.0207	92.4025	5.7782	0.7934	0.0028
L-Leucine	(M-H) -	130.0880	250.9260	6.9081	1.3271	0.0118
Hydroxyisocaproic acid	(M-H) -	131.0711	137.5360	1.9111	2.6679	0.0134
Hypoxanthine	(M-H) -	135.0321	169.3360	6.3508	0.7611	0.0003
2-Oxoadipic acid	(M-H2O-H) -	141.0180	365.9160	3.1031	1.1443	0.0390
L-Histidine	(M-H) -	154.0621	366.8935	2.1642	1.6983	0.0055
L-Phenylalanine	(M-H) -	164.0730	246.5370	5.5839	1.3528	0.0104
N-Acetyl-DL-methionine	(M-H) -	190.0548	184.5280	1.5021	1.7103	0.0081
L-Tryptophan	(M-H) -	203.0835	248.6450	2.8622	1.4084	0.0091
Indoxyl sulfate	(M-H) -	212.0031	26.7060	2.5906	0.5467	0.0310
sn-Glycerol 3-phosphoethanolamine	(M-H) -	214.0498	377.6670	1.2265	1.3431	0.0667
Chenodeoxycholate	(M-H) -	391.2871	144.2480	12.7681	4.9951	0.0373
Cholic acid	(M-H) -	407.2823	195.3125	9.8141	4.5789	0.0122
Glycochenodeoxycholate	(M-H) -	448.3090	193.6560	5.0633	2.1925	0.0473

4-Androsten-17.beta.-ol-3-one glucosiduronate	(M-H) -	463.2346	212.3140	1.1474	6.7003	0.0204
Taurochenodeoxycholate	(M-H) -	498.2921	132.3305	21.3265	5.1382	0.0001
Glycocholic acid	(2M-H) -	929.6165	227.2290	3.0253	15.9922	0.0194

Note. RT, retention time; FC, Fold Change, mean value of peak area obtained from LP/ mean value of peak area obtained from Con.

