Integrated analysis of multi-tissues lipidome and gut microbiome reveals microbiota-induced shifts on lipid metabolism in pigs

Chunlin Xie\textsuperscript{a, b, c}, Xiaoyan Zhu\textsuperscript{a, b, c}, Baoyang Xu\textsuperscript{a, b, c}, Yaorong Niu\textsuperscript{a, b, c}, Xuelei Zhang\textsuperscript{a, b, c}, Libao Ma\textsuperscript{a, b, c, *}, Xianghua Yan\textsuperscript{a, b, c, *}

\textsuperscript{a} State Key Laboratory of Agricultural Microbiology, Hubei Hongshan Laboratory, Frontiers Science Center for Animal Breeding and Sustainable Production, College of Animal Sciences and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China
\textsuperscript{b} The Cooperative Innovation Center of Sustainable Pig Production, Wuhan, Hubei 430070, China
\textsuperscript{c} Hubei Provincial Engineering Laboratory for Pig Precision Feeding and Feed Safety Technology, Wuhan, Hubei 430070, China

Abstract

Lipid metabolism is very important for meat quality in pigs. Accumulating evidence shows that gut microbiota can contribute to this physiological process. However, the gut microbiota that function in lipid metabolism and adipogenesis remains unclear. Here, we compared the characteristics of fat deposition and gut microbial community between Laiwu pigs and Duroc × (Landrace × Yorkshire) (DLY) pigs. Fecal microbiota transplantation (FMT) was performed to determine the possible impact of gut microbiota on lipid metabolism in pigs. An integrated analysis of the gut microbiome and lipidome of the small intestine, plasma, and liver was conducted to investigate the effects of FMT on host lipid metabolism. The comparative analysis of the gut microbiome showed higher abundance of Bacteroidetes (P = 0.0018) while lower abundance of Firmicutes (P = 0.012) in Laiwu pigs, and the microbial composition can be transferred from Laiwu pigs into DLY pigs. Transmission electron microscope and Oil red-O staining were performed to analyze the effects of FMT on lipid deposition in liver, the main target organ for lipid metabolism. The results showed that FMT significantly increased the number of lipid droplets (P = 0.0035) and lipid accumulation (P = 0.0026) in liver. Furthermore, integrated multi-tissues lipidome analysis demonstrated that the fatty acyls and glycerolphospholipids were significantly increased (P < 0.01) in intestine and liver, while glycerolipids and fatty acyls were reduced (P < 0.01) in plasma. In the small intestine, FMT increased (P < 0.01) the relative abundance of polyketides and prenol lipids but reduced (P < 0.01) the saccharolipids. Correlation analysis revealed the potential interactions between microbiota and lipid metabolites. Together, our results indicated that the gut microbiota may regulate the lipid metabolism and enhance the accumulation of lipid droplets in the liver of pigs.

1. Introduction

The commensal gut microbiota is a complex microbial ecosystem and is an essential component in the maintenance of lipid metabolism (Suarez-Zamorano et al., 2015). Several studies have indicated that hundreds of gut bacterial species across many taxa may play a critical role in lipid metabolism (Cotillard et al., 2013; Ghazalpour et al., 2016; Le Chatelier et al., 2013). For example, intestinal microbiota contributes to improved host energy and bile acid metabolism, and reduced diet-induced obesity upon cold exposure (Zietak et al., 2016). Administration of Lactobacillus gasseri restored fatty acid metabolism and glucose homeostasis in high fat diet-induced disorders (Bauer et al., 2018). The gut—liver
axis, as one of the most critical interactions between gut microbiota and extra-intestinal organs, represents a close functional and bidirectional link between the gut microbiota and the liver (Konturek et al., 2018). Short chain fatty acids (SCFA), by-products of microbial fermentation, are linked to hepatic lipid metabolism and energy homeostasis (Ringseis et al., 2020). In addition, the metabolites of carbohydrate, protein, lipids, and amino acids in diets are transported to the liver through the portal vein, and may then affect hepatic function (Tripathi et al., 2018). Thus, based on the close association between the gut microbiota and lipid metabolism in liver, the intestinal microbiota affected by the environment will impact lipid metabolism and glucose homeostasis through “gut—blood—liver” axis, thereby altering the fat deposition throughout the body.

Metabolic disorders such as obesity, diabetes, hepatic steatosis, and non-alcoholic fatty liver disease, which pose major challenges to health care, are closely associated with perturbed gut microbiota (Boulange et al., 2016; Guo et al., 2016; Leung et al., 2016; Turnbaugh et al., 2006). Murri et al. (2013) revealed that altered gut microbiome is strongly linked to insulin sensitivity and diabetes. Another study, reported by Chen et al. (2021), systematically investigated the close association between Prevotella copri and fat accumulation in pigs. This study indicated that host chronic inflammatory responses were activated by the metabolites of P. copri. Meanwhile, increasing fat deposition and altering serum metabolites associated with obesity were observed after P. copri administration in germ-free mice (Chen et al., 2021). Pigs are an important animal model to study human obesity-related diseases due to the similarities of phenotype and physiology with humans (Kim et al., 2004; Spurlock and Gabler, 2008).

