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Original Research Article

Secondary bile acids are associated with body lipid accumulation in obese pigs

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ABSTRACT

The aim of this study was to investigate the reasons for the differences in lipid accumulation between lean and obese pigs. The bile acids with varying levels within two types of pigs were found and then in vitro experiments were conducted to identify whether these bile acids can directly affect lipid accumulation. Fourteen pigs, including seven lean and seven obese pigs with body weights of approximately 80 kg, were fed the same diet at an amount approximately equivalent to 3% of their respective body weights daily for 42 d. In vitro, 3T3-L1 preadipocytes were cultured in medium with high glucose levels and were differentiated into mature adipocytes using differentiation medium. Then, bile acids were added to mature adipocytes for 4 d. The results showed that there was a difference in body lipids levels and gut microbiota composition between obese and lean pigs ($P < 0.05$). According to the results of gut microbial function prediction, the bile acid biosynthesis in colonic digesta of obese pigs were different from that in lean pig. Sixty-five bile acids were further screened by metabolomics, of which 4 were upregulated ($P < 0.05$) and 2 were downregulated ($P < 0.05$) in obese pigs compared to lean pigs. The results of the correlation analysis demonstrated that chenodeoxycholic acid-3-β-D-glucuronide (CDCA-3Gln) and ω-muricholic acid (ω-MCA) had a negative correlation with abdominal fat weight and abdominal fat rate, while isoallolithocholic acid (IALCA) was positively associated with crude fat in the liver and abdominal fat rate. There was a positive correlation between loin muscle area and CDCA-3Gln and ω-MCA ($P < 0.05$), however, IALCA and 3-oxodeoxycholic acid (3-oxo-DCA) were negatively associated with loin eye muscle area ($P < 0.05$). Isoallolithocholic acid increased the gene expression of peroxisome proliferator-activated receptor gamma (*PPARG*) and the number of lipid droplets ($P < 0.05$), promoting the lipid storage when IALCA was added to 3T3-L1 mature adipocytes in vitro. In conclusion, the concentration of bile acids, especially gut microbiota related-secondary bile acids, in obese pigs was different from that in lean pigs, which may contribute to lipid accumulation within obese pigs.

1. Introduction

The swine industry comprises different breeds of pigs, such as lean and obese pigs, which exhibit significant variations in phenotypic indicators such as growth performance and carcass traits (Quinious et al., 1995). Duroc × (Landrace × Yorkshire) pigs (DLY pigs), as lean crossbred pigs, show a high lean percentage and slaughter weight, while Chenghua pigs, an obese local breed originating in Sichuan Province, are characterized by a low lean percentage, high lipid storage and low slaughter rate (Jiang et al., 2012; Li et al., 2022). A high level of body lipids and a low slaughter rate are known risk factors causing economic losses in the swine industry (Marcon et al., 2019; Pettigrew et al., 2001). Previous studies suggested that the variation in phenotypic indicators between lean pigs and obese pigs was mainly attributed to differences in genetic background (Jiang et al., 2012). However, recent research indicates that diet and gut microbiota have a more prominent influence than genetics in explaining the differences in metabolism among

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individuals (Chen et al., 2022). Backhed et al. (2007) noted that germ-free mice were resistant to obesity when they were fed a high-fat diet. Fecal microbiota transplantation has the ability to transfer obesity-associated characteristics from donors to recipients (Duca et al., 2014). Dietary supplementation can reduce body lipid deposition and improve obesity by altering the structure and composition of gut microbiota (David et al., 2014; Genco et al., 2013; Waubant et al., 2019). These results show that gut microbiota plays key roles in body lipid accumulation.

The gut microbiota communicates to the host via microbial metabolites (Mithieux, 2018). Bile acid, one of the metabolites in the gut, is involved in fat digestion and lipid metabolism (Ahmad et al., 2019; Yu et al., 2019). Bile acids (BA) can be classified into primary BA and secondary BA (Di Ciaula et al., 2018). Primary BA are synthesized in the liver via the enzyme cholesterol 7α-hydroxylase (CYP7A1) or sterol 27-hydroxylase (CYP27A1), while secondary BA are produced in the gut as microbial metabolites of primary BA (Di Ciaula et al., 2018; Poland et al., 2021). Gut microorganisms have the ability to modify the hydroxyl groups of BA through processes such as oxidation, dehydroxylation, and isomerization (Doden et al., 2021). For example, 6α-hydroxylated BA including hyodeoxycholic acid (HDCA) and ω-muricholic acid (ω-MCA) are produced by the gut microbiota (Lin et al., 2020). Zheng observed that humans with obesity had lower levels of 6α-hydroxylated BA compared to lean humans (Zheng et al., 2021b). ω-MCA has been shown to improve glucose homeostasis, and HDCA can alleviate non-alcoholic fatty liver disease through the bile acid receptor farnesoid X receptor (FXR) (Kuang et al., 2023; Zheng et al., 2021a). In contrast to 6α-hydroxylated BA, Louca observed that elevated levels of isoursodeoxycholic acid (isoUDCA), as a secondary bile acid, were associated with hypertriglyceridemia and increased appetite (Louca et al., 2023). The decreased levels of iso-UDCA may play a role in promoting satiety and improving lipid regulation after bariatric surgery (Louca et al., 2023). These findings imply that gut microbiota related-secondary BA may promote or inhibit body lipid storage. However, the composition and concentration of BA in obese pigs and whether certain BA are related to lipid accumulation remain unclear. Thus, we conducted a comparative experiment to compare the differences in body fat storage, gut microbial structure, and bile acids profile between lean pigs (DLY pigs) and obese pigs (Chenghua pigs). The relationship between BA profile and lipid accumulation in finishing pigs was studied in this experiment. The deposition of body lipid in finishing pigs has been consider as a process of the enlargement of mature adipocytes, which are differentiated from preadipocytes (Anderson et al., 1973). Previous studies have demonstrated that the 3T3-L1 cell lines are easily cultured and the mature 3T3-L1 adipocytes can serve as an In vitro model to simulate lipid deposition in finishing pigs (Kim et al., 2019; Poulos et al., 2010). Therefore, in this study, the 3T3-L1 preadipocytes were cultured and differentiated to mature adipocytes in vitro. The 3T3-L1 mature adipocytes were used to investigate whether the BA can directly affect the lipid accumulation, following the screening of differential BA between lean and obese pigs, in order to provide additional evidence for the role of BA in lipid deposition of obese pigs.

