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Supplemental Information

Obese Ningxiang pig-derived microbiota rewires carnitine metabolism to promote muscle fatty acid deposition in lean DLY pigs

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Experimental details

Animal studies

All animal studies were performed in conformance with the institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University. 43 obese Ningxiang pigs (male, approximately 80 kg body weight) and 50 commercial lean DLY pigs (male, approximately 115 kg body weight) were selected from two abattoirs for muscle FA and gut microbial analyses.

Mutual fecal microbial transplantation between obese Ningxiang pigs and lean DLY pigs

Sixteen male DLY and 14 male Ningxiang pigs with a similar body weight (20.01±0.28) were co-housed in the same pig farm and fed the same diet. 60 mL Fresh fecal samples were immediately collected from the control DLY (DLY_{cont}) and Ningxiang pigs (NXP_{cont}) and mixed with 240 mL precooling normal saline for centrifugation (3000r/min, 5 min, 4 ℃). The supernatant of the microbial fluid was harvested and orally transplanted into DLY (DLY_{FMT}) and Ningxiang pigs (NXP_{FMT}) (Figure 2A). Fecal microbiota were isolated and transplanted every day for 14 weeks.

Isolation and culture of *L. reuteri* from Ningxiang pigs

Fresh fecal samples from an adult Ningxiang pig were collected and used for *L. reuteri* isolation. One g fecal sample was suspended in phosphate buffered saline (PBS) buffer and different diluted samples were plated on MAR medium (Changsha WellBio., 37 °C for 2–7 days) to isolate *L. reuteri*. Each single colony from each plate was purified in a new MAR plate, and the single bacterial colony was sequenced using two universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The 16S rRNA gene sequences were aligned to the NCBI nucleotide sequence database to identify *L. reuteri* strains. The isolated *L. reuteri* XL0930 was further stored at the China Center for Type Culture Collection (CCTCC M 20211240).

Colonization of obese Ningxiang pig-derived *L. reuteri* in DLY pigs

16 male DLY pigs (90-day old) were divided into two groups (n=8): control pigs received normal saline and *L. reuteri* treated pigs were orally administered 1×10^11CFU Ningxiang pig-derived *L. reuteri* XL0930 every day for 60 days (the dosage of *L. reuteri* XL0930 was accorded to previous study, data not show).

Inhibition or activation of SLC22A5 in murine model

6-week old ICR mice (male) were administered 40 mg/kg WY-14643 and 100 mg/kg mildronate for 14 weeks to activate or inhibit SLC22A5, respectively. WY-14643 (S8029), and mildronate (S4130) were obtained from Selleck Chemicals. SAT, peri-epididymal adipose tissue (PEAT), abdominal adipose tissue (AAT), perirenal adipose tissue (PAT), and epididymal fat (EAT) were separated and weighed.

Dietary L-carnitine supplementation in DLY pigs

16 male DLY pigs (74.24±1.77 body weight) were divided into 2 groups (n=8), control pigs were fed a basic diet and carnitine administrated pigs were fed the basic diet supplemented 100 mg/kg for 35 days [1].

Addition of L-carnitine in 3T3-L1 cells

3T3-L1 preadipocytes were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% (v/v) bovine calf serum (BCS, Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C under a humidified 5% CO_2 atmosphere. For differentiation, 10,000 cells/well were seeded in 96 well plates and allowed to reach confluence (termed Day -2). The 2-day post-confluent 3T3-L1 cells were induced in DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), 1 μM dexamethasone (Sigma), 10 μg/mL insulin (Sigma), and 10% fetal bovine serum (FBS, Gibco). After incubation for two days, the medium was replaced with maintenance DMEM medium containing 10% FBS and 10 μg/mL insulin for an additional six days. L-carnitine (0, 1, 10, 100, 1000, and 10,000 mM) was used to stimulate the cells during differentiation from day 0 to day 8. The effect of L-carnitine on the viability of differentiated 3T3-L1 cells was assessed using the MTT assay.