In the pig industry, intramuscular fat (IMF) and subcutaneous fat have great influence on pork quality and economic returns (Kucha et al., 2018). Previous studies have indicated that the IMF content has a positive correlation with the tenderness, juiciness, and sensory quality of pork (Fortin et al., 2005; Jung et al., 2015). However, the subcutaneous fat contains high amounts of saturated fatty acids and cholesterol, which is a major risk factor for atherosclerosis (Wolfram, 2003). Therefore, production of pork with reduced subcutaneous fat and increased IMF content would be advantageous for the pig industry. The Laiwu pig is a well-known Chinese indigenous breed, characterized by a strong ability to accumulate fat in body (Zeng et al., 2005). In contrast, the Duroc × (Landrace × Yorkshire) (DLY) pig is a widely adopted crossbreed with a faster growth rate but poor lipid storage (Liu et al., 2015). Hence, the significant difference in the characteristics of fat deposition between Laiwu pigs and DLY pigs makes them as ideal models to study the effects of microbiota on lipid metabolism and adipogenesis.

There are accumulating evidence that distinct gut microbiota composition between lean and obese humans can be transferred to mice via fecal microbiota transplantation (FMT) (Backhed et al., 2004; Ley et al., 2005), which has widely been adapted to reconstruct gut microbial communities and host functions (Kootte et al., 2017). In another study, obese Rongchang pigs exhibited a distinct gut microbial composition compared with lean Yorkshire pigs. Furthermore, transferred microbiota from pigs to mice remarkably affects fiber characteristics and fat deposition in skeletal muscle (Yan et al., 2016). All together, these studies have suggested a strong relation between microbiota and lipid deposition, and the lipid metabolic phenotype and fat storage are transferable via microbial transplantation. However, whether and how the gut microbiota in obese pigs regulates lipid metabolic properties and fat accumulation are poorly understood. The objective of this study was to compare the differences in the gut microbial communities between DLY pigs and Laiwu pigs and investigate the relationship between microbiota and lipid metabolism by transferring fecal microbiota from healthy Laiwu pigs into DLY pigs. Integrated multi-tissues lipidomic analysis was performed to reveal the lipid metabolites profile affected by FMT. The experimental design is illustrated in Fig. 1. To further investigate the relationships of microbiota and lipid metabolites, correlation analysis of microbiome and lipidome was performed.

2. Materials and methods

2.1. Animal ethics

The experiment was performed in the Hubei Provincial Engineering Laboratory for Pig Precision Feeding and Feed Safety Technology, and was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, Wuhan, China, under permit numbers HZAUSW-2019-012.

2.2. Experiment procedure and sampling

Feces were collected from mature healthy Laiwu pigs, and the fecal suspension was prepared according to a previously described protocol (Hu et al., 2018). Briefly, fresh feces were collected from 300-d-old healthy Laiwu pigs with similar body weight at the Laiwu Pig Conservation Farm, Shandong. The feces samples were diluted with sterile saline (2 mL sterile saline for 1 g feces) and homogenized in a standard blender to slurry. After that, the slurry was filtered with sterile gauze at least 3 times, followed by a 0.25-mm stainless steel sieve to remove the undigested particles. Subsequently, the filtrates were mixed with sterile glycerol at 10% final concentration and stored at −80 °C immediately to improve the survival rates of fecal microbiota. The count of the live microbes in the fecal suspension was performed with optical microscopy combined with methylene blue staining.

A total of 16 DLY piglets (initial body weight = 29.6 ± 0.18 kg) at the age of 70 days were randomly divided into 2 groups and each group was assigned to 4 pens. The experimental diets were formulated to meet the nutrient requirements recommended by the NRC (2012), and were mainly composed of corn, soybean meal, wheat bran, and rice bran (Table S1). All the pigs were housed in a standard environment with natural light regime, humidity of 50% to 60%, at a temperature of 25 ± 5 °C.

The pigs in control group were treated with 5 mL sterile saline, and the pigs in FMT group were treated with 5 mL fecal filtrates by oral administration every other day in the morning after a meal for 3 months. The pigs were not pre-treated with antibiotics to meet the requirement of antibiotic-free production. Pigs were free access to food and water throughout the experiment. Pigs were slaughtered at market weight. The fresh feces samples were collected from the rectum and stored at liquid nitrogen immediately for 16S rDNA sequencing. Blood samples were drawn from the antecubital vein and kept standing for 1 h at 4 °C, and then centrifuged at 3,000 × g for 15 min at 4 °C to obtained plasma samples. The small intestinal samples were cut from the middle of small intestine and frozen at liquid nitrogen immediately. Liver samples were fixated with 2.5% glutaraldehyde for transmission electron microscopy analysis. The liver tissue samples also embedded in optimal cutting temperature compound and frozen for Oil red-O staining.

2.3. Oil red-O staining

Frozen liver sections were placed in 0.25% Oil red-O solution in isopropanol for 8 to 10 min, then rinsed in 60% isopropanol and counterstained with hematoxylin for 1 min. The villi heights, crypts...
depths, and cross-sectional area of myofiber were measured using Image-Pro Plus software (version 6.0).

2.4. Transmission electron microscopy

For transmission electron microscopy analyzing, liver and muscle samples were collected and fixed in 2.5% glutaraldehyde prepared in 0.1 M Sorensen’s buffer (pH 7.4). Thereafter, the samples were treated with 1% osmium tetroxide in 0.1 M Sorensen’s buffer and were sequentially dehydrated in graded alcohols and propylene oxide, followed by infiltration in Spurrs or Epon. Ultrathin sections of the tissue samples were made using a diamond knife and were visualized with a transmission electron microscope.

