

Low Crude Protein Diet Affects the Intestinal Microbiome and Metabolome Differently in Barrows and Gilts

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Abstract

Background Low protein diets are commonly used in the growing-finishing pig stage of swine production; however, the effects of low dietary protein on the intestinal microbiota and their metabolites, and their association with pig sex, remain unclear. The present study aimed to assess the impact of a low crude protein (CP) diet on the gut microbiome and metabolome, and to reveal any relationship with sex.

Results Barrows and gilts (both $n=24$; initial body = 68.33 ± 0.881 kg) were allocated into two treatments according to sex. The four groups comprised two pairs of gilts and barrows fed with a high protein diet (HPD, CP 17% at stage \square ; CP 13% at stage \square) and a low protein diet (LPD, CP 15% at stage \square ; CP 11% at stage \square), respectively, for 51 d. Eight pigs in each group were slaughtered and their colon contents were collected. Intestinal microbiota and their metabolites were assessed using 16S rRNA sequencing and tandem mass spectrometry, respectively. The LPD increased intestinal microbiota species and richness indices significantly in both sexes compared with the HPD. The Sample Shannon index was significantly different between barrows and gilts. At the phylum level, the LPD increased the relative abundance of Actinobacteria significantly. The influence of dietary protein levels on Proteobacteria and Synergistetes were associated significantly with sex. At the genus level, Clostridiales, Neisseria, and Prevotellaceae were affected significantly by dietary protein levels. In the latter two genera, the effects were significantly different between barrows and gilts. Metabolomic profiling indicated that dietary protein levels mainly affected intestinal metabolites in gilts rather than barrows. A total of 434 differently expressed metabolites were identified in gilts fed the two protein diets. Correlation analysis identified that six differentially abundant microbiota communities were closely associated with twelve metabolites that were enriched for amino acids, inflammation, immune, and disease-related metabolic pathways.

Conclusions These results suggested that decreasing dietary protein contents benefitted the intestinal microbiota in growing-finishing pigs, which selectively affected the microbiota and metabolite profiles in gilts.

1 Introduction

The mammalian gastrointestinal tract is colonized by thousands of microbial strains that form highly dense, dynamically changing, and extremely complicated communities exceeding 100 trillion microbial cells [1]. These intestinal microorganisms, which metabolize dietary substances to obtain nutrients and energy while producing other metabolites, play vital roles in host physiology and nutrient metabolism [2]. Therefore, dietary nutrients containing proteins, lipids, and carbohydrates decide the available substrates for the host intestinal microbiota, and affect microbial populations and their metabolic activities. Alternatively, the intestinal microbiota and its metabolic substances that are changed by dietary nutrients could further affect host health and physiological function [3]. In particular, intestinal microbial-derived metabolites have been reported recently to control gut inflammatory responses [4], thus directly influencing a variety of aspects of animal and human health [5, 6], and regulating host immunity [7].

Therefore, revealing the relationship among dietary nutrients and intestinal microbiota and metabolites is important to improve the health and growth of mammals by regulating dietary compositions.

Protein is an essential nutritional component in human and animal diets throughout life processes. However, undigested excess dietary protein and amino acid will remain in the large intestine, and be fermented by abundant microbes, which can have unfavorable influences on the host [8, 9]. The microbial fermentation-derived metabolites from proteins are increasingly recognized to induce the inflammatory response, tissue permeability, and colitis in the gut, and are involved in the development of certain metabolic diseases and colorectal cancer [10]. Although both the contents and sources of protein have been demonstrated to change the gut microbiota and their metabolites [11], the protein contents, rather than sources, have a much greater impact [12]. Long-term excessive protein intake has been shown to shift the microbiota composition, and change microbial taxa and fermentation pathways [13]. A low protein diet or dietary protein consumption with high digestibility could reduce the amount of protein reaching the colon, thus limiting the available protein for fermenting bacteria [14, 15]. However, there have been inconsistent conclusions about the influence of dietary protein levels on the intestinal microbiota. Some studies have reported that low-protein intake causes beneficial changes to the gut microbiota [16, 17], while others have suggested that high protein consumption increased the gut microbiota diversity [18, 19].

In swine production, reducing dietary protein levels has marked benefits, including saving crude protein (CP) resources, lessening feed costs, and reducing nitrogen excretion in feces and urine. The recommendation for the dietary protein content decreased by 2% to 4% in the new edition of the NRC guidelines [20]. With the development of industrial synthetic amino acids (AA) technology, this nutritional strategy has become increasingly popular, especially in growing-finishing pigs, because pigs in this stage have very large populations, gain the most weight, consume large amounts of dietary protein, and excrete the maximum amount of nitrogen as slurry. Studies in piglets showed that dietary protein levels changed the intestinal microbiota compositions and microbially-derived metabolites [12]. In growing-finishing pigs, because of their relatively stable microbiota structure, studies focused on environmental influences of N extraction [21-23]. In fact, increasing studies indicate that the compositions and structure of the intestinal microbiota can change dynamically in response to many factors, including feed [24, 25]. Even in older life, the gut microbiota has the propensity of accelerating compositional change [26]. More significantly, we are interested in whether the impacts of dietary nutrients on intestine microbiota and metabolites are affected by sex. This is very important for precision nutrition of sex-specific feeding measures. Therefore, the objective of this study was to assess the influences of dietary protein levels on the profiles of intestinal microbiome and metabolome between barrows and gilts.

2 Materials And Methods

2.1 Animals and experimental treatments

A total of 24 barrows and 24 gilts (Duroc × Landrace × Yorkshire, 120 ± 2 days old) with an average initial body weight of 68.33 ± 0.881 kg were randomly assigned to one of two dietary treatments by sex (n = 12). Therefore, the following four groups in this experiment were treated: (1) BHP, barrows fed a high protein diet containing 17% crude protein (CP) in stage 1 and 15% CP in stage 2; (2) GHP: gilts fed a high protein diet containing 17% CP at stage 1 and 15% CP at stage 2; (3) BLP, barrows fed a low protein diet containing 13% CP at stage 1 and 11% CP at stage 2; and (4) GLP, gilts fed a low protein diet containing 13% CP at stage 1 and 11% CP at stage 2. Feed intake and body weight of every experimental animal were recorded using OSBORNE FIRE® performance testing systems Osborne Industries, Inc., Osborne, KS, USA) by recognizing the electronic ear mark during the feed eating time. Therefore, each treatment had one pen with 12 pigs. The experimental diets were formulated based on corn-soyabean meal and met the nutritional needs of growing-finishing pigs according to the National Research Council [20] (Table 1). All pigs had *ad libitum* access to food and clean drinking water. The experiment lasted 51 days.

2.2 Sample collection and preparation

At the end of the feeding trial, eight pigs that were the closest to the average body weight of those in their treatment group were selected and electronically stunned and exsanguinated. The gastrointestinal tract was removed after the pigs' abdominal cavities were opened. A colonic content sample of each pig was collected in 5 mL sterile frozen tubes, immediately flash-frozen in liquid nitrogen, and stored at -80 °C for further analysis.