On day 8, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with PBS, cells were stained with 0.5% Oil Red O solution in 60% isopropanol. After washing once with 60% isopropanol and twice with PBS, and cells were photographed under microscope (Zeiss). For quantification, the retained dye was solubilized in 100% isopropanol, and measured at absorbance of 510 nm using multifunctional microplate reader (TECAN).

Full-length 16S rRNA sequencing for gut commercial bacteria

The DNA from the ileal mucosas of Ningxiang and DLY pigs was extracted using the Soil/Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The universal primer sets 27F: AGRGTTTGATYNTGGCTCAG and 1492R: TASGGHTACCTTGTTASGACTT were used to amplify the full-length 16S rRNA gene from the genomic DNA. Bioinformatics analyses of α-diversity (Simpson, Chao1, and ACE indices), β-diversity, and bacterial abundance were performed using the BMK Cloud (Biomarker Technologies Co., Ltd., Beijing, China).

Metagenomics sequencing for gut microbiota

A paired-end library with an insertion size of 350 bp was constructed for 30 pig samples (Illumina, USA) and sequenced using a Novaseq 6000 platform (Illumina, USA). High-quality reads were obtained by filtering out adaptors, low-quality reads, and host genomic DNA contaminants from the raw data. The distance between samples, β-diversity (PCoA plot), α-diversity (Shannon and Simpson indexes), microbial abundance, Venn analysis, linear discriminant analysis effect size (LEfSe), and functional annotations (Kyoto Encyclopedia of Genes and Genomes, KEGG databases) were performed using the BMK Cloud (Biomarker Technologies Co., Ltd., Beijing, China).

GC-MS targeted metabolomics for fatty acid analysis

Muscle and chymous FA were determined using GC-MS-targeted metabolomics by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. First, muscle and chymous samples were treated with 1 mL chloroform and methanol (1:1, vol:vol), and the samples were preformed vortex ultrasound (30 min) and centrifugation (13000r/min, 10 min, 4 ℃). Next, the supernatant samples were dried with nitrogen and mixed with 0.5 mL methylation reagent (0.5 mol/L sodium hydroxide methanol solution) in water bath (60 °C for 30 min). The cooled samples were further added 0.5 mL n-hexane and the supernatant (n-hexane layer) samples were harvested for GC-MS (8890B-5977B GC/MSD, Agilent Technologies Inc. CA, UAS). Masshunter software v10.0.707.0, Agilent, USA) was used to quantify FA.

LC-MS untargeted metabolomics for muscle and intestinal metabolic profiles

Muscle and intestinal samples (50 mg) were mixed with 400 μL of methanol:water (4:1, v/v) solution for 30s and ultrasound at 40 kHz for 30 min at 5 ºC. The samples were placed at -20 ºC for 30 min to precipitate the proteins. After centrifugation at 13000 g at 4 ºC for 15 min, the supernatant was carefully transferred to sample vials for LC-MS/MS analysis (Thermo UHPLC-Q Exactive Mass Spectrometer). Statistically significant differences among groups were selected with a p-value less than 0.05, using the free online platform Majorbio Cloud Platform (www.majorbio.com).

Meat quality

Backfat thickness (mm) between the 6th and 7th ribs was measured using a Vernier caliper, post-mortem pH of the muscle at 45 min and 24 h was measured using a portable pH meter (Matthaus pH Star, Germany), and color parameters (L∗, lightness; a∗, redness, and b∗, yellowness) were determined in duplicate at 45 min post-mortem, using a CR-410 hand-held colorimeter (Kinica Minolta Sensing Inc., Osaka, Japan).

The mean value of the three measurements was used as the final result, and the intramuscular fat content was determined with petroleum ether using a Soxtec Extraction method. All meat quality traits were measured at the same anatomical location on longissimus muscle samples from the left carcass.