2.5. Lipidomic analysis

Un-targeted lipidomic analysis was performed by BGI-Shenzhen, China. The frozen samples of the small intestine and liver were thawed on ice, and then weighed (25 mg) and homogenized with 800 μL frozen homogenization solution (dichloromethane:methanol = 3:1) in a 1.5-ml tube. Two small steel balls were added to the sample tube, and grinded at 50 Hz for 2 min in the TissueLyser. The samples were placed in a refrigerator at −20 °C for 2 h followed by centrifugation with 30,000 × g at 4 °C for 15 min. Taken 650 μL supernatant into a new tube and centrifuged at 4 °C, 25,000 × g for 20 min, 450 μL of the supernatant was dried. A volume of 450 μL of lipid reconstituted buffer (isopropanol:acetonitrile:water = 2:1:1) was added and vortexed for 60 s, followed by centrifugation at 4 °C, 25,000 × g for 15 min. 410 μL supernatant was collected to a new Eppendorf tube and stored at −80 °C until use. The LC–MS analysis was performed on an ultra performance liquid chromatography (UPLC) system (Waters, UK) coupled with a high-resolution tandem mass spectrometer Xevo G2 XS QTOF (Waters, UK). In brief, 10 μL samples were injected, chromatographic separations were performed on an ACQUITY UPLC CSH C18 column (100 mm × 2.1 mm, 1.7 μm, Waters, UK) held at 55 °C. The flow rate was 0.4 mL/min, the gradient elution buffers were solvent A (acetonitrile:water = 60:40, 0.1% formate acid and 10 mM ammonium formate) and solvent B (2-propanol:acetonitrile = 90:10, 0.1% formate acid and 10 mM ammonium formate). The gradient elution conditions: 40% to 43% phase B held for 0 to 2 min; 50% to 54% phase B held for 2.1 to 7 min; 70% to 99% phase B held for 7.1 to 13 min; 40% phase B held for 13.1 to 15 min. The metabolites were detected with Xevo G2-XS QTOF (Waters, UK) mass spectrometer. The Q-TOF was operated in both positive and negative ion modes. The capillary and sampling cone voltages were set at 3.0 kV and 40.0 V for positive ion mode, 2 kV and 40 V for negative ion mode. The Centroid MSE mode was used for acquiring data with an acquisition rate of 5 scans every second. In positive mode, the TOF mass range was from 100 to 2,000 Da; In negative mode, the TOF mass range was from 50 to 2,000 Da. The fragmentor voltage was 19 to 45 eV. The quality control sample was acquired after every 10 samples to evaluate the stability of the LC–MS. The mass spectrometry data were processed and analyzed using Progenesis QI (version 2.2, Waters, UK) software and package metaX in R (v3.11). The LipidMaps and KEGG database were used for metabolite identification.

2.6. Fecal microbiome 16S rDNA sequencing

Fecal samples were collected from Laiwu pigs, DLY pigs, and FMT recipient pigs, and stored at −80 °C until analysis. The bacterial DNA was isolated using Stool DNA Kit (Tiangen, DP712) according to
the manufacturer’s recommendations. The quality of DNA samples was test before used to construct libraries. The primers 515F (5’-GTCCAGCMGCCGCGGTAA-3’) and 806R (5’-GGAC-TACHVGGGTWTCTAAT-3’) were used to amplify the V3–V4 hypervariable variable region of the bacterial 16S rRNA gene. The 16S rDNA gene V3–V4 sequencing was performed by BGI-Shenzhen using an Illumina HiSeq/MiSeq platform. The reads with sequencing adapters, N base, poly base, low quality etc. were filtered out to clean data. The consensus sequence was generated by using FLASH (Fast Length Adjustment of Short reads, v1.2.11) when the 2 paired end reads overlapped. The clean tags were clustered into Operational Taxonomic Unit (OTU) with a 97% similarity by using USEARCH (v7.0.1090). The Ribosomal Database Project (RDP) Classifier (v2.2) trained on the database Greengene was used to classify the OTU representative sequences at a confidence threshold of 0.5. The OTU not assigned or not assigned to the target species were removed. The Beta diversity was calculated by software QIIME (v1.80). Bray–Curtis and weighted UniFrac were used to measure beta diversity. Furthermore, the principal coordinate analysis (PCoA) was performed by QIIME (v1.80) software to exhibit the differences between the samples according to the matrix of weighted UniFrac distance. The significant difference at the level of phylum, class, order, family, genus, and species between 2 groups were analyzed by Metastats (http://metastats.cbcb.umd.edu/) and R (v3.1.1). The adjust P-value was calculated based on Benjamini-Hochberg false discovery rate correction of function ‘p.adjust’ in the stats package of R (v3.1.1). The DNA sequences reported in this paper are publicly available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive and are accessible under BioProject accession number PRJNA683194.

2.7. Correlation analysis

Correlations among metabolites in tissues were estimated based on Pearson’s test. All possible correlations between microbial species and the metabolites in liver were calculated based on Spearman’s test. Calculations and visualization were performed in R (v3.1.1).

2.8. Statistical analysis

Statistical analyses were performed in GraphPad Prism software (v7.0). The significant difference between groups was conducted by unpaired two-tailed Student t-test, P < 0.05 was considered statistically significant. In the statistical analysis of lipidomic data, false discovery rate (FDR) correction was performed to obtain the adjust P-value. The Spearman correlation between microbiota and metabolites was analyzed by the “cor” of R (v3.1.1). Metastats (http://metastats.cbcb.umd.edu/) was applied to identify the differentially abundant taxa of the fecal microbiome. The data were shown as mean ± SEM.