2. Materials and methods

All experimental procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (20211028).

2.1. Animals, experimental design and sample collection

Fourteen pigs, consisting of seven lean pigs (DLY pigs) and seven obese pigs (Chenghua pigs), with body weights of approximately 80 kg, were individually housed in fourteen pens within an environmentally controlled room. All pigs had free access to water and were fed 3 times per day at 08:00, 14:00 and 20:00 o'clock. Notably, according to the National Research Council (NRC, 2012) and the Chinese National Feeding Standard (GB/T 39235-2020), there are inconsistencies across the energy requirements of lean and obese pigs. To minimize the influence of food or energy intake on experimental results, both types of pigs in this experiment were provided the diet with identical nutrient composition, and the experiment also referred to the standard operating procedures of digestion and metabolism trials. All pigs were fed the diet with an amount approximately equivalent to 3% of their respective body weights daily, allowing them to engage in their normal activities without receiving excessive energy. The chemical compositions of the basal diet are listed in Table S1 and the nutrient levels are calculated levels. The corn, soybean meal, wheat bran and others utilized in the formulation were consistent with the raw materials listed in the Chinese feed database (2020). Based on the nutritional parameters of these raw materials in the database and their proportions in the formula, the nutrient levels were calculated. The trial lasted for 42 d. On d 1 and 43 of the trial, all pigs were weighed. The average daily weight gain and the ratio of weight gain to feed were calculated.

At the end of the trial, all pigs were fasted overnight, and blood was collected in vacutainers. Serum samples were obtained after centrifugation (3500 × *g*, 10 min) and stored at −20 °C. The pigs were slaughtered via electrical stunning and exsanguinated according to standard commercial procedures. The tissue of left lateral liver lobe and the digesta from the middle part of colon were collected and then stored at −80 °C.

2.2. Reagents, cell culture and differentiation

The 3T3-L1 preadipocyte cell line was obtained from American type culture collection (CL-173, American Type Culture Collection, Manassas, USA). Isoallolithocholic acid was obtained from MedChemExpress (MedChemExpress, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), penicillin−streptomycin solution and foetal bovine serum (FBS) were purchased from Thermo Scientific (Thermo Scientific, Shanghai, China). Dexamethasone (Dex) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (Sigma-Aldrich, USA). Recombinant human insulin was purchased from Yuanye (Yuanye Bio-Technology Co., Ltd, Shanghai, China). All the reagents used in this study were of analytical grade.

The 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin solution until they had achieved confluence. To induce differentiation, 100% confluent cells were maintained for 2 d and then placed in DMEM containing 10% FBS, 10 μg/mL insulin, 1 μmol/L dexamethasone, and 0.5 mmol/L IBMX (day 0) for 2 d (Green et al., 2016). The cell medium was changed to DMEM containing 10% FBS and 10 μg/mL insulin, and two days later, the cell medium was replaced with DMEM containing 10% FBS and cells were incubated in this medium for 6 d. Isoallolithocholic acid (0, 2.5, 5, 10, 20, 40 and 80 μmol/L) was added to the cell medium after pre-adipocytes were differentiated into the mature adipocytes.

2.3. Carcass traits measurement and analysis of serum lipid metabolism-related indexes

Carcass weight was measured and used to calculate dressing percentage. The abdominal fat was stripped and weighed, and the abdominal fat rate was calculated by the abdominal fat weight and carcass weight. The formula for estimating lean meat percentage (%) = 60.30-0.847x + 0.147y, where x = fat depth (mm) and

y = muscle depth (mm) (Giblin et al., 2015). The loin eye muscle area at the 10th rib was measured according to the following equation: loin eye muscle area (cm^2) = loin eye muscle width (cm) × loin eye muscle height (cm) × 0.7. The content of crude fat in the longissimus dorsi and liver was determined according to the Soxhlet extraction method (Khoddami et al., 2011).

The concentrations of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in serum were measured by commercial assay kits from Jiancheng Bioengineering Institute (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. Analysis of BA in colonic digesta by ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS)

The BA in the samples were analysed using targeted metabolomics at a commercial company (MetWare Biotechnology Co., Ltd., Wuhan, China). Liquid samples (50 μL) were mixed with 10 μL of internal standard mixed liquid (concentration: 1 μg/mL) and 200 μL of 20% methanol acetonitrile. The mixture was shaken for 10 min at 2500 revolutions per minute and then placed in a refrigerator at −20 °C for 10 min. Centrifugation was performed at 13,523 × g at 4 °C for 10 min. The supernatant was collected and concentrated. The concentrate was then dissolved in 50% methanol water, and the sample was analysed using UPLC–MS/MS for BA profile analysis (Huang et al., 2011).

MetWare Database (Wuhan Metware Biotechnology Co., Ltd., Wuhan, China) is constructed based on standard products, and allows for qualitative analysis of mass spectrometry data to be carried out. Quantification was performed using multiple reaction monitoring by triple quadrupole mass spectrometry. After obtaining the mass spectrometry data for different samples, the chromatographic peaks of all target compounds were integrated, and quantitative analysis was conducted using standard curves. Standard solutions with various concentrations (0.1, 0.2, 0.4, 1, 2, 4, 10, 20, 40, 100, 200, 400 and 1000 ng/mL) were prepared, and the peak intensity data of quantitative signals corresponding to each concentration of the standard compounds were obtained. Sixty-five substances, including taurolithocholic acid-3-sulfate (Toronto Research Chemicals, NY, Canada), dehydrolithocholic acid (zzstandard, Shanghai, China), isoallolithocholic acid (zzstandard), 3-oxodeoxycholic acid (TRC), and others, were used as external standards for quantification. The internal bile acid standards, such as Cholic acid-d4 (IR-14894), Glycolithocholic acid-d4 (IR-14913), Deoxycholic acid-d4 (IR-14896), and others were obtained from Isoreag (Shanghai ZZBio Co., Ltd., Shanghai, China) or Scrbio (Shanghai, China).