Figures

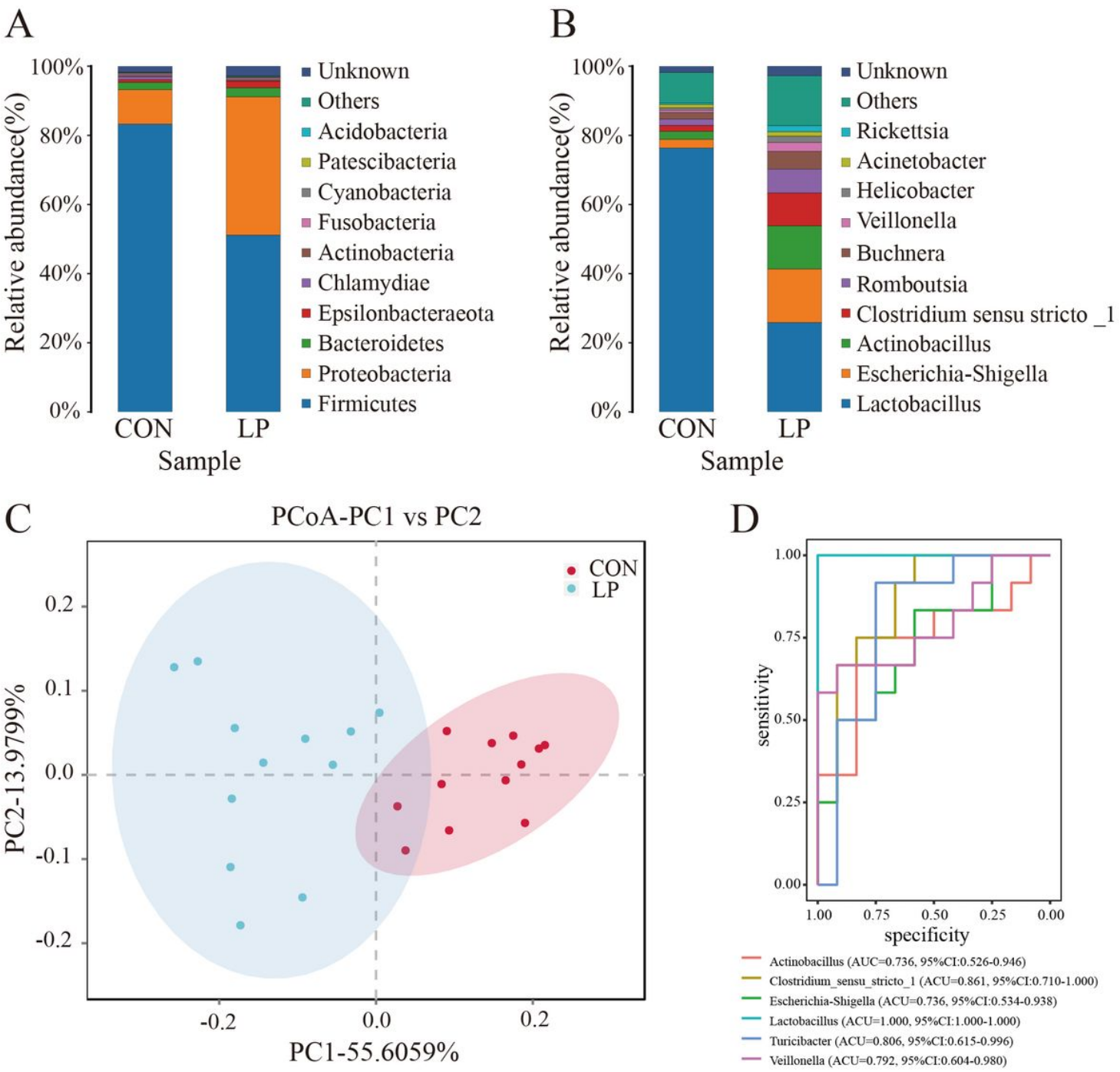

Figure 1

Classification of the bacterial community composition) by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON). (A) Phylum level. (B) genus level. (C) PCoA plot. (D) UPGMA tree. The relative abundance of the top 10 phylum and genus of jejunal microbiome composition profiles revealed by 16S rRNA sequencing (each color represents one bacterial). PCoA plot and UPGMA tree using the weighted unifrac similarity method.