3. Results

3.1. A significant difference in fecal microbiota composition between Laiwu pigs and DLY pigs

Laiwu pigs exhibit a significantly higher intramuscular fat and body fat content than imported commercial DLY pigs (Wang et al., 2020). To identify whether the excellent performance of fat deposition in Laiwu pigs is associated with specific microbiota composition, we performed 16S rDNA sequencing of feces samples collected from finishing Laiwu pigs and DLY pigs. We observed that the microbiota communities in Laiwu pigs were clearly segregated from those in DLY pigs based on principal component analysis (PCA, Fig. 2A). The comparison of the Bray–Curtis distance within Laiwu pigs and DLY pigs showed that the fecal microbiota composition in DLY pigs exhibited greater variability compared to Laiwu pigs (Fig. 2B). Alpha diversity analysis showed significantly increased community richness and diversity in Laiwu pigs, as indicated by increased Chao index (P < 0.0001), ace index (P < 0.0001), and observed species (P < 0.01) (Fig. 2C–E). However, the Laiwu pig showed a reduction in good coverage (P < 0.001) and there were no significant differences in Shannon and Simpson indices (Fig. 2F–H). The hierarchical cluster tree showed a closer distance within the group, further confirmed a distinct difference of gut microbiome community between DLY pigs and Laiwu pigs (Fig. 2I). To further investigate the phylotype distribution at the phylum level, we compared the community profile (Fig. 2I). The dominant phyla in all samples were Bacteroidetes, Firmicutes, Saprochaetes, and Proteobacteria, and a significantly higher proportion of Bacteroidetes (55.4% versus 29%, P < 0.01) were observed in Laiwu pigs compared to DLY pigs. In contrast, DLY pigs exhibited a higher relative abundance of Firmicutes (60.4% versus 38.0%, P < 0.01) and Proteobacteria (1.68% versus 0.66%, P < 0.01) compared to Laiwu pigs.

3.2. The gut microbiota-induced lipid droplets accumulation in liver

To investigate the relationship between microbiota and fat deposition, fecal microbiota collected from Laiwu pigs was transferred into DLY pigs every other day from 70 days to marked weight. Transmission electron microscopy (TEM) analysis was performed and revealed that, compared with control hepatic tissue (Fig. 3A, upper panels), the hepatic tissue displayed a significant (P < 0.05) increase in number of lipid droplets (Fig. 3A and B) after FMT. Consistent with our TEM analysis, Oil red-O staining revealed that hepatic tissue in FMT pigs showed more lipid droplets (Fig. 3C and D). Our results indicated that FMT augmented the hepatic lipid deposition and the phenotype of lipid deposition in liver were closely associated with gut microbiota and can be transferred via FMT. Metabolites reside at the important interface between microbiota and lipid metabolism in different tissues. Here, we combined lipidomic data from more than one tissue to reveal how lipid metabolic pathways are gated to microbiota administration and how tissue-specific and intra-tissues dynamics correlate after FMT.

3.3. Relationship among microbiota-mediated multi-tissues lipidomic profiling

Lipidomic technologies widely be used to elucidate their regulation, function, and physiological impact and to discover how these lipids interact to influence specific biological processes (Jha et al., 2018). To determine whether the increase of hepatic lipid deposition is correlated with shifts of lipid metabolites, we used untargeted lipidomic analysis to measure the shifts of FMT-modulated lipid metabolites profiling on 3 tissues: small intestine, plasma, and liver. Principal coordinate analysis based on UniFrac distance showed distinct clustering of metabolites between Ctrl and FMT group in all tissues (Fig. 4A–C). Overall, total of 9,836, 6,060, and 14,472 filtered ions were detected in the intestine, plasma, and liver, respectively. Out of these, 125, 232, and 289 lipid metabolites were altered by FMT in the intestine, plasma, and liver, respectively (Table S2). Heatmap of cluster analysis further indicated that the remarkable changes in lipid metabolites were induced by FMT. The volcano plots comparing Ctrl and FMT showed significantly altered metabolites, and hepatic metabolites were upregulated in FMT pigs. The intestinal microbiome is an important factor for maintenance of lipid metabolic homeostasis.
While class distribution was similar across tissues (Fig. 5A), relative metabolite abundance reflected tissue composition; these tissues revealed mostly glycerophospholipids (25.75% to 37.33%, Fig. 5B). The impact of FMT varied among tissues (Fig. 5C and D). While more than 4,000 total metabolites were detected in both intestine and liver, more than 2-fold metabolites were altered by
FMT in plasma than in intestine (242 significantly impacted in plasma versus 100 in intestine; Fig. 5C and D). Intestinal metabolites showed more resistant to FMT-induced alterations than plasma and liver (Fig. 5C). The metabolites affected by FMT showed a tissue specificity. For example, glycerophospholipids comprised 37.33% of intestinal metabolites and made up only 15% altered by FMT, while glycerolipids comprised only 16.62% on plasma yet 36.36% altered by FMT (Fig. 5B and D). To characterize the correlations among the metabolites of different classes, we analyzed the correlations of altered metabolites in 3 tissues, respectively. Comparative analysis of intra-tissue metabolites’ correlations indicated that glycerophospholipids had strongest correlation with other metabolites in 3 tissues (Fig. 5E). The different lipid species in plasma exhibited an increased variability compared to the intestine and liver. We defined 4, 6, and 3 distinct lipid clusters in the intestine, plasma, and liver, respectively, using hierarchical clustering (Fig. 5F). Moreover, within significant different lipid metabolites, the correlation between the lipid profiles appeared higher variability in liver compared to the other tissues (Fig. 5F). Previously, our analysis characterized the lipidomic landscape in different tissues and uncovered marked effects of FMT on the lipid metabolites profile of different tissues.