The concentration ratio of the external standard and the internal standard was used as the horizontal coordinate, while the peak area ratio of the external standard and the internal standard was used as the vertical coordinate to draw the standard curves for different BA (García-Cañaveras et al., 2012; John et al., 2014; Perwaiz et al., 2001).

2.5. 16S rRNA gene sequencing

The microbial DNA of colonic digesta was isolated with an E.Z.N.A. Stool DNA Kit (Omega, Norcross, GA, USA) according to the manufacturer's protocol. The DNA samples were sent to the Novogene Company (Novogene, Beijing, China), and the DNA concentration and purity were monitored on 1% agarose gels. Using the obtained concentration, DNA was diluted to l μg/μL with sterile water. The V3−V4 variable regions of the bacterial 16S rRNA gene were amplified to analyse the gut microbiota. PCRs were conducted using 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs, USA), along with 0.2 μmol/L of forward and reverse primers and approximately 10 ng of template DNA.

Paired-end reads were assigned to samples based on their unique barcodes and were truncated by removing the barcode and primer sequences. Quality filtering was performed on the raw tags using specific filtering conditions to obtain high-quality clean tags, following the QIIME quality control process. The tags were compared to the reference database (Silva database) using the UCHIME algorithm to identify and remove chimeric sequences (Bokulich et al., 2013; Caporaso et al., 2010; Magoc et al., 2011).

2.6. Cell viability assay, oil red O staining and lipid levels determination

3T3-L1 cells were differentiated into mature adipocytes and then treated with dimethyl sulfoxide (DMSO; 80 μmol/L) or IALCA (2.5, 5, 10, 20, 40 and 80 μmol/L) for 4 d. To identify the effect of IALCA on adipocyte survival, cell viability was determined using a Cell Counting Kit-8 cell assay (Beyotime Biotechnology, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

Mature adipocytes were fixed with 4% paraformaldehyde for 1 h at 37 °C, followed by staining with Oil red O solution (Solarbio, Beijing, China) for 1 h. Stained lipid droplets were observed and photographed using an inverted contrast microscope at 20× magnification. Then, 1 mL of isopropanol was added to extract the Oil red O dye, and the optical density (OD) value of the extracted solution was measured at a wavelength of 530 nm using a spectrophotometer (NanoDrop spectrophotometer; Thermo Scientific, Fremont, CA, USA).

2.7. Measurement of lipid metabolism-related genes by real-time quantitative PCR (RT-qPCR)

Liver tissue was homogenized, and RNA was extracted using RNAiso Reagent (TaKaRa Biotechnology, Dalian, China). Total RNA was isolated from 3T3-L1 adipocytes using TRIzol Reagent (TaKaRa) according to the manufacturer's instructions. cDNA was prepared using the PrimeScript RT reagent Kit, and RT–qPCR was carried out using SYBR Green PCR Master Mix (EZBioscience, Roseville, USA) following the manufacturer's protocol. The primer sequences used are listed in Table S2. The cycle threshold (Ct) values of the experimental genes were normalized against those of the stably expressed housekeeping gene β-actin.

2.8. Statistical analysis

The data were analysed by Student's t test or one-way ANOVA using IBM SPSS Statistics 23 software, and the data are expressed as the mean ± standard deviation (SD). For the microbiota analysis, alpha diversity was assessed using Chao1 and Shannon indexes, which were calculated using QIIME (version 1.9.1). LEfSe was applied to identify microbes of different taxa using the default parameters (linear discriminant analysis [LDA] score >3 and $P < 0.05$). Microbial function analysis was performed using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt 2). The predicted genes and their respective functions were aligned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the differences between groups were analysed using R software (v3.5.1). Student's t-test was used to assess the differences between groups for the pathways with data conforming to normal distribution. For the pathways with data not conforming to normal distribution, the Wilcoxon test was used to assess the differences between groups, with FDR correction performed on the P-value. The difference was considered

significant when the *P*-value is less than 0.05 and the difference was considered highly significant when the *P*-value is less than 0.01.

For the BA profiles analysis, the orthogonal partial least squares-discriminant analysis (OPLS-DA) model was constructed to distinguish colonic digesta with different BA and to discover potential biomarkers. The reliability of the OPLS-DA model was evaluated by R^2Y (explained Y variation), R^2X (explained X variation) and Q^2 (predicted Y variation) (Kang et al., 2022). Based on the results of OPLS-DA, the multivariate analysis of variable importance in projection was used to screen the bile acids with varying levels between groups, and the differential BA were further screened by combining the *P*-value or fold change in univariate analysis. Spearman's correlation analysis and linear regression analysis were conducted to clarify the relationship between colonic BA profiles and lipid levels in the body.

3. Results

3.1. Obese pigs showed a high level of body lipids

From Table 1, Obese pigs showed lower final weight, average daily gain and gain to feed ratio than that of lean pigs. There was low lean meat percentage and loin eye muscle area in obese pigs, while the abdominal fat weight and abdominal fat rate were higher than those of lean pigs (Table 2). Compared with lean pigs, obese pigs exhibited higher intramuscular and liver crude fat content (Table 2). From Table 2, obese pigs tended to have an increased concentration of serum LDL-C compared with lean pigs ($P = 0.09$).

3.2. The composition and function of gut microbiota in obese pigs were different from those in lean pigs

The colonic digesta of obese pigs tended to have an increased Shannon index ($P = 0.07$) (Fig. 1B). As shown in Fig. 1C, obese pigs showed a high relative abundance of Spirochaetota and a low relative abundance of Cyanobacteria at the phylum level. At the genus level, in Fig. 1D-E, the relative abundances of *Treponem*, *Christensenellaceae_R-7_group* and *UCG-002* were higher in obese pigs than in lean pigs. Microbial function prediction revealed that the primary and secondary BA biosynthesis pathways were predicted to be inhibited in obese pigs compared to lean pigs, and there were no significant differences in other predicted pathways between lean and obese pigs (Fig. 1F).

3.3. BA profiles in obese pigs differed from those in lean pigs

There was a clear separation of BA profiles between the samples from lean pigs and obese pigs (Fig. 2A). The R^2X, R^2Y and Q^2 values were 0.652, 0.994 and 0.772, respectively, as shown in Fig. 2B, representing the high prediction ability of OPLS-DA. As shown in Fig. 2C, obese pigs exhibited high levels of IALCA ($P < 0.01$), 6,7-diketolithocholic acid (6,7-DKLCA), dehydrolithocholic acid (DLCA) and 3-oxo-DCA, while low levels of CDCA-3Gln and ω-MCA ($P < 0.01$) were observed in obese pigs compared with lean pigs.