2.3 16S rRNA amplicon sequencing

Microbial genomic DNA from colonic content samples was extracted using the cetyltrimethylammonium bromide (CATB) /sodium dodecyl sulfate (SDS) method. The DNA concentration and purity of all DNA samples was estimated using a NanoDrop1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and 1% agarose gel electrophoresis. According to the concentration, DNA was diluted to 1 ng/μL using sterile water.

Amplifcons were generated by PCR of the hypervariable region V3-V4 (341F-806R) of the bacterial 16S rRNA gene using Phusion® High-Fidelity PCR Master Mix with GC Buffer (NEB, Ipswich, MA, USA). The obtained PCR products were purified using GeneJET kit (Thermo Scientific, Waltham, MA, USA). The purified products were used to construct a DNA library using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific). Sequencing was performed on a Thermo Fisher Ion S5TMXL platform (Thermo Fisher Scientific, Waltham, MA, USA).

2.4 Untargeted metabolomics analysis

Colon content samples were homogenized in prechilled methanol and 0.1% formic acid by thorough vortexing. The homogenates were incubated on ice for 5 min and then centrifuged at 5000 × g, at 4 °C for 5 min. The supernatant samples were diluted to final concentration 60% aqueous methanol. Subsequently, the samples were transferred to Eppendorf tubes with 0.22 μm filter and were centrifuged

at $5000 \times g$, at 4 °C for 10 min. Finally, the filtrates were injected into the liquid chromatography with tandem mass spectrometry (LC-MS-MS) system for analysis.

LC-MS/MS analyses were performed using an ultra-high performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific). Samples were injected onto a Hyperil Gold column (100 × 2.1 mm, 1.9 μm) using a 16 min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% formic acid in Water) and eluent B (methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2–100% B, 12.0 min; 100% B, 14.0 min; 100–2% B, 14.1 min; 2% B, 16 min. A Q Exactive HF-X mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, a capillary temperature of 320 °C, a sheath gas flow rate of 35 arb, and an aux gas flow rate of 10 arb.

2.5 Data processing

Microbiome. Raw data were processed according to the Cuadapt [27] quality control process. Then the reads were compared with the reference database [28] using the UCHIME algorithm [29] to detect chimeric sequences, which were removed [30]. Finally, clean data were obtained and used for subsequent analysis. Sequence analysis was performed by Uparse software version 7.0.1001 according to a previously published method [31]. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic units (OTUs). OTU abundance information was normalized using a standard of the sequence number corresponding to the sample with the least sequences. Analyses of alpha diversity (α -diversity) and unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis were performed based on the normalized data. Five indices including, observed species, Chao1, Shannon index, Simpson index, and ACE were calculated using QIIME Version1.7.0 [32]. The top 10 most abundant communities at the phylum and genus levels were defined as predominant bacteria and compared among different treatments.

Metabolomics. The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.0 (CD 3.0, Thermo Fisher Scientific) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: Retention time tolerance, 0.2 minutes; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, 100000. Next, the peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. The peaks were then matched with the mzCloud (<https://www.mzcloud.org/>) and ChemSpider (<http://www.chemspider.com/>) databases to obtain the accurate qualitative and relative quantitative results for principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Based on the differential metabolites identified by comparing GHP and GLP, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (<http://www.genome.jp/kegg/>) analysis was conducted to investigate the metabolomics pathways affected by protein level.

2.6 Statistical analysis

Statistical analysis was performed using the General Linear Model (GLM) procedures in SPSS V.19.0 (IBM Corp., Armonk, NY, USA) for a 2×2 factorial arrangement of treatments. The statistical model consisted of the fixed effects of dietary protein levels and pig sexes and their interactions. Single comparisons between GHP and GLP were performed using Student's t test in SPSS. The correlation between significantly changed bacteria (at the genus level) and metabolites in GHP and GLP were analyzed by Spearman's rank correlation test using GraphPad Prim V.8.0 (GraphPad Software, San Diego, CA, USA). The results were expressed as mean and SEM. The $P < 0.05$ was considered significant. All the indices were analyzed with pig as the experimental unit (n=8).

3 Results

3.1 DNA sequence coverage in colonic content

As shown in Table S1, 2,668,457 V3-V4 16S rRNA valid sequences reads were obtained from 32 samples, including 78,848 (GHP), 88,485 (GLP), 85,353 (BHP), and 80,871 (BLP) raw reads. After removing chimeric sequences, 74,565, 82,306, 80,145, and 75,997 clean reads remained in the GHP, GLP, BHP, and BLP groups, respectively. The effective proportion of clean reads was 90.14–97.79%. The GC% among the clean reads was 70.16–82.57%. The results showed that there were no dramatic differences in the number clean reads among the groups.

The Venn diagram analysis of OTUs is shown in Fig.1. There were 342 common OTUs among all groups (Fig. 1A), and 20, 40, 73, and 129 unique OTUs were identified in the GHP, GLP, BHP, and BLP groups, respectively. From the perspective of dietary crude protein levels (Fig. 1B), there were 396 common OTUs in the gilt groups, 81 unique OTUs in the GHP group, and 772 in the GLP group. Similarly, there were 1015 common OTUs in barrow groups, and 164 unique OTUs in the BHP group, and 244 in the BLP group. These results suggested that there were fewer common OTUs in gilts than in barrows. With respect to pig sex (Fig. 1C), there were 425 common OTUs in the HP groups, with 804 unique OTUs in the BHP group, and 90 in the GHP group. Similarly, there were 1060 common OTUs in the LP groups, with 264 unique OTUs in the BLP group, and 158 in the GLP group. The results suggested that there were fewer common OTUs in the HP groups than in the LP groups.

3.2 Observed species, microbial α -diversity, and cluster analysis

As shown in Table 2, the low protein diet increased the number of observed species significantly compared with high protein diet ($P < 0.05$). In contrast, pig sex had no significant effects ($P > 0.05$). However, pig sex, rather than dietary protein levels, affected the Shannon index significantly ($P < 0.05$) and was higher in barrows than in gilts. Neither the dietary protein level nor sex affected the Simpson index. Sample richness indices (ACE and Chao1) were higher ($P < 0.05$) in the pigs fed the low protein diet. Both indices showed no remarkable differences ($P > 0.05$) between gilts and barrows. In terms of all

the α -diversity indices, no significant interactions ($P > 0.05$) were found between dietary protein levels and pig sex.

As shown in Fig.2, the UPGMA cluster of community structures at the phylum level were analyzed among the four treatments. The results showed that the GLP and BLP groups were the closest, and then they clustered together with BHP, followed by GHP, indicating that dietary protein levels had greater effects on microbial community structures than pig sex.