3.4. Comparison of the multi-tissues lipidome under FMT

In order to provide the specific insights into the effects of FMT on the shifts of lipid metabolism, we ranked and visualized the top 50 most differentially abundant metabolites by fold change (Table S3–5). In the liver, clustering heatmap showed that out of the 32 increased lipids in FMT, 14 (43%) were classified as glycerophospholipids (Fig. 6A). The most changed lipids in liver were norlithocholic acid (Fig. 6B) and cyclophosphamide (Fig. 6C) by P-value ($P = 0.0008$) and fold change (FMT:Ctrl = 8.66), respectively. As expected, the FMT increased the total relative abundance of glycerophospholipids ($P < 0.01$) in the differential lipid metabolites (Fig. 6D). Moreover, FMT increased the relative abundance of fatty acyls ($P < 0.01$), prenol lipids ($P < 0.05$), saccharolipids ($P < 0.05$), and sterol lipids ($P < 0.05$), while decreased the relative abundance of...
of glycerolipids and polyketides ($P < 0.05$) in the differential lipid metabolites of liver (Fig. 6D).

In the intestine, out of the 37 increased lipids, 12 (32%) classified as fatty acyls, and only 3 were glycerophospholipids (Fig. S1). However, there was no significant difference in the fatty acyls ($P > 0.05$) of the total differential lipids between FMT and Ctrl pigs. FMT increased the relative abundance of glycerophospholipids ($P < 0.05$), polyketides ($P < 0.01$), and prenol lipids ($P < 0.01$), while decreased ($P < 0.01$) the relative abundance of saccharolipids (Fig. 6D). Interestingly, in the plasma of FMT pigs, 47 lipids were...
decreased in the top 50 most differentially metabolites, only 3 lipid metabolites (22:6 cholesteryl ester, orsellinic acid, and PS [P-20:0/20:5]) were increased. Out of these decreased lipids, 25 (53%) were classified as glycerolipids (Fig. S2). As expected, the total relative abundance of glycerolipids in the differential metabolites of plasma were decreased. Moreover, plasma from FMT pigs showed decreased levels of fatty acyls ($P < 0.01$), glycerophospholipids ($P < 0.01$), polyketides ($P < 0.05$), and prenol lipids ($P < 0.01$) than...
Fig. 6. Fecal microbiota transplantation (FMT) induced shifts on lipid metabolites in intestine, plasma, and liver. (A) The top 50 significant different lipid metabolites by fold change in the liver, clustered using Euclidean distance. The most changed features in FMT pigs were (B) norlithocholic acid and (C) cyclophosphamide. (D) Comparison the relative abundance of the main classes of lipids in the differential metabolites. Statistical analysis was performed using unpaired Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

FA = fatty acyls; GL = glycerolipids; GP = glycerophospholipids; PK = polyketides; PR = prenol lipids; SL = saccharolipids; SP = sphingolipids; ST = sterol lipids.
those Ctrl pigs, while increased (P < 0.05) the level of sphingolipids (Fig. 6D). Taken together, these data revealed a distinct lipid metabolism response in different tissues under microbial mediation.

3.5. Difference in microbial community composition after FMT treatment

We investigated the microbial alterations associated with increased hepatic lipid deposition using 16S rDNA sequencing analysis of fecal microbiota in pigs after FMT experiment. Bray Curtis-based PCA differentiated FMT pigs from the Ctrl pigs (Fig. 7A). The analysis of PCA based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway further indicated that a significant difference in the microbial community between FMT pigs and Ctrl pigs and suggested that FMT caused variation in the function of microbiota (Fig. 7B). At class level, all pigs in the FMT and Ctrl were dominated by Bacteroidia and Clostridia, which belongs to Bacteroidetes and Firmicutes respectively (Fig. 6C). The result also indicated that FMT caused shifts from DLY pigs to Laiwu pigs in the bacterial community structure (Fig. 7D). For instance, the relative abundance of Clostridia in Ctrl pigs was 51.3%, and it was reduced to 35.4% in FMT pigs, which is close to the levels of DLY pigs (37.4%). Consistently, FMT-derived fecal samples showed significant increase in Bacteroidia (46.6%), which had higher relative abundance in fecal samples of Laiwu pigs than DLY pigs (55.4% versus 33.8%). At genus level, the difference in the microbial composition induced by FMT was shown in Fig. 53. The gut microbial community in FMT pigs had higher proportions of Pyramidobacter.