Table 1
Differences in growth performance between lean and obese pigs.[1]

Items	Lean pigs	Obese pigs
1–42 d		
Initial weight, kg	81.00 ± 5.10	82.29 ± 3.55
Final weight, kg	118.71 ± 6.40^{A}	105.29 ± 7.43^{B}
ADFI, g/d	3000 ± 115^{A}	2569 ± 73^{B}
ADG, g/d	898 ± 54^{A}	548 ± 110^{B}
G/F	0.30 ± 0.02^{A}	0.21 ± 0.04^{B}

ADFI = average daily feed intake; ADG = average daily gain; G/F = gain-to-feed ratio.
Within a row, different uppercase letter superscripts indicate highly significant differences ($P < 0.01$).
[1] Seven pigs per group.

Table 2
Differences in the body fat level and the serum lipid metabolism-related parameters between lean and obese pigs.[1]

Items	Lean pigs	Obese pigs
Body fat level		
Carcass weight, kg	86.39 ± 5.60^{A}	73.34 ± 4.92^{B}
Loin muscle area, cm^2	56.71 ± 13.20^{A}	28.84 ± 6.16^{B}
Lean meat percentage, %	52.47 ± 3.66^{a}	45.60 ± 2.04^{b}
Abdominal fat weight, kg	1.63 ± 0.48^{b}	2.75 ± 0.90^{a}
Abdominal fat rate, %	1.91 ± 0.64^{B}	3.71 ± 1.00^{A}
Intramuscular crude fat, %	1.73 ± 0.51^{b}	2.52 ± 0.73^{a}
Liver crude fat, %	2.04 ± 0.67^{B}	3.81 ± 1.32^{A}
Serum lipid metabolism-related parameters		
TC, mmol/L	2.84 ± 0.55	3.05 ± 0.37
TG, mmol/L	0.32 ± 0.17	0.34 ± 0.09
HDL-C, mmol/L	2.04 ± 0.38	1.78 ± 0.47
LDL-C, mmol/L	2.60 ± 0.49	3.18 ± 0.66
TBA, μmol/L	12.15 ± 6.85	7.25 ± 6.08

TC = total cholesterol; TG = total triglyceride; HDL-C = high density lipoprotein cholesterol; LDL-C =low density lipoprotein cholesterol; TBA = total bile acids.
Within a row, different lowercase letter superscripts indicate significant differences ($P < 0.05$) and different uppercase letter superscripts indicate highly significant differences ($P < 0.01$).
[1] Seven pigs per group.

3.4. Lipid accumulation in obese pigs strongly correlated with BA

As shown in Fig. 3, IALCA was positively associated with abdominal fat rate and the crude fat in the liver and longissimus dorsi, but it was significantly negatively associated with loin eye muscle area. The loin eye muscle area also negatively correlated with 3-oxo-DCA. Chenodeoxycholic acid-3-β-D-glucuronide and ω-MCA were significantly negatively associated with the crude fat of the liver, abdominal fat weight and abdominal fat rate, and there was a significant positive correlation between eye muscle area and CDCA-3Gln and ω-MCA.

3.5. IALCA promoted adipogenesis in 3T3-L1 adipocytes

From Fig. S1, successful adipogenic differentiation was characterized by the presence of lipid droplets in adipocyte cells and a more rounded cell morphology. Cell survival was tested using the CCK8 assay, and the results showed that IALCA (2.5, 5, 10, 20 or 40 μmol/L) had no effect on adipocyte survival (Fig. 4A). Oil red O staining was used to detect intracellular lipid droplets in 3T3-L1 adipocytes treated with IALCA. Isoallolithocholic acid (2.5 and 5 μmol/L) had no significant effect on the formation of lipid droplets, but adipocytes treated with 10, 20 and 40 μmol/L IALCA for 4 d showed a clear increase in lipid accumulation (Fig. 4 B–F and Fig. S2).

3.6. The BA receptor pathway in obese pigs differed from that in lean pigs

Obese pigs showed lower relative *CYP27A1* and *FXR* gene expression and tended to decrease sterol regulatory element-binding protein-1c (*ChREBP*) gene expression ($P = 0.079$) in the liver than lean pigs (Fig. 5). In vitro, IALCA increased the gene expression of *PPARG* and decreased leptin gene expression in adipocytes (Fig. 6).