3.3 Relative abundance of the predominant microbial community induced by dietary protein levels in the colonic content of barrows and gilts

The results for the relative abundance of top 10 members of the microbial community structure in the colonic contents at different levels (phylum and genus) are shown Fig.3 and Table 3 and Table 4. At the phylum level, reducing dietary protein levels significantly increased the abundance of *Actinobacteria* ($P < 0.05$) and decreased the abundance of unidentified bacteria ($P < 0.01$). No remarkable differences ($P > 0.05$) were found between gilts and barrows. For *Proteobacteria*, *Gracilibacteria*, and *Synergistetes*, there were significant interactions ($P < 0.05$) between dietary protein levels and pig sex. At the genus level, the top three microbial community, including unidentified *Clostridiales* ($P < 0.05$), *Neisseria* ($P < 0.05$), and unidentified *Prevotellaceae* ($P = 0.00$) were significantly affected by dietary protein levels. The abundance of unidentified *Prevotellaceae* was also different ($P < 0.01$) between gilts and barrows. In addition, dietary protein levels and pig sex showed significant interactions in *Neisseria* ($P < 0.05$) and unidentified *Prevotellaceae* ($P = 0.00$). In addition, the abundances of *Gracilibacteria* ($P < 0.05$) and unidentified bacteria ($P = 0.00$) were significantly affected by dietary protein levels, and showed significant interactions ($P < 0.05$) between dietary protein levels and pig sex.

3.4 Metabolome profiles and PCA of the main metabolites in the colonic content of barrows and gilts induced by dietary protein levels

To reveal the effects of dietary protein levels and pig sex on intestinal metabolic profiles, LC-MS was used to analyze the metabolome of the colonic content. As shown in Table S2, the score plot of LC-MS (electrospray ionization negative (ESI-)) data with 2037 metabolite signals and LC-MS (ESI+) data with 3844 metabolite signals were detected.

From the perspective of dietary protein levels (Fig. 4A), the PCA results showed that dietary protein levels had a robust influence on main metabolites of pigs, especially between GHP and GLP groups, in which the metabolic communities were clustered. From the perspective of pig sex (Fig. 4B), the PCA results showed that the main metabolites between the two pairs of BHP and GHP, and BLP and GLP groups were mixed together. Especially for barrows, there was a marked variation among samples even in the same group. In contrast, samples of gilts were more gathered. The PLS-DA score plots (Fig. 5) also showed that the GHP and GLP groups were well-separated, suggesting that dietary protein levels caused more significant biochemical changes in gilts compared with that in barrows. These results suggested that main metabolites between gilts and barrows and within barrows fed different dietary protein levels had

no significant differences. Therefore, in this study subsequent analysis on microbial different metabolites-related results mainly focused on the experimental gilts fed the HP and LP diets.

3.5 Identification and KEGG analysis of differently abundant metabolites in colonic content of gilts fed the high protein and low protein diets

Furthermore, the parameters of variable importance of projection (VIP) >1.0 and adjusted $q < 0.05$ were used to detect differentially abundant metabolites in response to different dietary protein levels in gilts. As shown in Fig.6 and Table S3, compared with those in the GHP group, a total of 156 differentially abundant metabolites in LC-MS (ESI-) were identified in the GLP group, including 32 increased and 124 decreased abundant metabolites. Similarly, 278 metabolites in LC-MS (ESI+) were identified, including 126 increased and 156 decreased abundant metabolites. These results suggested that the low protein diet induced more decreased and fewer increased abundant metabolites.

The KEGG was used to analyze the pathways of the differentially abundant metabolites between the two gilt groups. As shown in Fig.7 and Table S4, the metabolic pathways of the Phosphotransferase system (PTS), Ascorbate and aldarate metabolism, the HIF-1 signaling pathway, and Asthma and Glutathione metabolism were associated with four metabolites in LC-MS (ESI-) and were significantly affected by dietary protein levels. Inflammatory mediator regulation of TRP channels, the Fc epsilon RI signaling pathway, Linoleic acid metabolism, Degradation of aromatic compounds, and Biosynthesis of alkaloids derived from histidine and purine were associated with nine metabolites in LC-MS (ESI+) and were significantly affected by dietary protein levels. Interestingly, the metabolite vitamin C (also named ascorbic acid), was enriched and regulated the pathways of PTS, Ascorbate and aldarate metabolism, the HIF-1 signaling, and Glutathione metabolism.

3.6 Correlation between the predominant microbial community and differentially abundant metabolites in the colonic content of gilts induced by dietary protein levels

There were six genus-level microbial communities whose proportions showed significant differences in response to dietary protein levels (Table S5). Compared with the GHP group, the proportions of unidentified *Clostridiales* ($P < 0.05$) and *Terrisporobacter* ($P < 0.01$) were significantly increased in GLP and the proportions of the remaining communities, *Neisseria* ($P < 0.05$), unidentified *Prevotellaceae* ($P = 0.00$), *Gracilibacteria* ($P < 0.05$), and unidentified bacteria ($P < 0.01$), were decreased.

To further reveal the crosstalk between the microbiota and the host, the six communities were selected and used to analyze the correlation with 12 changed metabolites that were enriched in above KEGG analysis. As shown in Fig.8, the proportion of unidentified *Clostridiales* was associated positively with the levels of the Platelet-activating factor ($P < 0.05$), Cinnamaldehyde ($P < 0.01$), Carbazole ($P < 0.01$), and Arachidonic acid ($P < 0.01$). *Neisseria* was associated positively with Vitamin C ($P < 0.01$), Histamine ($P < 0.01$), Naphthalene ($P < 0.01$), and Acetophenone ($P < 0.01$), and negatively with Cinnamaldehyde ($P < 0.05$) and 3-Phenylpropanoic acid ($P < 0.05$). Unidentified *Prevotellaceae* was associated positively with Vitamin C ($P < 0.05$), D-Mannose 6-phosphate ($P < 0.05$), Dihomo-gamma-linolenic acid ($P < 0.05$),

Naphthalene ($P < 0.05$) and Dolichotheleine ($P < 0.05$), but negatively with Platelet-activating factor ($P < 0.01$), Cinnamaldehyde ($P < 0.01$), 3-Phenylpropanoic acid ($P < 0.01$), Carbazole ($P < 0.01$), and Arachidonic acid ($P < 0.01$). The proportion of *Terrisporobacter* was associated positively with Platelet-activating factor ($P < 0.01$), Cinnamaldehyde ($P < 0.01$), Carbazole ($P < 0.01$), and Arachidonic acid ($P < 0.01$), but negatively with Vitamin C ($P < 0.05$) and Histamine ($P < 0.01$). *Gracilibacteria* were associated positively with Vitamin C ($P < 0.01$), Histamine ($P < 0.01$), Dihomo-gamma-linolenic acid ($P < 0.01$), Naphthalene ($P < 0.01$), and Acetophenone ($P < 0.01$), but negatively with Cinnamaldehyde ($P < 0.05$) and 3-Phenylpropanoic acid ($P < 0.01$). Unidentified bacteria were associated positively with Histamine ($P < 0.01$) and Naphthalene ($p < 0.05$) but negatively with Platelet-activating factor ($P < 0.05$), Cinnamaldehyde ($P < 0.01$), 3-Phenylpropanoic acid ($P < 0.01$), Carbazole ($P < 0.01$), Arachidonic acid ($P < 0.01$), and Dihomo-gamma-linolenic acid ($P < 0.01$).