To identify the specific microbes that affect fat deposition, we compared the relative abundance of fecal microbial species identified in Ctrl pigs and FMT pigs. Differentially abundant species between the 2 groups were performed using metatransit analysis and the data showed that the relative abundance of 5 bacterial species (Bacteroides uniformis, Treponema pectinovorum, Sphaerochaeta globosa, Hydrogenoanaerobacterium saccharovorans, and Pyramidobacter pisolens) in FMT pigs were significantly higher than that in Ctrl pigs (Fig. 7E). In contrast, seven bacterial species (Cloestridium methylpentosum, Blautia wexlerae, Vampirovibrio chlorellavorus, Corynebacterium stationis, Robinsoniella peoriensis, Escherichia, and Actinobacillus porcinus) exhibited lower relative abundance in FMT pigs (Fig. 7E).

3.6. Correlation analysis between microbial species and lipid metabolites

To gain insights into the relationships between identified specific bacterial species and differential lipid metabolites, we performed an integrated microbiome and lipidome analysis based on Spearman’s correlations. The differential metabolites were screened with the P-value < 0.05 and the fold change > 1.5. The correlation heatmap showed that the glycerophospholipids was main class affected by FMT in liver (Fig. 8). We observed a strong, positive correlation between up-regulated bacterial species and glycerophospholipids-classified metabolites (Fig. 8), suggesting that the up-regulated bacterial species might strongly linked to the increased lipid accumulation in liver. The 4-hydroxynonenal and sabinene hydrate in intestine showed a significant positive correlation with the microbial species which were increased in FMT pigs (Fig. 5A4). Nevertheless, glycerolipids was the most covered metabolite changed by FMT in plasma (Fig. 5A8) and showed a strong negative correlation with the increased microbial species in FMT pigs. Of note, Treponema pectinovorum shown strong correlation with most of metabolites which altered by FMT in 3 tissues. On the contrary, C. stationis showed significant positive correlations with the most of glycerolipids which were decreased in the plasma after FMT (Fig. S4B). Therefore, these data suggested microbiota change was strongly related to peripheral region lipid metabolites and would affect hepatic lipid metabolism and deposition.

4. Discussion

Laiwu pigs and DLY pigs exhibit notable differences in fat deposition (Liu et al., 2015; Zeng et al., 2005). Some studies have reported that Chinese indigenous pig breeds (obese type) showed distinct microbial communities from commercial pig breeds (Xiao et al., 2018; Yang et al., 2014). The Bama sows, which exhibit many properties such as high intramuscular fat content and excellent meat quality, showed higher gene copy of Bacteroidetes compared to Duruc pigs (Yang et al., 2014). Consistent with previous studies (Allen et al., 2011; Yan et al., 2016), the fecal microbiota divided into 2 different clusters between Laiwu pig and DLY pig, and dominated by Bacteroidetes, Firmicutes, Spirochaetes, and Proteobacteria both in Laiwu and DLY pig. In addition, a higher relative abundance of Bacteroides was observed in Laiwu pigs and their FMT recipients. Bacteroides, predominant intestinal symbiotic bacterium, have sophisticated ability to degrade polysaccharides for energy acquisition (Sommenburg et al., 2009). The significantly higher abundance of Bacteroidetes in Laiwu pigs and their microbiota recipients may be related to the excellent roughage tolerance of Chinese indigenous pig breeds. A significant reduction in abundance of Clostridia was observed in pigs with FMT administered. The decrease in Clostridia communities was also observed in the fecal microbiome of obese adults compared to normal adults (Li et al., 2021). It was reported that Clostridia is associated with fat absorption and alleviation of obesity in mice (Petersen et al., 2019). Moreover, the significantly reduced proportion of Clostridia in diabetic patients suggests that their reduction is associated with pathogenic properties (Qin et al., 2012). Thus, the transplantation of fecal microbiota from healthy Laiwu pigs might improve the health of DLY pigs by decreasing the relative abundance of Clostridia.

Mounting evidence confirmed that the gut microbiome responds to environmental stimuli in ways that impact a variety of metabolic properties including carbohydrate, lipid, and protein metabolism (Holscher, 2017). A strong association between microbial communities and lipid metabolism in humans and animals were established in recent years, but the mechanisms and causal relationship remain unclear. A previous study has shown that the gut microbial inoculation induces expression of stearoyl-Coenzyme A desaturase (Scd1) and ELOVL fatty acid elongase 5 (Elov5) resulted in significant alterations in glycerophospholipid acyl-chain profiles in liver (Kindt et al., 2018). Glycerophospholipids are critical components of cellular membrane and exhibits high structural diversity, which strongly links to biological and physiological functions of animal (Antonny et al., 2015). Our data also shown that the lipid metabolites altered by FMT were mainly classified into glycerophospholipids in liver and showed positive correlations with B. uniformis, Treponema pectinovorum, S. globosa, H. saccharovorans, and P. pisolens (Fig. 8). A previous study reported that the abundance of Bifidobacterium pseudolongum was strongly correlated with the glycerophospholipid metabolism (Zhao et al., 2020). Thus, it can be speculated that the hepatic glycerophospholipid metabolism is significantly modulated by gut microbiota. However, the mechanism of glycerophospholipid synthesis promoted by FMT as identified in this study needs to be clarified in further study.