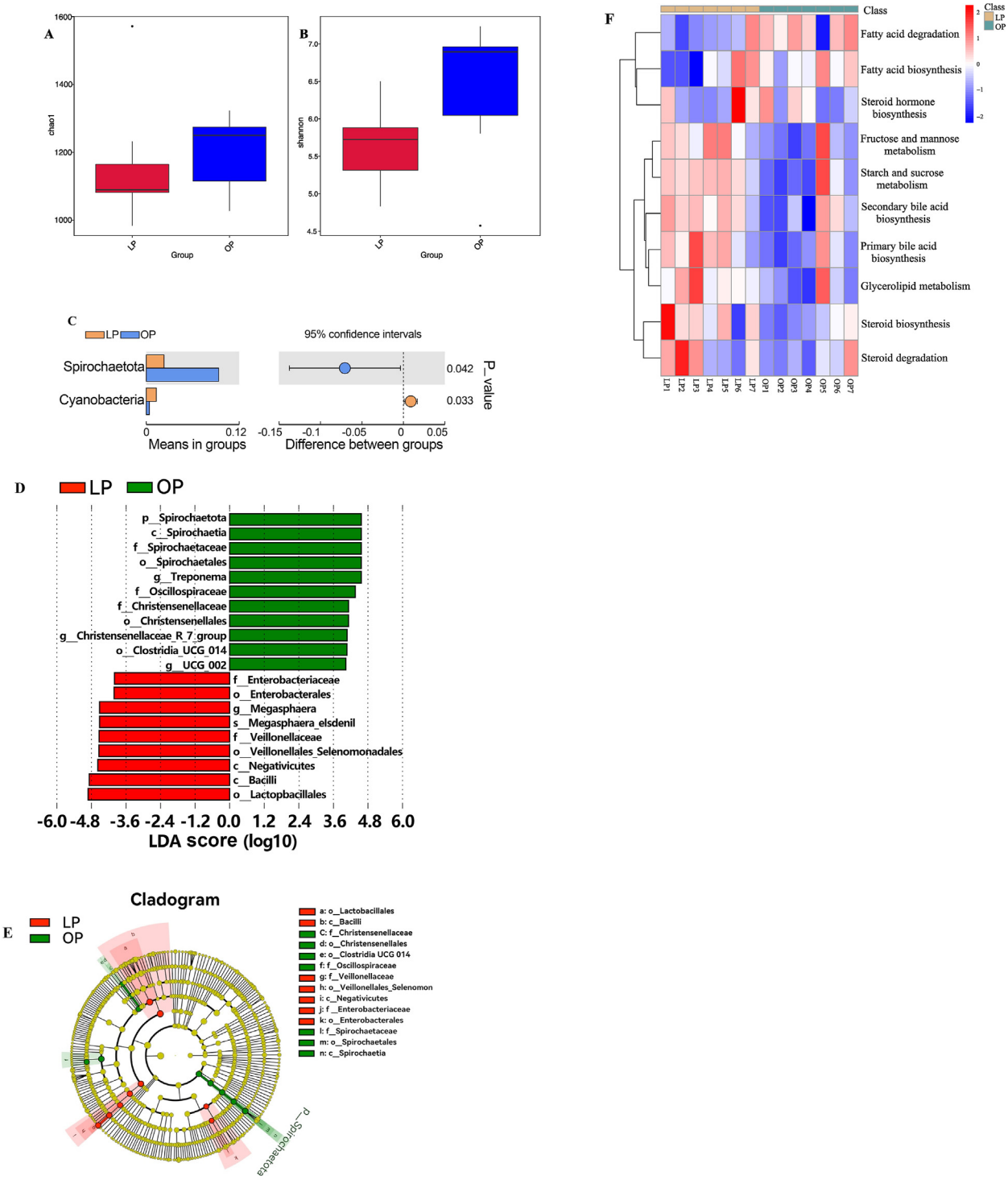

Fig. 1. Differences in composition and function of colonic microbiota between lean and obese pigs. (A-B) Differences in α-diversity (Chao1 and Shannon indexes) of colon between lean and obese pigs. (C) Differences in microbial abundance at the phylum level of colon between lean and obese pigs. (D) Linear discriminant analysis (LDA) effect size (LEfSe) analysis revealed significant bacterial differences in colonic microbiota between lean and obese pigs. (E) Cladogram representation of gut microbiota taxa differences between lean and obese pigs. (F) Differences in lipid metabolism-related pathway between lean and obese pigs. LP = lean pigs; OP = obese pigs.

4. Discussion

Chinese indigenous pig breeds, such as Chenghua or Min pigs (obese pigs), are unattractive to many farmers due to their poor growth performance and low lean meat percentage (Li et al., 2022). It is necessary to find measures to reduce the body lipid storage of obese pigs. In this study, compared to lean pigs, obese pigs showed low average daily gain and lean meat percentage but high abdominal fat weight and abdominal fat rate. Current evidence demonstrates that the higher the loin muscle area is, the higher the lean meat percentage in pigs (Honeyman et al., 2003). The data on lipid accumulation-related indexes show that obese pigs have a low

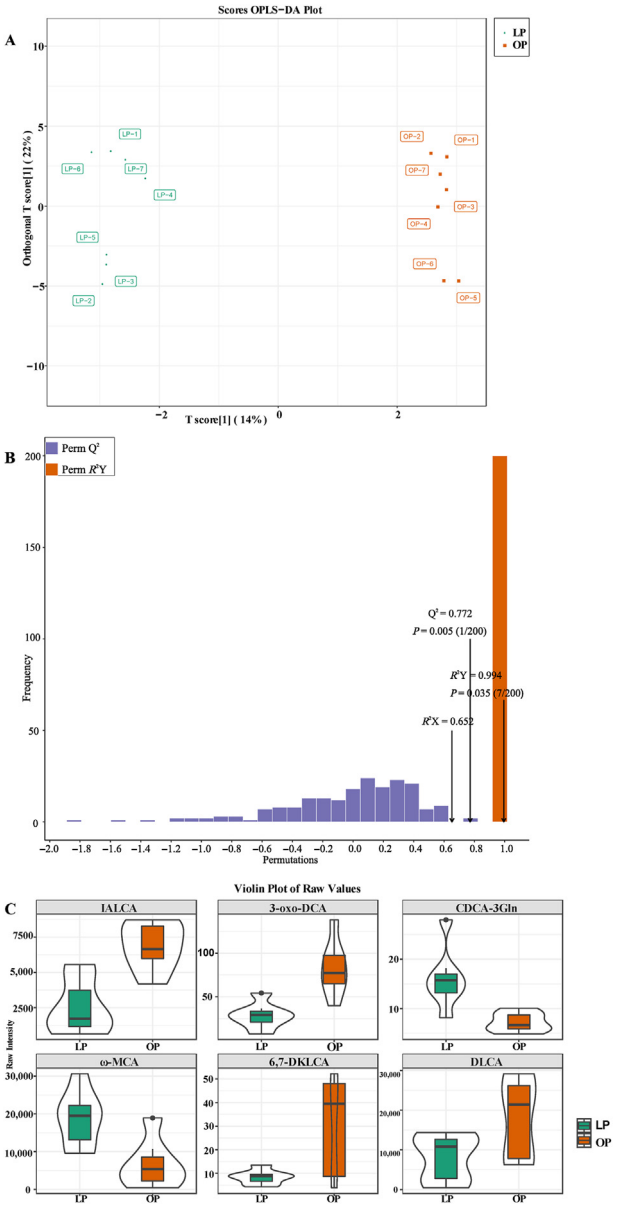

Fig. 2. The differential bile acids in colonic digesta between lean and obese pigs (ng/g). (A and B) OPLS-DA score and OPLS-Permutation. OPLS-DA = orthogonal partial least squares-discriminant analysis; R^2Y = explained Y variation; R^2X = explained X variation; Q^2 = predicted Y variation. (C) The differential bile acids in colonic digesta between lean and obese pigs. IALCA = isoallolithocholic acid; 3-oxo-DCA = 3-oxodeoxycholic acid; CDCA-3Gln = chenodeoxycholic acid-3-β-D-glucuronide; ω-MCA = ω-muricholic acid; 6,7-DKLCA = 6,7-diketolithocholic acid; DLCA = dehydrolithocholic acid; LP = lean pigs; OP = obese pigs.

loin muscle area, and a high intramuscular and liver crude fat contents. These results indicated that obese pigs had a higher body lipid level than lean pigs, which is consistent with previous findings.