4 Discussion

Dietary protein intake is indispensable for humans and animals to maintain health and growth. However, inappropriate protein consumption, including both excess and limitation, has adverse effects on the body, especially the intestinal microbiota, which is very sensible to subtle environmental changes. Previous studies reported that dietary protein levels had no significant effects on microbiota diversity; they did alter its composition in the hindgut of growing-finishing pigs [33-37]. However, our results revealed that the low protein diet not only changed the microbiota composition at the genus level, but also increased microbial species and improved community diversity and richness in growing-finishing pigs, which might be related to the dietary protein content used and the reduction levels. The microbiota was also demonstrated to be affected by intestinal sampling points. Dietary protein levels changed the ileal microbiota diversity rather than that in feces in weaned piglets [38]. Moderate lowering of the dietary protein concentration also improved the α -diversity of that intestinal microbial community in growing pigs [39, 40]. However, an excessive protein decrease, i.e., a very low protein intake, was detrimental to the microbiota structure [41, 42]. Therefore, our results demonstrated that the dietary protein content also affected the intestinal microbiota diversity in pigs at the growing-finishing stage, and suggested that the low protein diet formulated in the present study was appropriate and helpful to improve intestinal microbial health in growing-finishing pigs.

In terms of intestinal microbiota composition, our results demonstrated the four main phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Fusobacteria* in all groups accounted for over 77% of all bacteria phyla. The former three phyla were also found to be the most abundant bacterial communities in the feces of piglets [43]. We found the proportion of *Actinobacteria* increased when the dietary protein level was lower. Although the role of *Actinobacteria* in the intestines is unclear, they are probiotics and could produce biological active substances with anti-inflammatory and antibacterial properties [44]. Our results revealed that the low protein diet mainly increased the proportion of *Clostridiales*, and decreased *Prevotellaceae*, *Neisseria* and *Gracilibacteria*. Similarly, the abundances of *Clostridiales* were decreased by feeding high protein diets in the large intestinal content of rats and in the feces of dogs [45, 46]. However, this was inconsistent with the previous results [47, 48] which showed that a low protein diet

decreased the relative abundance of *Clostridium* in weaned piglets. The difference was attributed to the *Clostridiales*, which were detected in growing-finishing pigs and piglets belong to different genera, suggesting they probably played different roles. Interestingly, a previous study found that the proportion of *Prevotella* in fecal samples was lower in piglets fed with a low protein diet than in those fed a high protein diet, but was higher in the ileal digesta [49]. The inconsistency on the microbiota from different intestinal sampling sections was also demonstrated by Rist et al [50]. This variation might reflect the specific functions of bacteria in different intestinal segments. Therefore, the roles of the differentially abundant bacteria in the gut should be investigated in further studies.

Although dietary protein levels had greater influence than pig sex, our results revealed there were also the differences of microbiota diversity and communities between barrows and gilts. More interestingly, the two factors showed obvious interactions in microbial community structure. The interactions resulted in the influences of dietary protein levels or pig sex being compromised or strengthened, or even offset or reversed. Specifically, at the phylum level the relative abundances of both *Proteobacteria* and *Gracilibacteria* were lower in the GLP group than in the GHP group, whereas *Proteobacteria* was higher in the BLP group than in the BHP group. *Gracilibacteria* showed no significant difference between barrow groups. By contrast, *Synergistetes* showed no significant difference between gilt groups, but was lower in the BLP group than in the BHP group. Thus, the low protein diet decreased the abundance of *Proteobacteria* and *Gracilibacteria*, and increased the abundance of *Synergistetes* in gilts, but the effects were entirely contrary in barrows. At the genus level, the relative abundances of *Neisseria*, *Prevotellaceae*, *Gracilibacteria*, and unidentified bacteria were higher in the GLP group than in the GHP group and higher in the GHP group than in the BHP group, but all four communities showed no significant differences between the BLP and BHP groups, or between the GLP and BLP groups. These interactions also suggested that the low protein diet had a greater influence on intestinal microbial communities in gilts than in barrows. Thus, the present study revealed the effects of combining two factors of pig sex and dietary protein content on the intestinal microbiota, which indicated new feeding strategies could be developed, such as designing different dietary protein concentrations according to pig sex to ensuring intestinal microbiota health. In addition, our results suggested that it is necessary to use single sex animals for intestinal microbiota investigations to remove sex-related inherent differences.

The mammalian gastrointestinal microbial flora could produce a myriad of specialized metabolites that the microflora use to communicate with their host and that could affect host health and even potentially be used to cure diseases [51]. Dietary interventions had major influences on the metabolic composition and abundance of the intestinal microbiome [52]. Previous studies demonstrated the roles of dietary starch, fat/fiber [53, 54] and fermented feed [55] in growing-finishing pigs. In the present study, PCA and PLS-DA analysis showed that colonic metabolites were influenced by both dietary protein levels and pig sex, in which the GHP and GLP groups had a clear separation compared with the other pairs of groups. These results indicated a low protein diet specifically shifted the metabolic profile in gilts. Interestingly, an increase in protein consumption for 10 weeks had no influences on the fecal microbiota and volatile metabolites in healthy older men [52]. Notably, in the above study, all the participants were men rather than women. In contrast, another study on female pigs rather than male also proved that sanitary

conditions affected the colonic microbiome and metabolome [56]. The influences of the dietary protein content on intestinal microbial metabolome have been rarely reported, not to mention additional considerations of sex. The present study was the first time to reveal the female-specific influences on intestinal microbial metabolome in swine.

In recent years, two types of protein metabolites produced by intestinal microbes, biogenic amines and short chain fatty acids (SCFAs), which are generated by deamination and decarboxylation, respectively have been studied [57]. However, the present study found the SCFA levels were not different between the GHP and GLP groups. In fact, previous studies demonstrated that the main SCFAs, such as acetate, propionate, and butyrate, are generated in the hindgut from carbohydrates fermentation rather than proteins. Branched chain fatty acids also belong to the SCFAs, which account for about 5%-10% of total SCFAs, are produced by the microbiota exclusively from branched chain amino acids (BCAAs) [58, 59]. To meet all the essential amino acids needs of experimental pigs, in this study, the low protein diet was formulated by the addition of two BCAAs, Ile and Val, which means there were almost the same amounts of undigested and unabsorbed BCAAs entering the large intestine in the GHP and GLP groups. Therefore, this is the reason why differences in the SCFA concentrations were not detected between the two groups. Pearson's correlation analysis showed that the relative abundances of bacteria at the genus level were closely associated with the concentrations of 12 specific metabolites. Among these metabolites, histamine showed the largest decrease. The polyamine histamine is a well-known pro-inflammatory factor [60]. Consistently, KEGG analysis also found that several inflammation-related pathways were enriched. These results suggested that the intestinal *Neisseria* and *Gracilibacteria*, which are histamine-producing bacteria, probably induced inflammation by the increase of histamine concentration in gilts fed with the high protein diet. In addition to amino acid metabolism, the present study found that the dietary protein content also regulated the proportions of certain special bacteria and their metabolites, such as fatty acids, vitamin C, and platelet-activating factor, which might have subsequent influences on immune, diseases and lipid related metabolism processes.