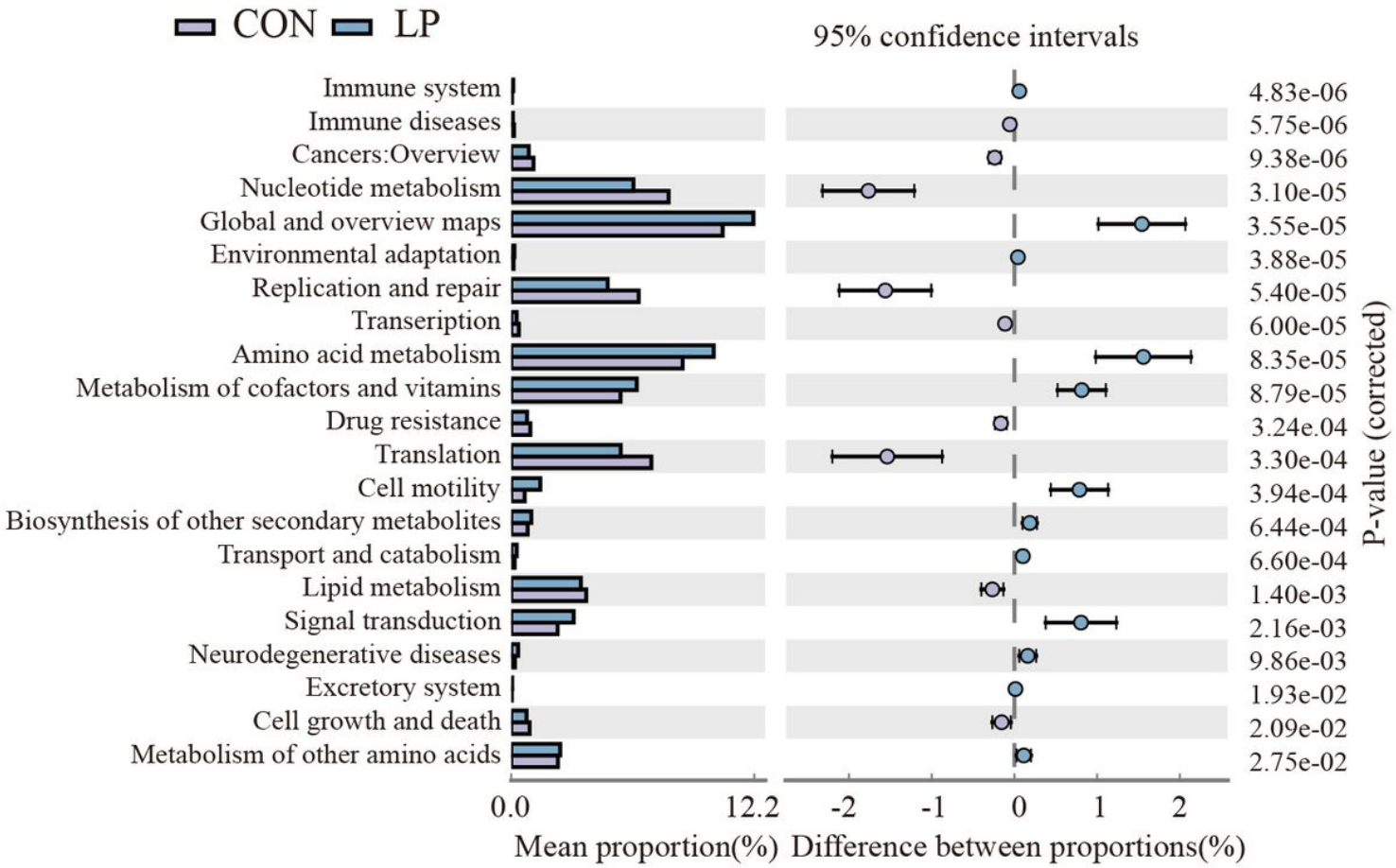

Figure 2

Predicted microbial functions using PICRUSt by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON) when bacteria differed.