The differences in body lipid accumulation between lean and obese pigs was thought to be caused by several factors such as genetic background, diet and gut microbiota (Jiang et al., 2012; Yang et al., 2015). A new study was conducted to investigate the factors contributing to inter-individual variability in metabolism (Chen et al., 2022). The study involved 1,368 extensively phenotyped individuals, and the researchers analyzed 1,183 plasma metabolites (Chen et al., 2022). The findings revealed that diet and the gut microbiome have a more significant impact on metabolism variability than genetics (Chen et al., 2022). Gut microbiota, as an important influencing factor, has been considered a key target in regulating lipid metabolism (He et al., 2017). In addition, the gut microbiota is more readily identified and is more controllable than are genetic factors (David et al., 2014). In this study, we analysed the colonic microbial composition of lean and obese pigs. Alpha-diversity indexes, including the Shannon and Chao1 indexes, reflect the abundance and consistency of gut microbiota. Xie noted that there was a high Chao1 index in Laiwu pigs, an indigenous pig breeds of China, compared with lean pigs (Xie et al., 2022). Similar to Xie's results, obese pigs showed a higher Shannon index and gut microbiota species richness than did lean pigs. Analyzing the gut microbes at the phylum or genus level can aid in learning the ecological functions and metabolic levels of gut microbiota (Adak et al., 2019). Zhou reported that Spirochaetota, which is mainly involved in carbohydrate fermentation, was enriched in Chinese native pig breeds (Zhou et al., 2020). Consistent with previous studies, we also found a higher relative abundance of Spirochaetota in obese pigs than in lean pigs. Chen demonstrated a strong positive correlation between the lipids in the brain of rats and the presence of Spirochaetes in the gut (Chen et al., 2018). Hallowell noted a higher abundance of Spirochaetes in pigs that were fed a high-fat diet compared to those in the ad libitum and limit fed groups (Hallowell et al., 2021). This suggests that Spirochaetes at the phylum level may be associated with lipid storage. At the genus level, the relative abundance of *Christensenellaceae_R-7_group* and *UCG-002* was higher in obese pigs, and the evolutionary branching diagram showed that *Christensenellaceae_R-7_group* and *UCG-002* belong to the same *Clostridia* order. Gut microbiota has been considered to regulate host physiology via microbial metabolites (Mithieux, 2018). To further identify the function of gut microbiota, we conducted PICRUSt 2 analysis and found that there were significant differences in primary and secondary BA biosynthesis in lean and obese pigs.

Bile acids, which are steroid substances derived from hepatic cholesterol and metabolized by gut microbiota, can be categorized into primary and secondary BA (Guzior et al., 2021; Li et al., 2012). In this study, we compared the differences in BA concentration between lean and obese pigs. The BA profile data from lean and obese pigs showed significant differences in OPLS-DA scores, and the differential metabolites in the 65 BA identified in the pigs were identified by combining the OPLS-DA results and the fold change or *P*-value of the single variable analysis. Compared with lean pigs, obese pigs showed low levels of CDCA-3Gln and ω-MCA in the colonic digesta and high levels of several BA, including IALCA, 6,7-DKLCA, DLCA and 3-oxo-DCA. The PubChem database was applied to obtain the chemical structure of BA, and we further analysed the structure of these BA. Chenodeoxycholic acid-3-β-D-glucuronide, in which chenodeoxycholic acid (CDCA) is linked with glutamic acid, is a conjugated BA, and the remaining six BA are secondary and unconjugated BA (Guzior et al., 2021). Numerous studies have shown that *Clostridia* can secrete bile salt hydrolase (BSH), which is involved in the conversion of conjugated BA to secondary BA (Staley et al., 2017). In this study, we found that obese pigs exhibited a higher relative abundance of microorganisms belonging to the *Clostridia* order. ω-MCA, as secondary BA, is converted from CDCA, and hydroxylation occurs at the C6 position of ω-MCA (Jia et al., 2018; Thakare et al., 2018). Makki found that oligofructose can enrich bacteria involved in 6α-hydroxylated BA production (ω-MCA) and elevated the level of 6α-hydroxylated BA, subsequently improving body weight in mice fed a Western-style diet (Makki et al., 2022). In this study, obese pigs demonstrated lower 6α-hydroxylated BA levels and higher levels of IALCA, 6,7-DKLCA and DLCA than lean pigs. Isoallolithocholic acid, 6,7-DKLCA and DLCA