5 Conclusions

Combined intestinal microbiome and metabolome analysis demonstrated that the dietary protein content selectively altered the gut microbial diversity, composition, and metabolic profiles in the colon of growing-finishing pigs. Decreasing dietary protein levels increased the intestinal microbial community species and improved α -diversity. The influences of dietary protein on the composition of intestinal microbes were significantly different between barrows and gilts. In particular, the concentrations of intestinal metabolites in gilts, including histamine, fatty acids, vitamin C, and platelet-activating factor, which involve amino acids, immune, and inflammation related metabolism, were altered by changing the dietary protein levels. These findings provide new evidences for feeding strategy of protein precision nutrition by sex in growing-finishing pigs.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the data used in the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Ziwei Xu was the Principal Investigator of the relevant project (2021C02007) and is the corresponding author. All co-authors were variously involved in completion of this paper: Xin Tao designed the study, in charge of this study and was the Principal Investigator of the relevant project (2018C02035), and wrote the manuscript. Bo Deng designed this study together, was the Principal Investigator of the relevant project (2018C02044) and joined in part of this study. Qizhi Yuan performed animal feeding experiment. Xiaoming Men and Jie Wu joined in part of this study.

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Figures

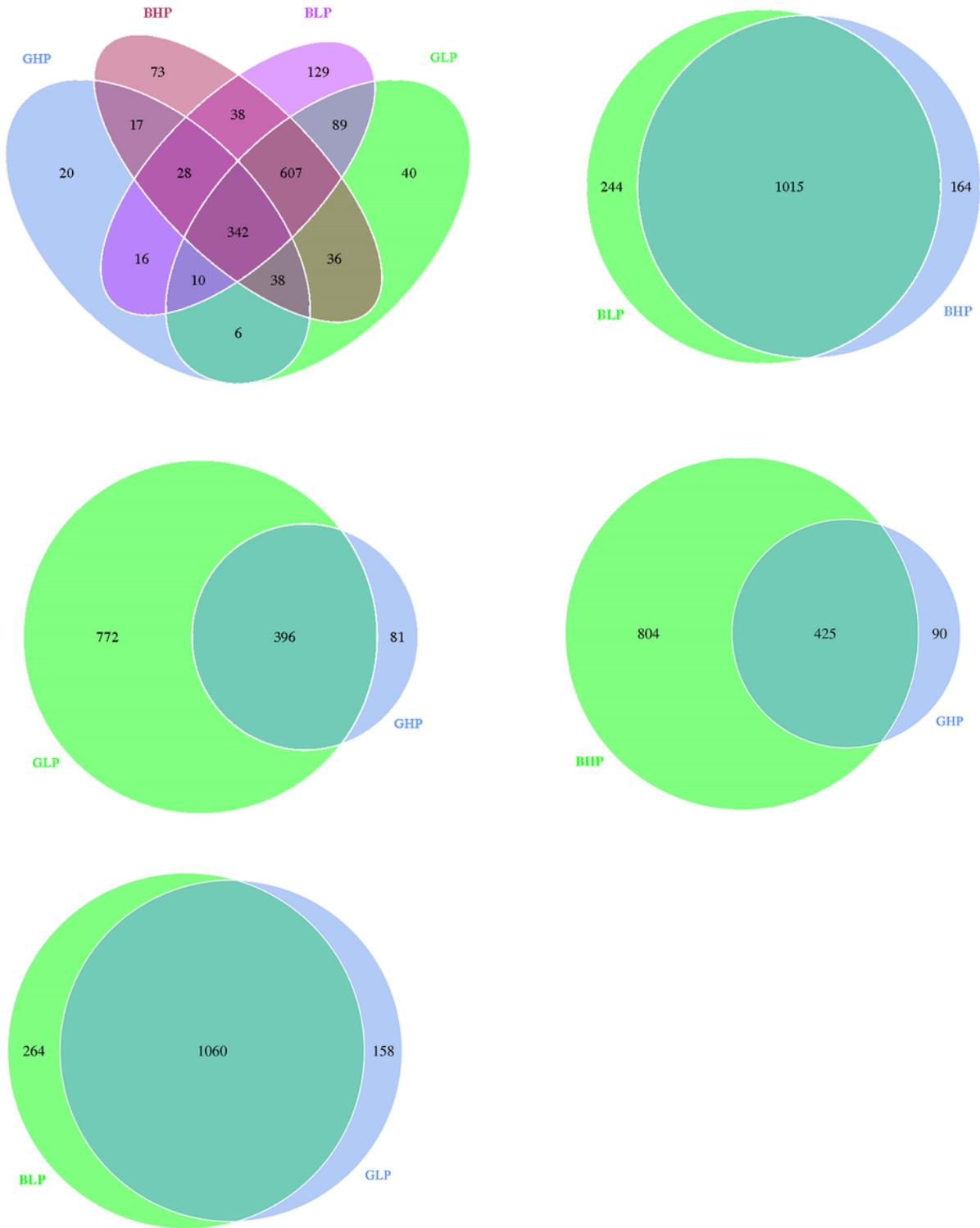


Figure 1

Venn diagram showing the unique and shared OTUs in the different groups. A, a comparison among all the treatments; B, the effect of dietary crude protein levels; C, the effect of pig sex