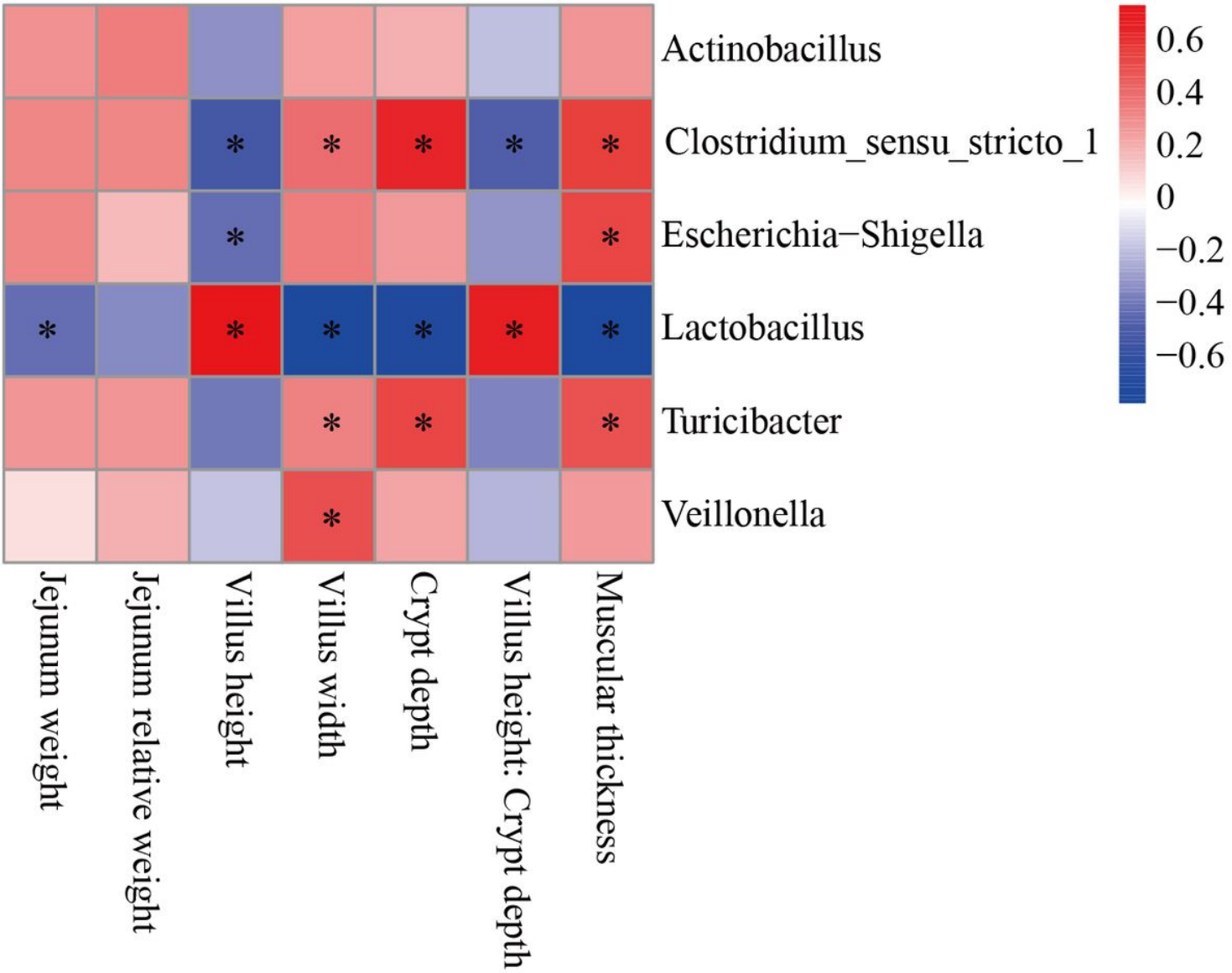

Figure 3

Correlations between differential genera and morphological traits at the jejunum by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP; N=12) or 14% crude protein (CON; N=12). Each row in the graph represents a genus, each column represents a morphological trait, and each lattice represents a Spearman correlation coefficient between a genus and a morphological trait. Red represents a positive correlation, while blue represents a negative correlation. *Significant correlation between the LP and CON groups ($P < 0.05$).