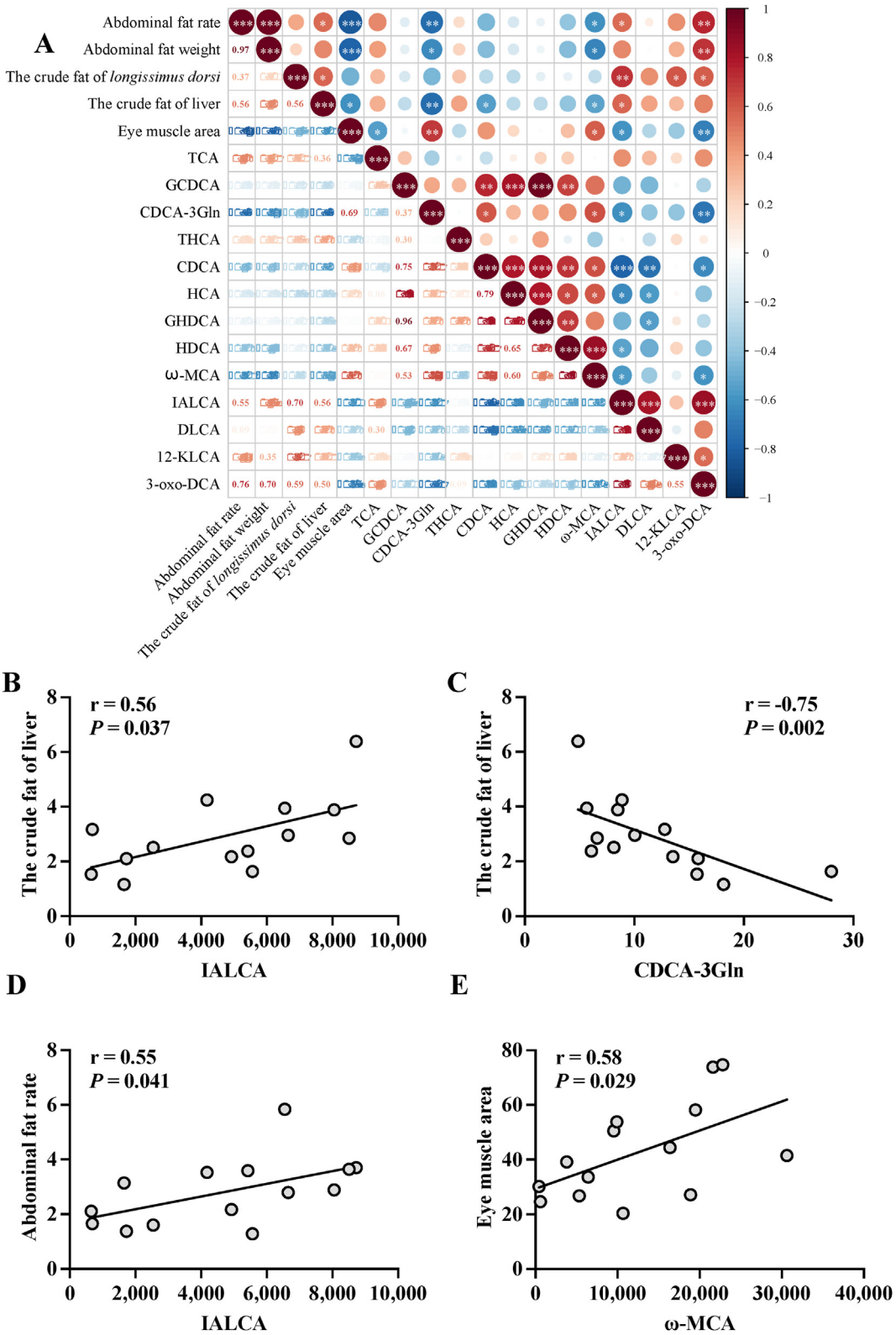

Fig. 3. The correlation between bile acids and metabolic indexes. (A) The correlation between bile acids, abdominal fat rate, abdominal fat weight, eye muscle area and the crude fat in liver and longissimus dorsi. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B-E) The linear-regression model between bile acids and metabolic indexes. r = the linear correlation coefficient; IALCA = isoallolithocholic acid; 3-oxo-DCA = 3-oxodeoxycholic acid; CDCA-3Gln = chenodeoxycholic acid-3-β-D-glucuronide; ω-MCA = ω-muricholic acid; 6,7-DKLCA = 6,7-diketolithocholic acid; TCA = taurocholic acid; GCDCA = glycochenodeoxycholic acid; THCA = taurohyocholic acid; CDCA = chenodeoxycholic acid; HCA = hyocholic acid; GHDCA = glycohyodeoxycholic acid; HDCA = hyodeoxycholic acid; 12-KLCA = 12-ketolithocholic acid.