Fatty acyls are synthesized by chain elongation of acetyl-CoA with malonyl-CoA groups under the elongases, which are important energy sources and building blocks of complex lipids (Xicoy et al., 2019). The esterification of fatty acyls with glycerol gives rise to the glycerolipids and the fatty acyls usually derived from
Fig. 7. Fecal microbiota transplantation (FMT) shifts gut microbial composition in DLY pigs. (A) A principal components analysis (PCA) score plot showing a distinct microbial composition between Ctrl pigs and FMT pigs, each data point represents one pig. (B) Scatterplot from PCA based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis by Phyloween Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) in intestinal bacterial communities of pigs. (C) Clustering analysis of the stool microbiota composition at class level. The hierarchical cluster tree on the left represented the clustering of subjects. The bar plot on the right represented the relative abundance of the bacteria. (D) Relative abundance of Bacteroidia and Clostridia, statistical analysis was performed using unpaired Student’s t-test, \(* P < 0.05\). (E) Comparison of the relative abundances of different bacterial species of Ctrl pigs and FMT treated pigs, statistical difference was assessed through metastats analysis. DLY = Duroc \(\times\) (Landrace \(\times\) Yorkshire); Ctrl = control.
phospholipids or triglycerides. Our data has shown that FMT reduced the relative level of glycerolipids and increased the glycerophospholipids and fatty acyls in liver. These results perhaps indicate that microbiota facilitate the conversion of glycerolipids to fatty acyls. Furthermore, in the plasma of FMT pigs, the levels of 7 classes of lipids were reduced except sphingolipids, but 5 classes of lipids were increased in liver. The results suggested that the microorganisms promote the accumulation of lipids in the liver and reduce the catabolism of fat. Lipids in sphingolipids play important roles in regulating inflammation and immunity (Hannun and Obeid, 2018). Gut microbiota could have several sphingolipids-related effects. The production of sphingolipid by Bacteroides species promotes intestinal health and the deficiency of bacterial sphingolipid correlates with inflammatory bowel diseases in

![Correlation heatmap for hepatic metabolites and bacteria](image)

**Fig. 8.** Heatmap showing the spearman correlation between significant difference bacteria’s relative abundance at species level and metabolites in liver. The spearman correlation matrix was constructed in R using the heatmap2 function, *P* < 0.05, **P** < 0.01, red and blue indicate positive and negative correlations, respectively. FA – fatty acyls; GL – glycerolipids; GP – glycerophospholipids; LP – lipids and lipid-like molecules; PK – polyketides; PR – prenol lipids; ST – sterol lipids; SP – sphingolipids.

![Integrative diagram of the effects of gut microbiota on lipid metabolism](image)

**Fig. 9.** Integrative diagram of the effects of gut microbiota on lipid metabolism. Red arrows represent the up regulation in experimental group and green arrows represent the down regulation in experimental group. Microbiome: Bacteroides uniformis, Treponema pectinovorum, Sphaerochaeta globosa, Hydrogenoanaerobacterium saccharovorans, and Pyraminobacter piscolens.
humans (Brown et al., 2019). Bacteroidetes are the most dominant members known to synthesize sphingolipids in animal intestine (Yatsunenko et al., 2012). In our results, a significantly higher proportions of Bacteroidetes in Laiwu pigs and FMT pigs combined with the increased of sphingolipids in plasma of FMT pigs revealed that the characteristic of bowel disease resistance in Chinese indigenous pig breeds may be related to the production of sphingolipids by Bacteroidetes species.

5. Conclusions

In this study, we compared the fecal microbiome between Laiwu pigs and DLY pigs using 16S rDNA sequencing analysis and found that obese Laiwu pigs exhibit a distinct microbial composition compared to lean DLY pigs. Furthermore, we transferred fecal microbiota from Laiwu pigs into DLY pigs and found that the FMT replicates the characteristics of hepatic lipid droplet deposition of the donors. The integrated multi-tissues lipidomic analysis indicated that gut microbiota plays a critical role in mediating lipid metabolism and revealed a remarkable distinct lipid metabolism response in different tissues. The correlation analysis between microbiota and metabolites enhanced our ability to screen the specific bacterial species with potential biological significance in lipid accumulation. The proposed results are summarized in Fig. 9.

In summary, our microbial transplantation studies highlight that we can leverage multi-tissues lipidomic analysis to advance understanding the interactions of lipid metabolic profile and microbial communities to identify potential biomarkers involved in lipid deposition of pigs.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that could inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

This work was supported by the Natural Science Foundation of China (31925037).

Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.janinu.2022.04.011.