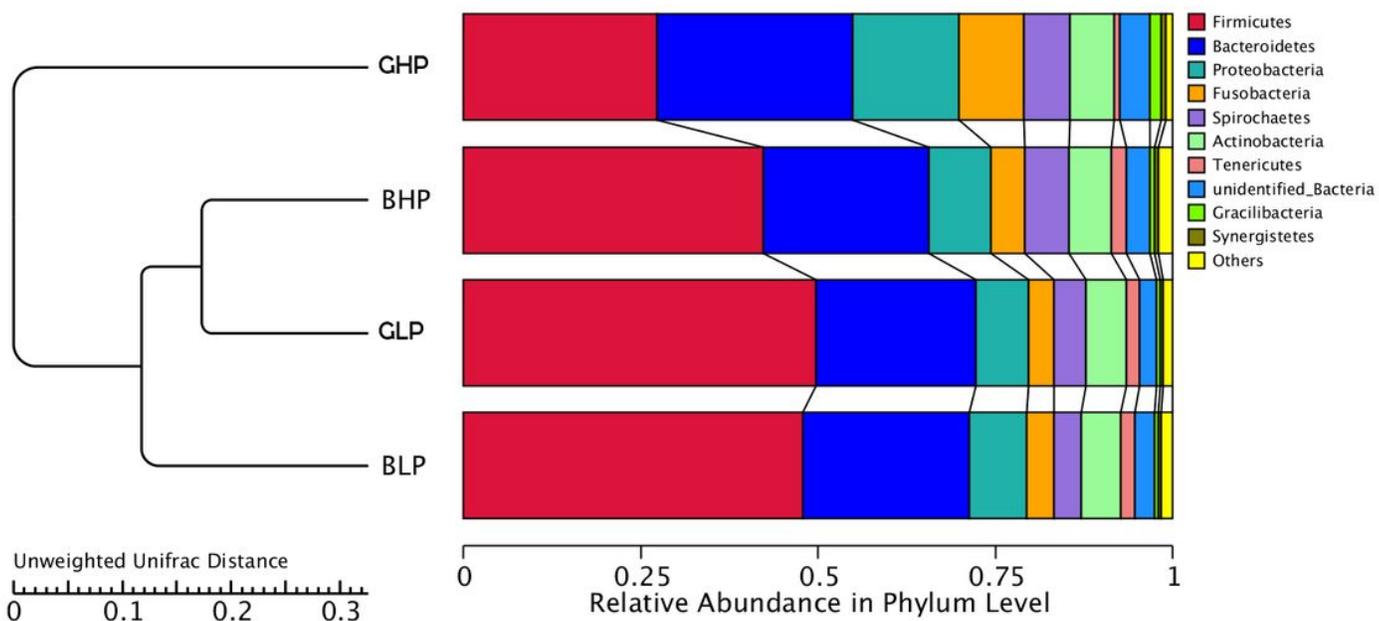


Figure 2

A UPGMA cluster tree based on weighted unifrac distances of the OTU community

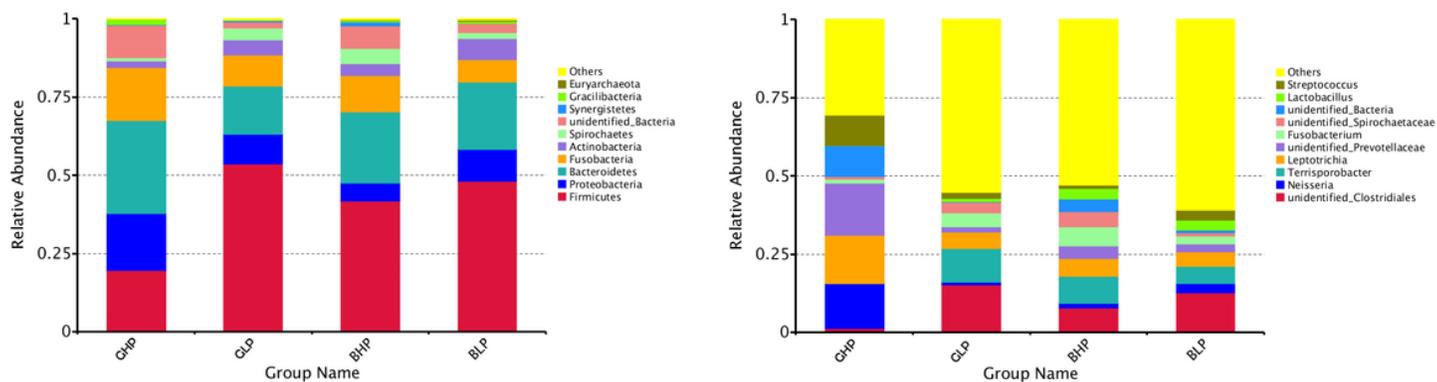


Figure 3

Relative abundance of microbial community (Top 10) structure in the colonic content at different levels. A, Phylum level; B, Genus level

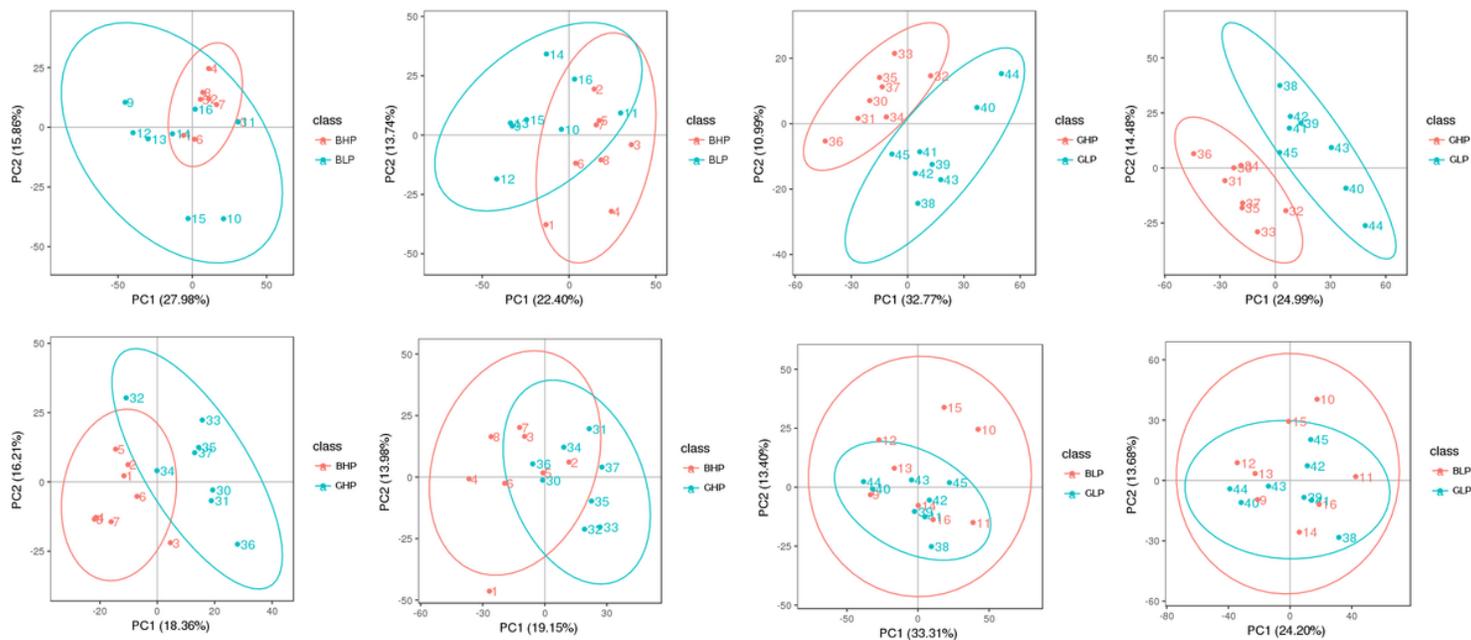


Figure 4

PCA score plot of colonic metabolomic data for barrows and gilts fed low and high protein diets. A, Effects of dietary protein levels; B, Effects of pig sex