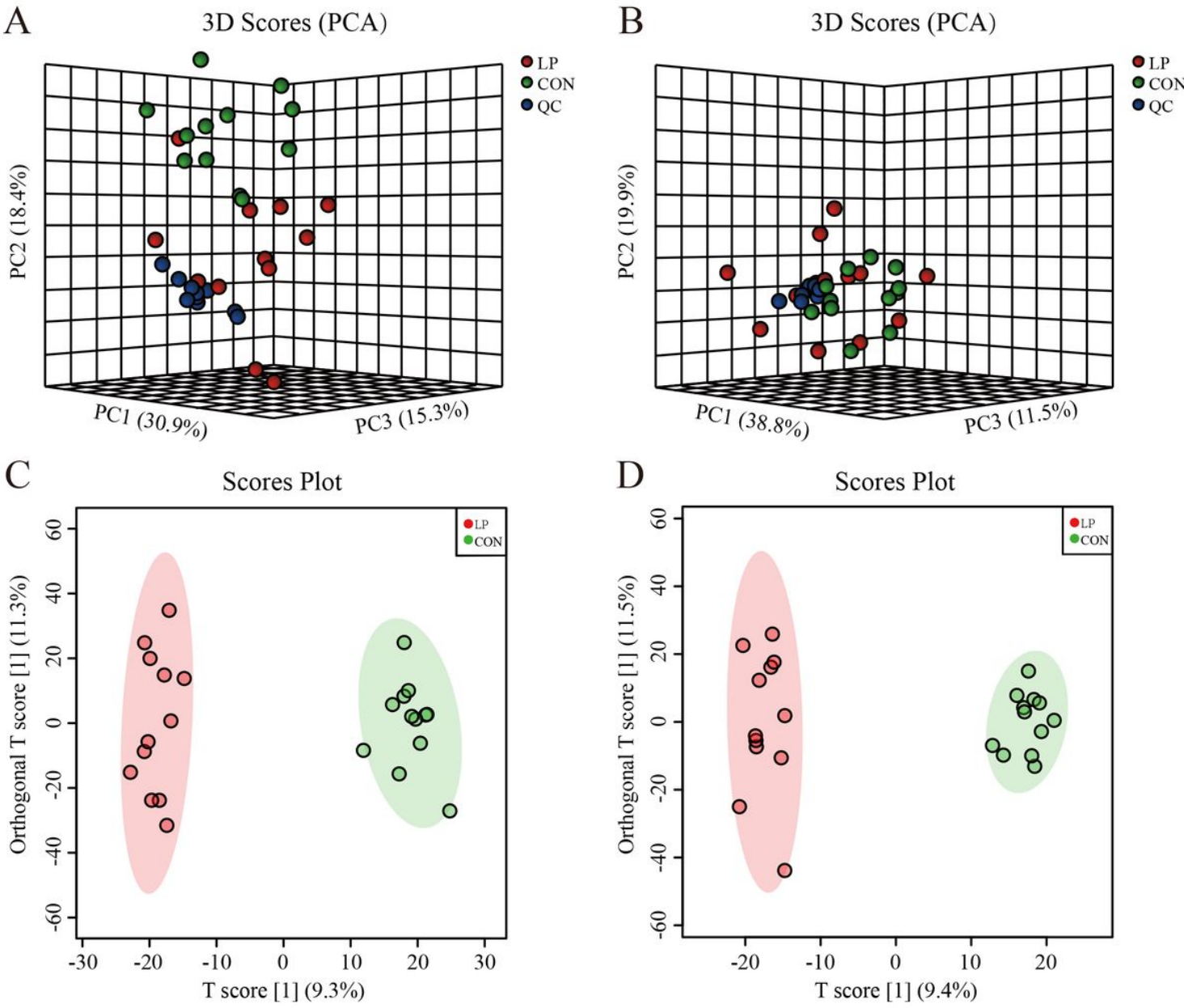

Figure 4

Plot of 3D-PCA and OPLS-DA score, and volcano plot of jejunal metabolites by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON) for comparisons following (A, C, E) positive and (B, D, F) negative mode ionization.