Gene expression

Gene expression was determined using real-time PCR. Total RNA from intestinal and adipose tissues was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then treated with DNase I (Invitrogen). The Reverse transcription was conducted at 37 °C for 15 min and 95 °C for 5 s. A comparative relationship between reaction cycles (CT) was used to determine gene expression relative to the β-actin control (housekeeping gene). The primers used in this study and the relative abundances of target genes were calculated according to previous studies [2-5].

Muscle total L-carnitine determination

Total muscle L-carnitine levels were determined using an ELISA kit. Muscle samples (1 g) were mixed with 9 ml of extraction solution (n-butanol: methanol: water=5:25:70 V:V:V) to homogenize the specimens adequately using a homogenizer. The samples were centrifuged for approximately 20 minutes (2000-3000 rpm), and the supernatant was collected for ELISA testing according to a previously described procedure.

Statistical analysis

All values are presented as the mean ± SEM. Differences among the groups were compared by Student's t-test using IBM SPSS Statistics 22 software, and the differences were considered significant at $P < 0.05$. Pearson correlation analysis was used to evaluate the potential associations between gut microbiota, metabolites, and muscle FA, with a value of $P < 0.1$ means the significance. All figures were drawn using the GraphPad PRISM 9 software.

References

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2. Gong, S., Yin, Y., Han, M., et al. (2023). Dietary leucine and fish oil cooperatively regulate skeletal myofiber type transformation via the CaMKII signaling pathway of pigs. Food Funct. ***14***, 133-147.
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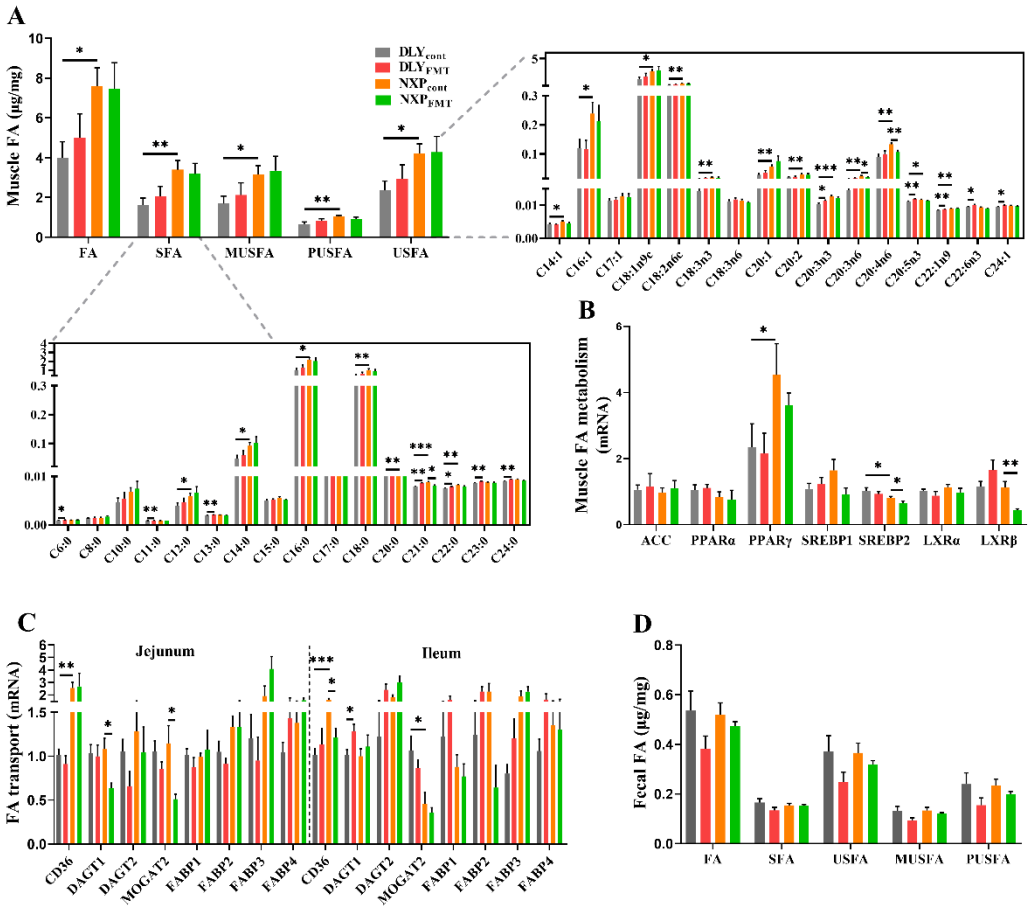
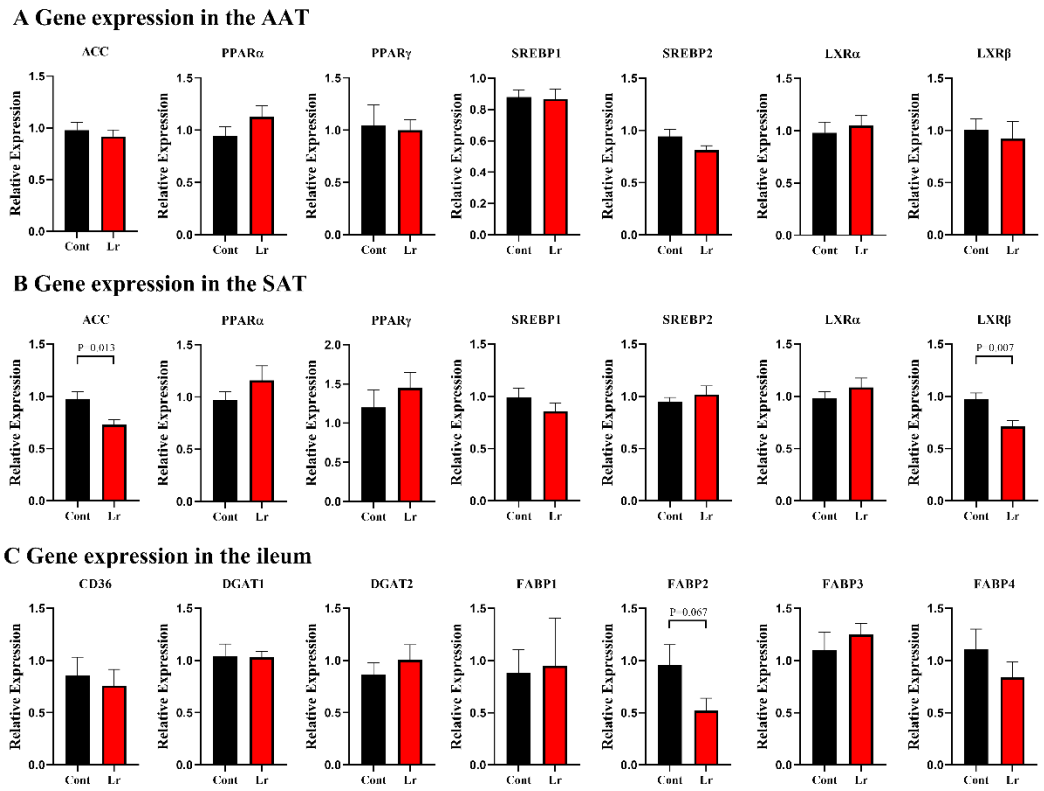
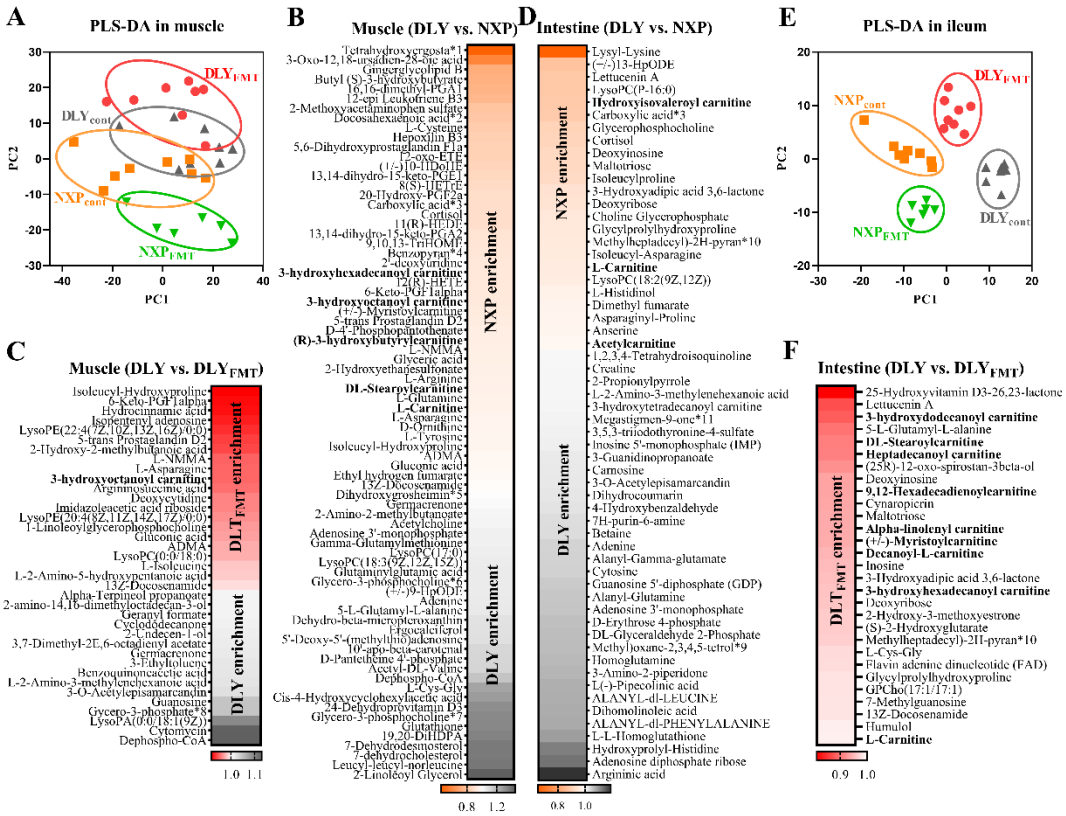
Supplementary figures