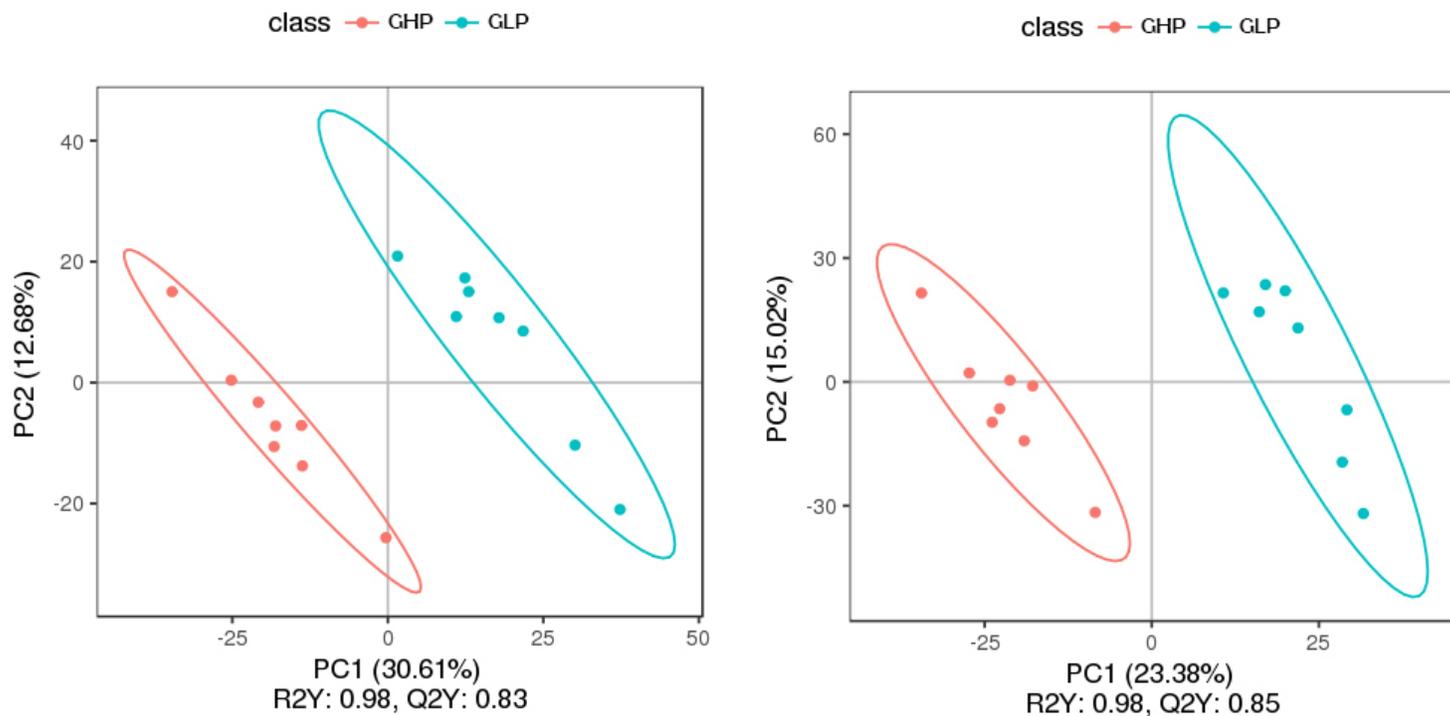


Figure 5

PLS-DA score plot of colonic metabolomic data from gilts fed low and high protein diets

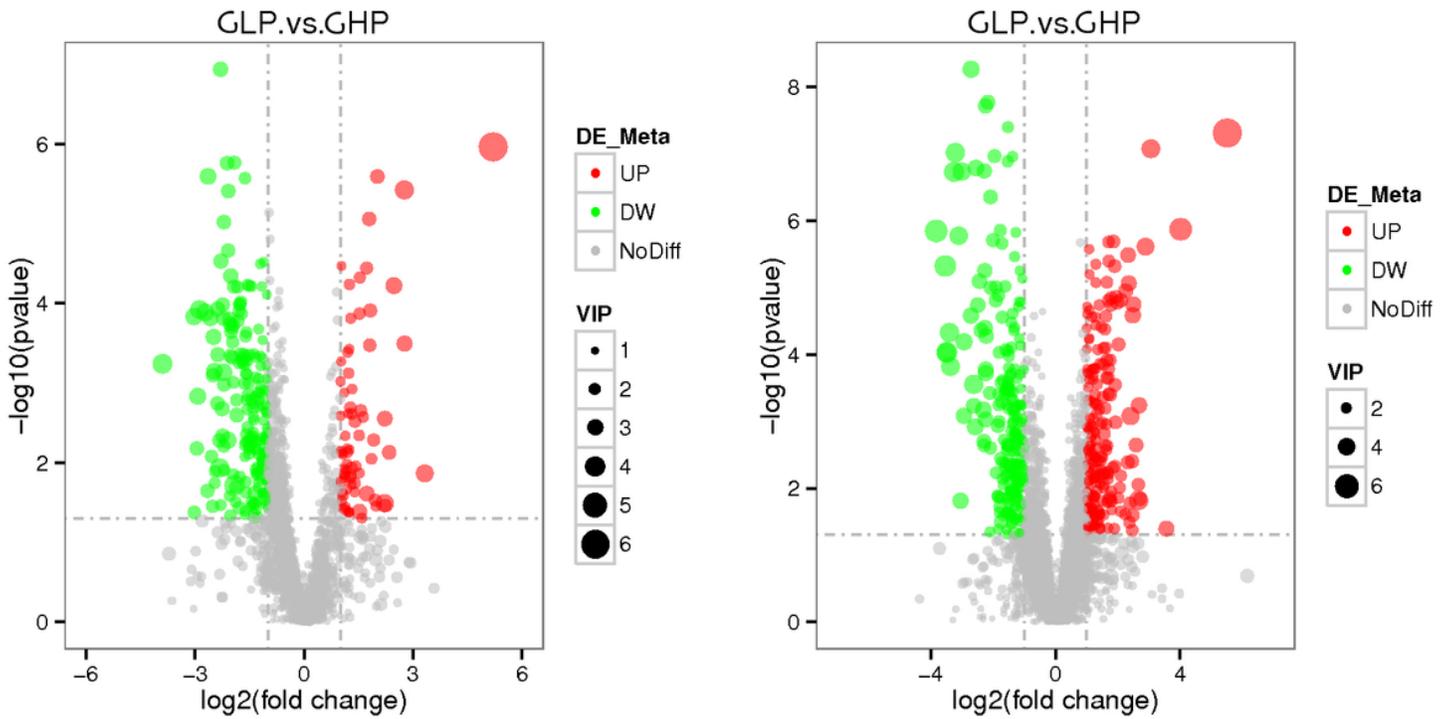


Figure 6

Score volcano plots of colonic metabolomic data from gilts fed low and high protein diets