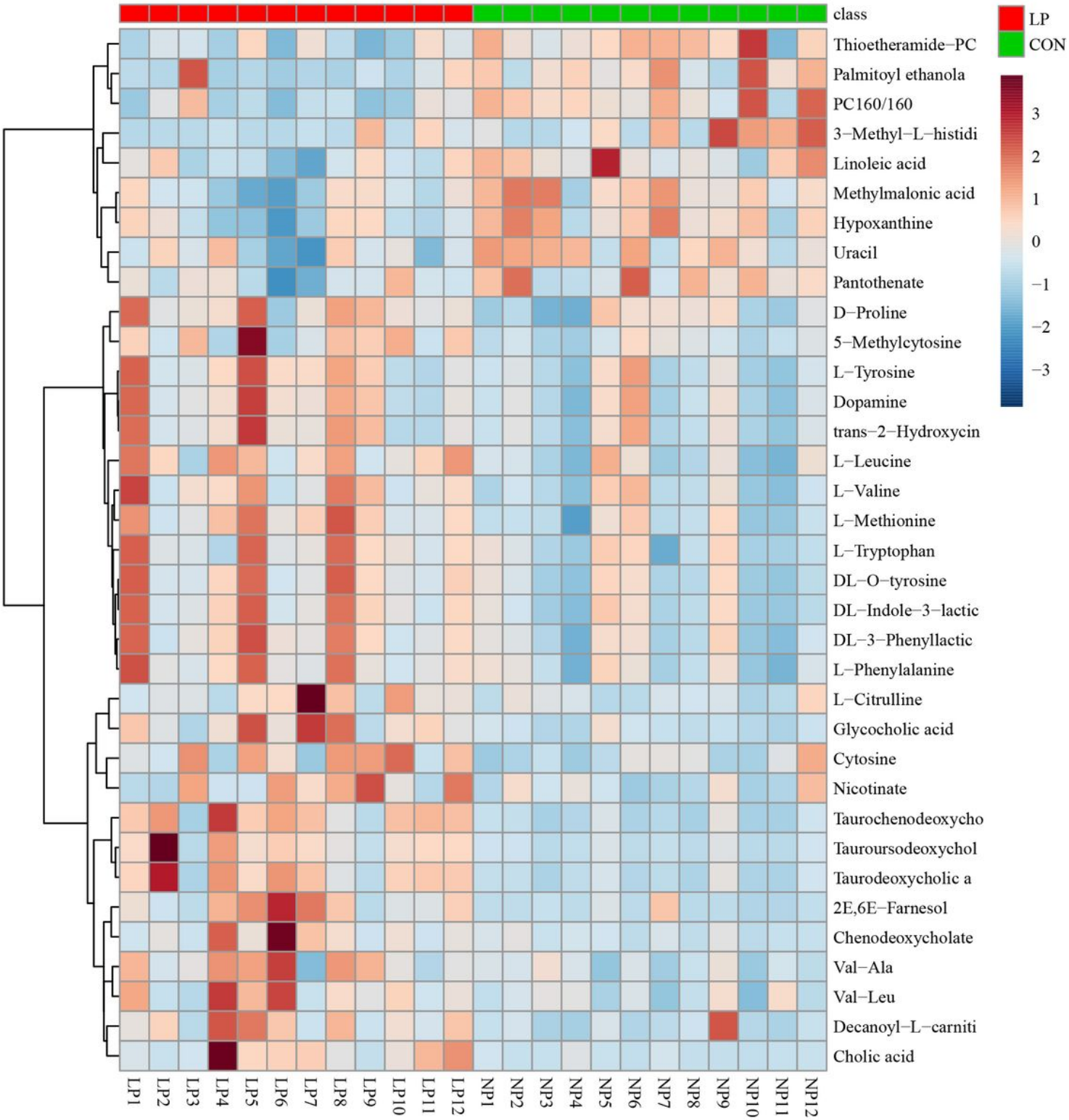

Figure 5

Hierarchical clustering analysis for identification of different metabolites by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON) following positive mode ionization. Each column in the figure represents a sample, each row represents a metabolite, and the color indicates the relative amounts of metabolites expressed in the group; Red indicates that the metabolite is expressed at high levels, and blue indicates lower expression.