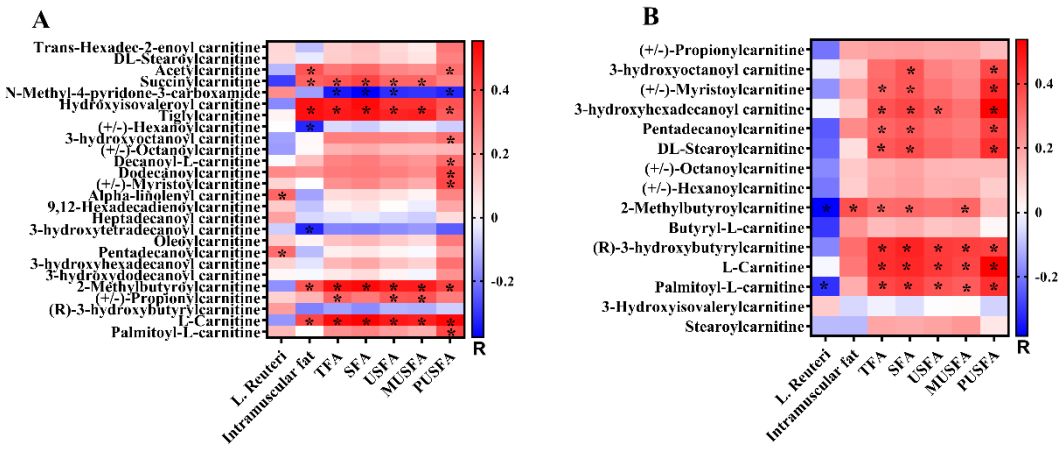
Supplementary Figure 1 Fecal microbiota transplantation affected muscle and fecal FA concentrations. A, Muscle FA; B, Muscle FA metabolic genes; C, Intestinal FA transport related genes; D, Fecal FA concentrations.