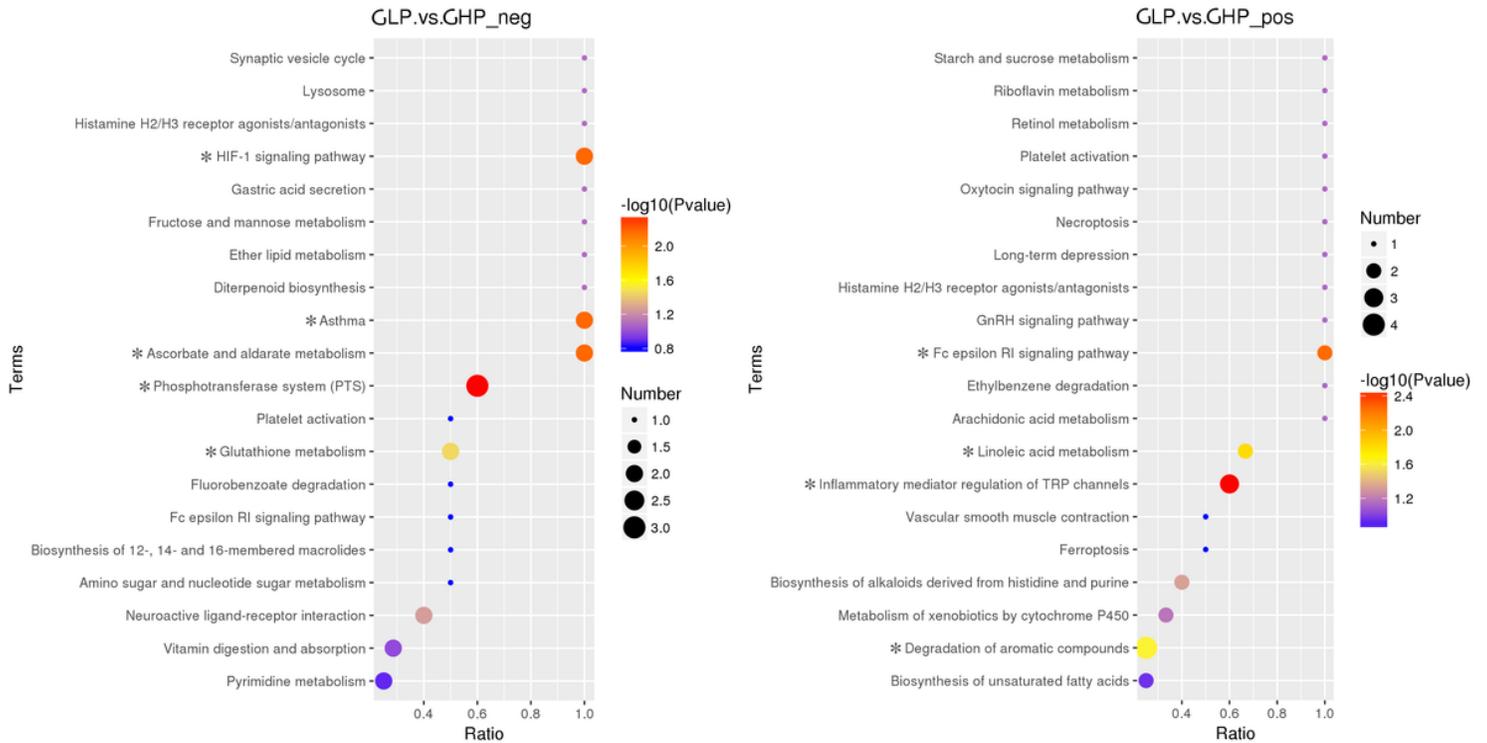


Figure 7

KEGG pathway enrichment of the differentially abundant metabolites between the two gilt groups. * $P < 0.05$, A, LC-MS (ESI-); B, LC-MS (ESI+)

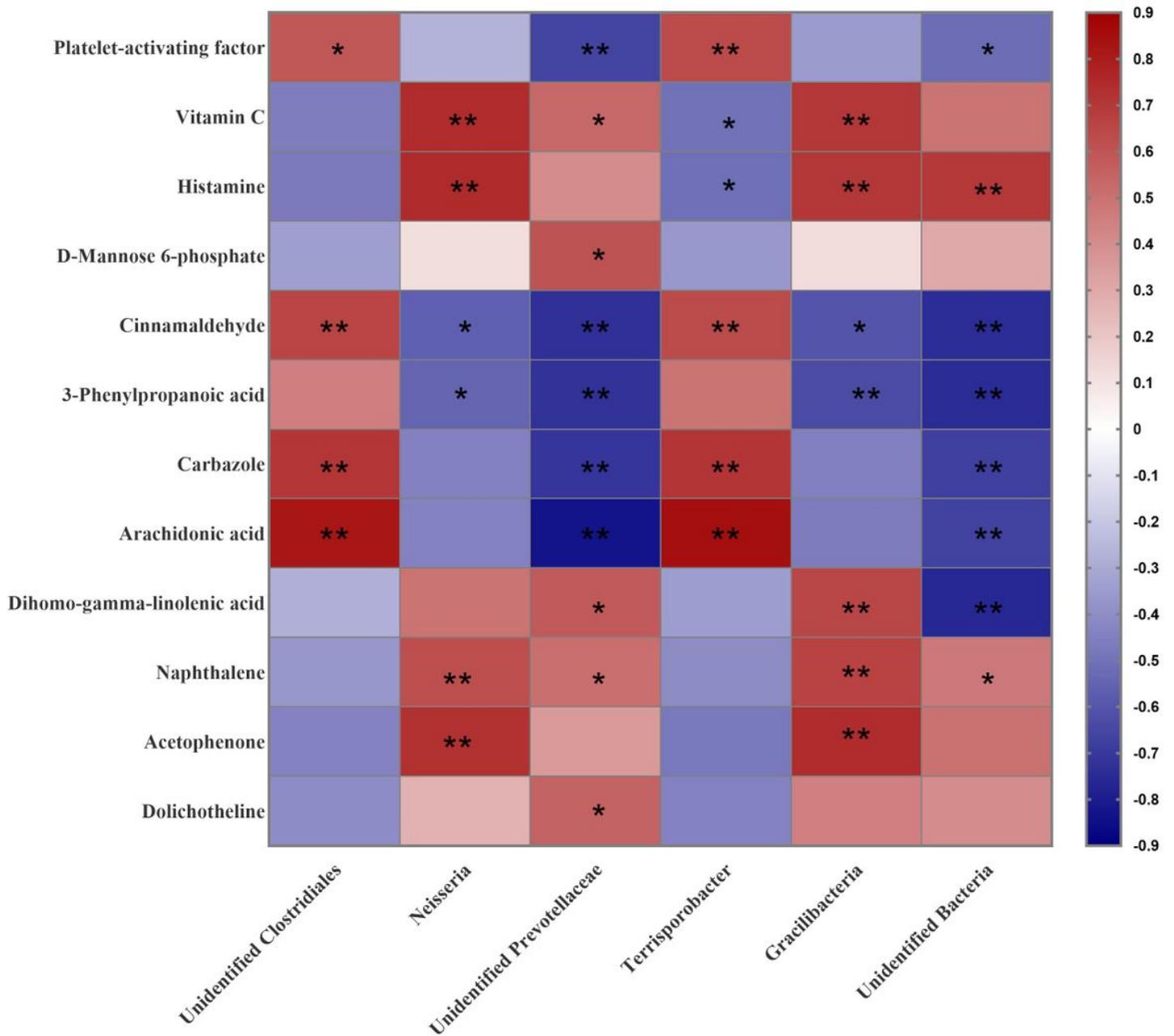


Figure 8

Correlation analysis between the predominant microbial community at genus level (relative abundance) and differentially abundant metabolites in the colonic content of gilts induced by different dietary protein levels

Supplementary Files

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