References

Allen HK, Looff T, Bayles DO, Humphrey S, Levine UY, Alt D, et al. Antibiotics in feed induce prohages in swine fecal microorganisms. mBio 2011;2.
Antony B, Vanni S, Shindou H, Ferreira T. From zero to six double bonds: phospholipid unsaturation and organelle function. Trends Cell Biol 2015;25:427–36.
Backhed F, Ding H, Wang T, Hooper LV, Koh KY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci USA 2004;101:15718–23.
Bauer PV, Duca FA, Waise TMZ, Dranse HJ, Rasmussen BA, Puri A, et al. Lactobacillus gasseri in the upper small intestine impacts an ACSL3-dependent fatty acid-sensing pathway regulating whole-body glucose homeostasis. Cell Metab 2013;17:572–87, e576.
Boulange CL, Neves AL, Chiloux J, Nicholson JK, Dumas ME. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Med 2016;8:42.
Brown EM, Ke X, Hitchcock D, Jeanfavre S, Avila-Pacheco J, Nakata T, et al. Bacteroides-derived sphingolipids are critical for maintaining intestinal homeostasis and symbiosis. Cell Host Microbe 2019;25:668–80, e667.
Chen C, Fang S, Wei H, He M, Fu H, Xiong X, et al. Prevotella copri increases fat accumulation in pigs fed with formula diet. Microbiome 2021;9:175.
Coltillard A, Kennedy SP, Long JC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. Nature 2013;500:585–8.
Fortin A, Robertson WM, Tong AK. The eating quality of Canadian pork and its relationship with intramuscular fat. Meat Sci 2005;69:297–305.
Chazalpour J, Cardona F, Lai KP, Chen J. Integrated omics analysis reveals the altered of gut microbe-metabolites in obese adults. Brief Bioinform 2021;22(3):bbaa165.
Jha P, McDevitt MT, Gupta R, Quiros PM, Falony G, et al. Effect of gut microbiota transplantation in pigs. Front Microbiol 2018;9:1328.
Kucha CT, Liu L, Ngadi MO. Non-destructive spectroscopic techniques and multivariate analysis for assessment of fat quality in pork and pork products: a review. Sensors (Basel) 2018;18.
Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013;500:541–6.
Leung C, Rivera L, Furness JB, Angus PW. The role of the gut microbiota in NAFLD. Nat Rev Gastroenterol Hepatol 2016;13:412–25.
Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 2006;103:11070–5.
Li R, Huang X, Liang X, Su M, Lai KP, Chen J. Integrated omics analysis reveals the alteration of gut microbe-metabolites in obese adults. Brief Bioinform 2021;22(3):bbaa165.
Wei H, Xiong X, Yang J, Zhou L, Yang B, Li H, et al. Genome-wide association analyses for meat quality traits in Chinese Erhualian pigs and a Western Duroc x [Landrace x Yorkshire] commercial population. Genet Sel Evol 2015;47:44.
Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Sorribes E, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC Med 2013;11.
Petersen C, Bell R, Køag KA, Lee SH, Soto R, Ghazarian A, et al. T cell-mediated regulation of the microbiota protects against obesity. Science 2019;365:340–4.
Qin J, Li YR, Cai ZM, Li SH, Zhu JF, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 2012;490:55–60.
Ringseis R, Gesner DK, Eder K. The gut-liver axis in the control of energy metabolism and food intake in animals. Annu Rev Anim Biosci 2020;8:295–319.
Sonnenburg JL, Xu J, Leip DD, Chen CH, Westover BP, Weatherford J, et al. Glycan metabolism, gut microbiota and host metabolism. Curr Opin Lipidol 2016;27:722.
Tripathi A, Debelius J, Brenner DA, Karin M, Loomba R, Schnabl B, et al. The gut-liver axis: how do gut bacteria influence the liver? Gut 2018;67:172–84.
Vanhooren S, Vernooy L, Van de Wiele T, Desreumaux D, De Preter K, Van Herreweghe L, et al. Microbiota and prebiotics and the gastrointestinal microbiota. Gut 2014;63:184–95.
Xianghua Yan

Author contributions

Chunlin Xie: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing - Original Draft.
Xiaoyan Zhu: Investigation, Visualization.
Xiaobin Xu: Investigation, Visualization.
Yaorong Niu: Investigation, Validation.
Chunlin Xie: Investigation, Visualization.
Xuelei Zhang: Formal analysis, Investigation, Visualization.
Yaorong Niu: Investigation, Visualization.
Kottert H: Conceptualization, Supervision, Project administration, Resources.
Xianghua Yan: Conceptualization, Supervision, Project administration, Funding acquisition.

Animal Nutrition 10 (2022) 288–293
Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JL. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.

Wang H, Wang J, Yang DD, Liu ZL, Zeng YQ, Chen W. Expression of lipid metabolism genes provides new insights into intramuscular fat deposition in Laiwu pigs. Asian-Australas J Anim Sci 2020;33:390–7.

Wolfram G. Dietary fatty acids and coronary heart disease. Eur J Med Res 2003;8:321–4.

Xiao Y, Kong F, Xiang Y, Zhou W, Wang J, Yang H, et al. Comparative biogeography of the gut microbiome between Jinhua and Landrace pigs. Sci Rep 2018;8:5985.

Xicoi H, Wieringa B, Martens CJM. The role of lipids in Parkinson’s disease. Cells 2019;8.

Yan H, Diao H, Xiao Y, Li W, Yu B, He J, et al. Gut microbiota can transfer fiber characteristics and lipid metabolic profiles of skeletal muscle from pigs to germ-free mice. Sci Rep 2016;6:31786.

Yang L, Bian G, Su Y, Zhu W. Comparison of faecal microbial community of lantang, bama, erhuai, mianzhu, xiaomeishan, duroc, landrace, and yorkshire sows. Asian-Australas J Anim Sci 2014;27:898–906.

Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222–30.

Zeng QY, Wang GL, Wei SD, Wang LY, Yang HL, Cao HF, et al. Studies on carcass and meat quality performance of crossbred pigs with graded proportions of Laiwu Black genes. Yi Chuan 2005;27:65–9.

Zhao MJ, Jiang ZL, Cai HY, Li Y, Mo QF, Deng LL, et al. Modulation of the gut microbiota during high-dose glycerol monolaurate-mediated amelioration of obesity in mice fed a high-fat diet. mBio 2020;11.

Zietak M, Kovatcheva-Datchary P, Markiewicz LH, Stahlman M, Kozak LP, Backhed F. Altered microbiota contributes to reduced diet-induced obesity upon cold exposure. Cell Metab 2018;23:1216–23.