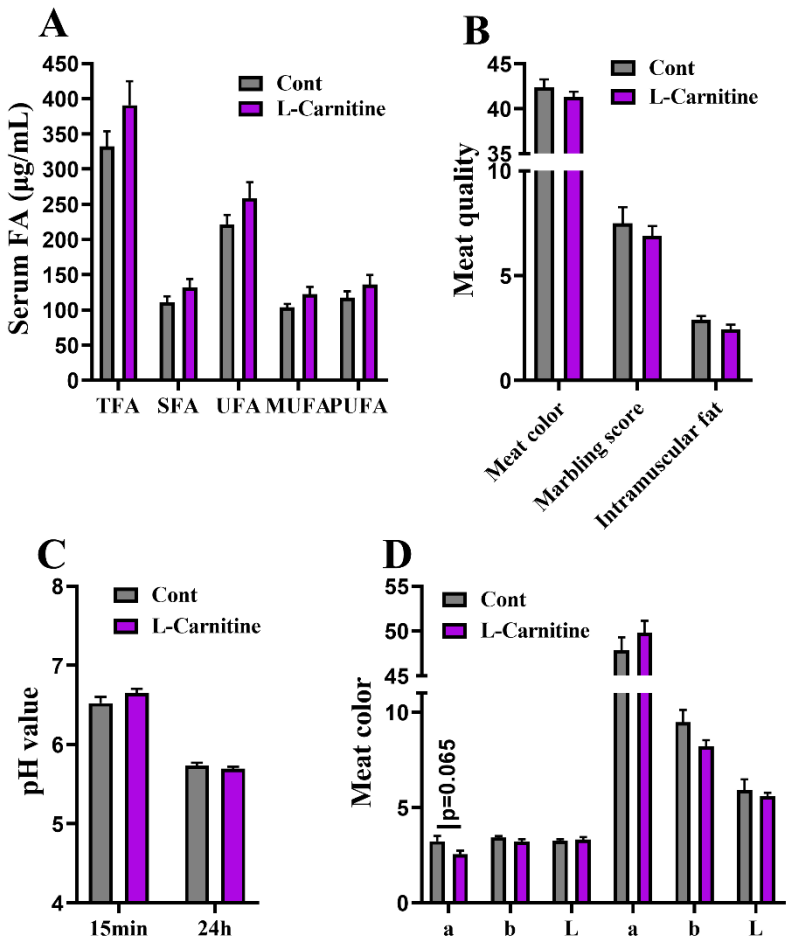
Supplementary Figure 2 Effects of obese Ningxiang pig-derived *L. reuteri* (Lr) on gene expressions in lean pigs. A, Lipid metabolic genes in the AAT; B, Lipid metabolic genes in the SAT; C, Intestinal FA transport related genes.



Supplementary Figure 3 Fecal microbiota transplantation reprogramed muscle and intestinal metabolic profiles in Ningxiang and DLY pigs. A PLS-DA in the muscle; B-C Differentiated metabolites in the muscle; D Differentiated metabolites in the intestine between DLY and Ningxiang pigs; E PLS-DA in the intestine; F Differentiated metabolites in the intestine between DLY and DLY_{FMT} datasets.



Supplementary Figure 4 Correlations between carnitine and FA. A, Correlations between carnitine and FA in the intestine; B, Correlations between carnitine and FA in the muscle.



Supplementary Figure 5 Effects of dietary L-carnitine on meat quality in lean DLY pigs. A, Serum FA level; B, Meat quality; C, pH value of muscle; D, Meat color.