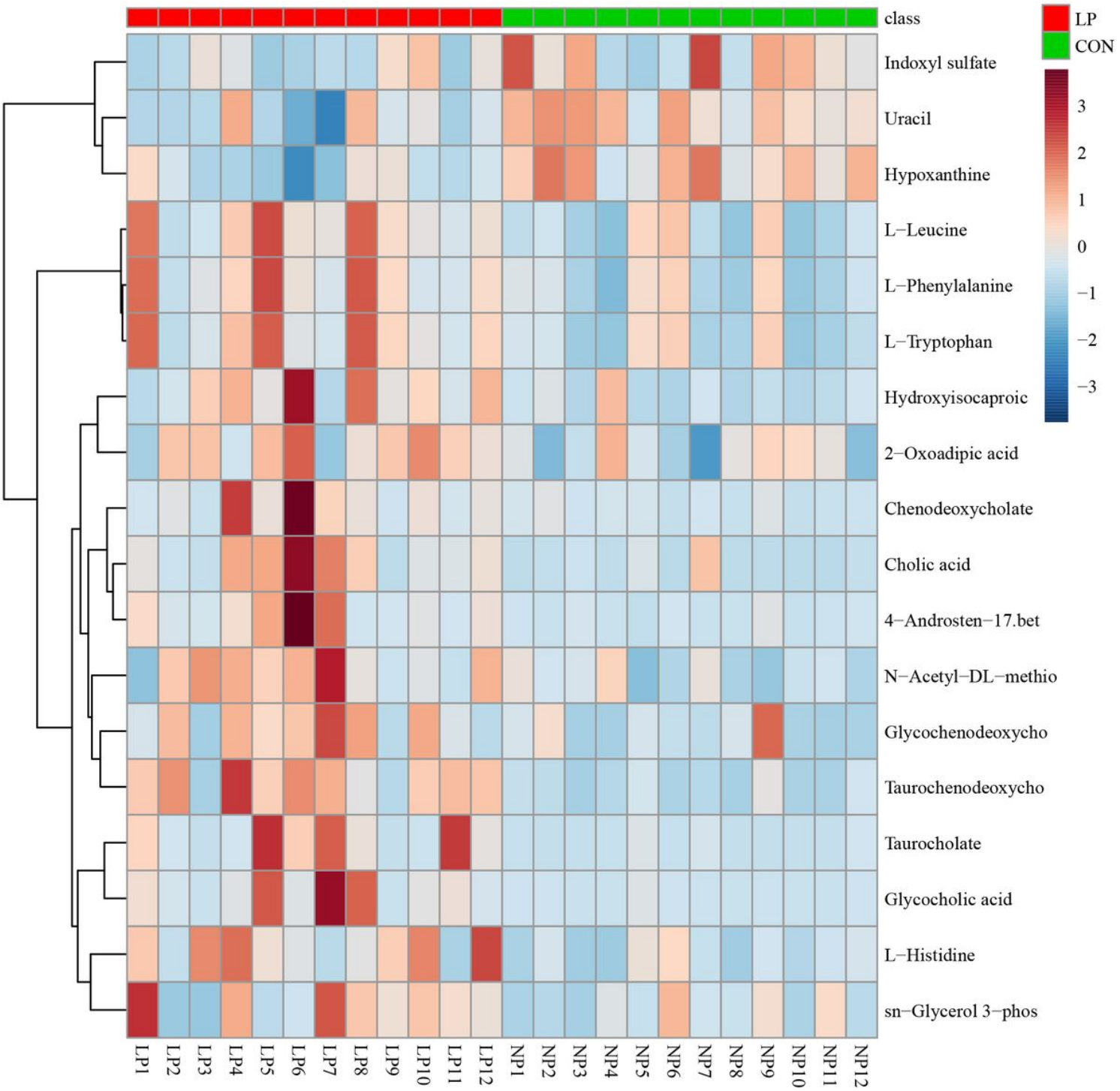

Figure 6

Hierarchical clustering analysis for identification of different metabolites by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP; red) or 14% crude protein (CON; green) following negative mode ionization. Each column in the figure represents a sample, each row represents a metabolite, and the color indicates the relative amounts of metabolites expressed in the group; Red indicates that the metabolite is expressed at high levels, and blue indicates lower expression.

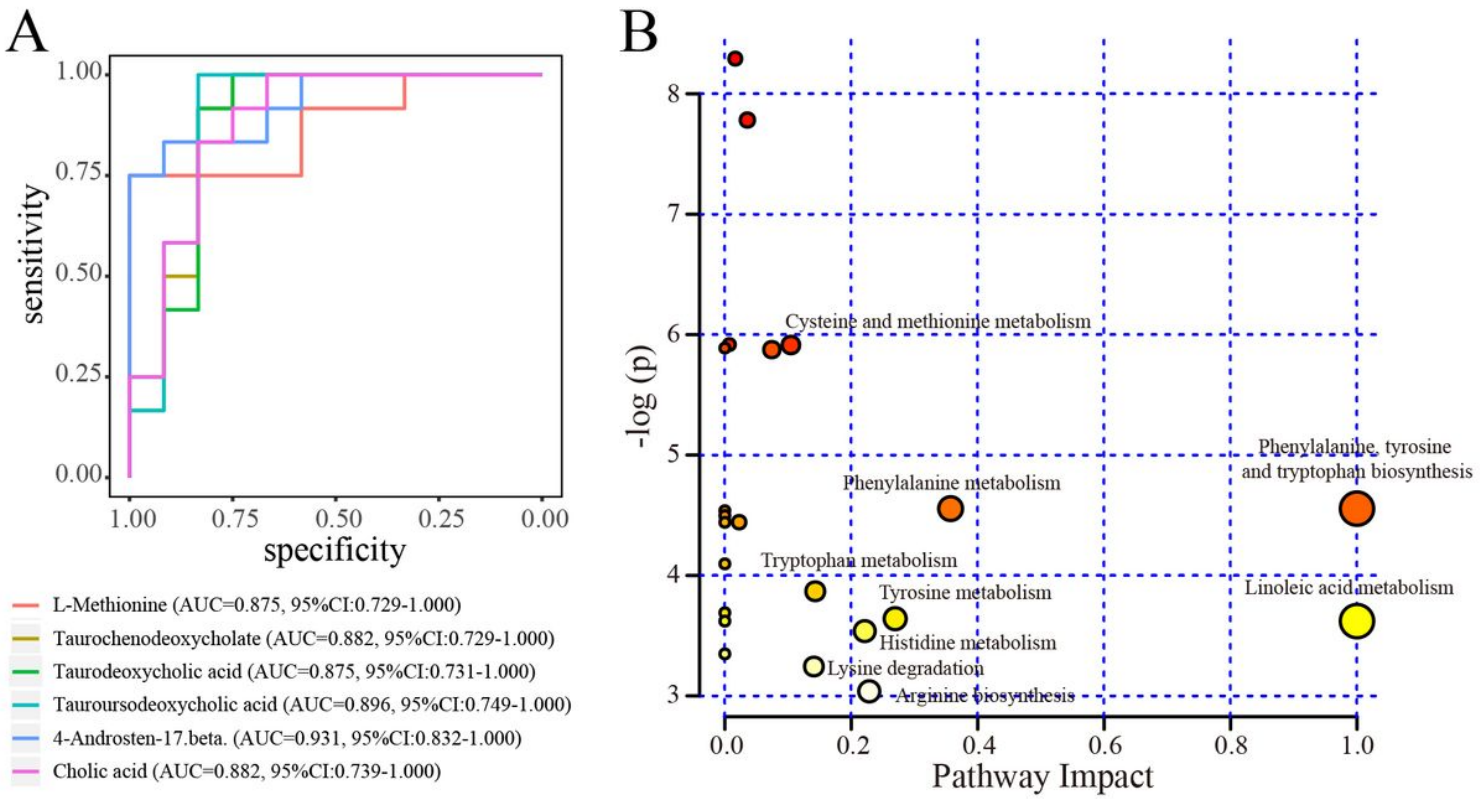

Figure 7

Metabolic pathway enrichment analysis following positive and negative mode ionization to provide metabolite overview by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON).

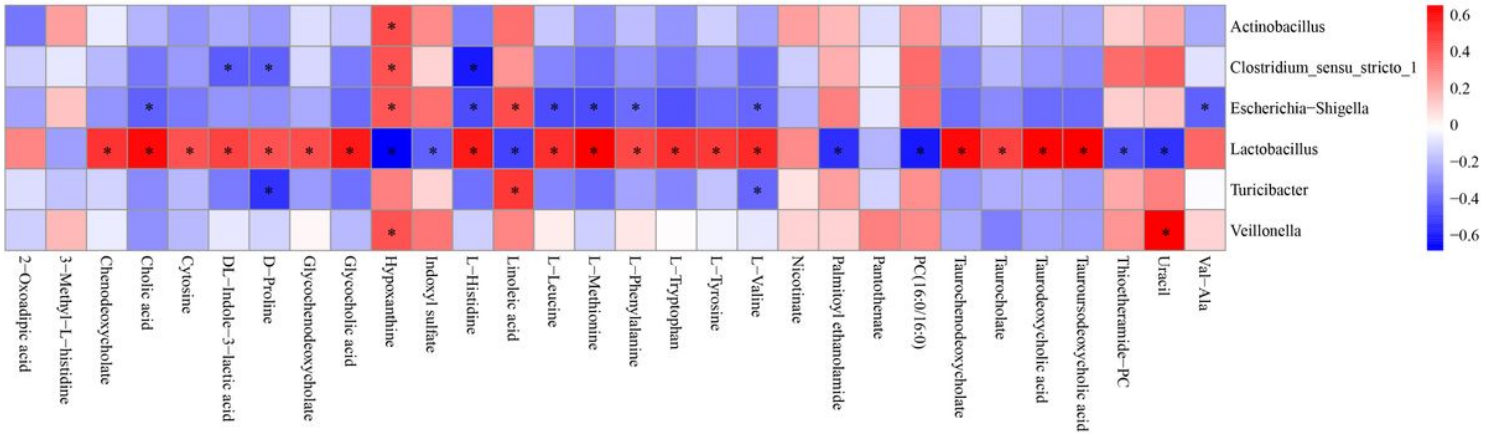

Figure 8

Correlation analysis between genera and metabolite concentrations (VIP > 2) by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON). Each row in the graph represents a genus, each column represents a metabolite, and each lattice represents a Pearson correlation coefficient between a component and a metabolite. Red represents a positive correlation, while blue represents a negative correlation. *Significant correlation between the LP and Con groups ($P < 0.05$).