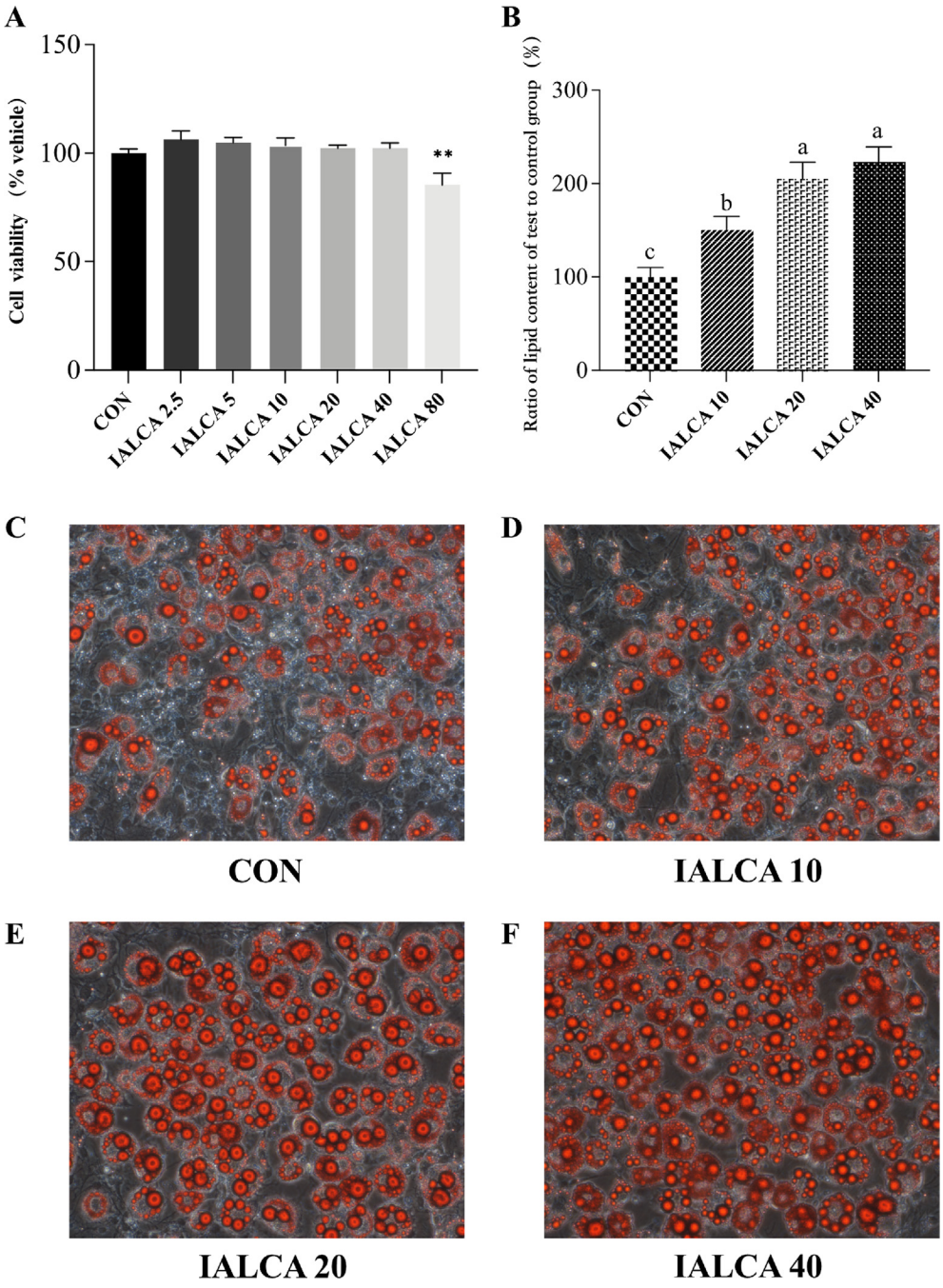

Fig. 4. Effect of isoallolithocholic acid (IALCA; 0, 10, 20, and 40 μmol/L) on lipid accumulation in 3T3-L1 adipocytes. (A) Effect of IALCA on the cell viability of 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with different doses (0, 2.5, 5, 10, 20, 40 and 80 μmol/L) of IALCA for 96 h; $n = 6$. **, $P < 0.01$. (B) The determination of lipid level by isopropanol extraction; $n = 3$. Bars without a common superscript differ at $P < 0.05$. (C–F) The lipid droplets stained with Oil red O staining and subsequently photographed using an inverted contrast microscope at 20× magnification; $n = 3$.

are derivatives of the secondary BA lithocholic acid (LCA), and 3-oxo-DCA is converted from deoxycholic acid (DCA) by gut microbiota (Jia et al., 2018; Wei et al., 2020). These results suggest that the difference in BA profile between lean and obese pigs is due to gut microbiota.

It is unclear whether the gut microbiota related-bile acids participate in lipid accumulation in obese pig. Chen et al. (2020) found that the hepatic lipid levels in subjects with obesity is positively associated with DCA and taurodeoxycholic acid. Jiao et al. (2018) presented that the absolute concentration or percent quantity of serum DCA was higher in patients with nonalcoholic fatty liver disease (NAFLD) than in healthy controls. Smirnova et al. (2022) further noted that the level of DCA derivatives (dehydrocholic and 7,12-diketolithocholic acid) increased with increasing NAFLD severity. In addition to DCA, the concentration of LCA was reported to be enriched in adolescents with obesity (Liu et al., 2022). On the other hand, 6α-hydroxylated BA has been reported to improve lipid storage and obesity as mentioned above (Makki et al., 2022). To clarify the relationship between BA and the levels of body lipids, correlation analysis and in vitro cell culture experiments were conducted in this study. The results showed that ω-MCA and CDCA-3Gln negatively correlate with the crude lipid

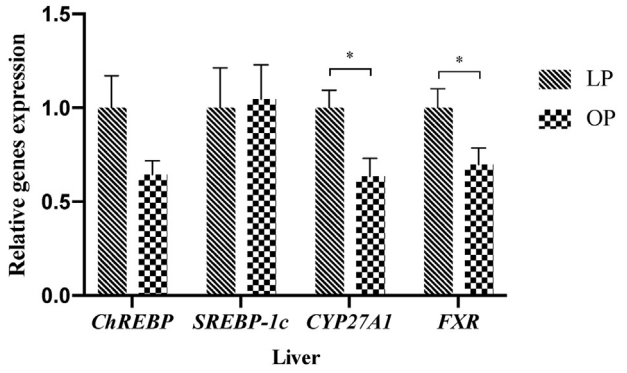

Fig. 5. Differences in lipid metabolism-related genes expression levels of liver between lean and obese pigs. *ChREBP* = carbohydrate response element binding protein; *SREBP-1c* = sterol regulatory element-binding protein-1c; *CYP27A1* = sterol 27-hydroxylase; *FXR* = farnesoid X receptor. *, $P < 0.05$; LP = lean pigs; OP = obese pigs.

content of the longissimus dorsi, abdominal fat weight and abdominal fat rate, and a significant positive correlation was observed between eye muscle area and CDCA-3Gln and ω-MCA. In contrast, the loin eye muscle area was negatively correlated with 3-oxo-DCA. Isoallolithocholic acid was also significantly negatively associated with loin eye muscle area, but it was positively correlated with abdominal fat rate and the crude fat in the liver and longissimus dorsi. Notably, correlation analysis does not mean causation, and previous studies showed secondary bile acids in the colon can diffuse passively across the colonic epithelium and reach the portal circulation (Gillard et al., 2023). In order to explore whether there is a direct connection between bile acids and lipid accumulation, we conducted the in vitro experiments to add bile acids in adipocytes. Mature adipocytes, which are capable of storing lipids, were obtained from 3T3-L1 preadipocytes, and then IALCA was added to them. More lipid droplets were found after IALCA treatment than after control treatment. PPARG, a nuclear receptor, is responsible for the formation of lipid droplets in adipocytes (Burstein, 2005). Leptin, secreted by adipose tissue, can inhibit adipogenesis (Gimeno et al., 2005). We found that IALCA enhanced the gene expression *PPARG* and reduced the gene expression of leptin in 3T3-L1 adipocytes. These results suggest that secondary BA in obese pigs may contribute to the body lipid accumulation.

This raises the question of how these secondary BA affect host lipid metabolism. Many studies have found that BA can regulate lipid metabolism via nonreceptor and receptor-mediated mechanisms (Fuchs et al., 2022). For example, FXR and Takeda G-protein-coupled receptor 5 (TGR5) are recognized as classic bile acid receptors (Fuchs et al., 2022; Zhang et al., 2016). The activation of hepatic FXR can reduce liver fat accumulation by inhibiting the activity of ChREBP and sterol regulatory element-binding protein-

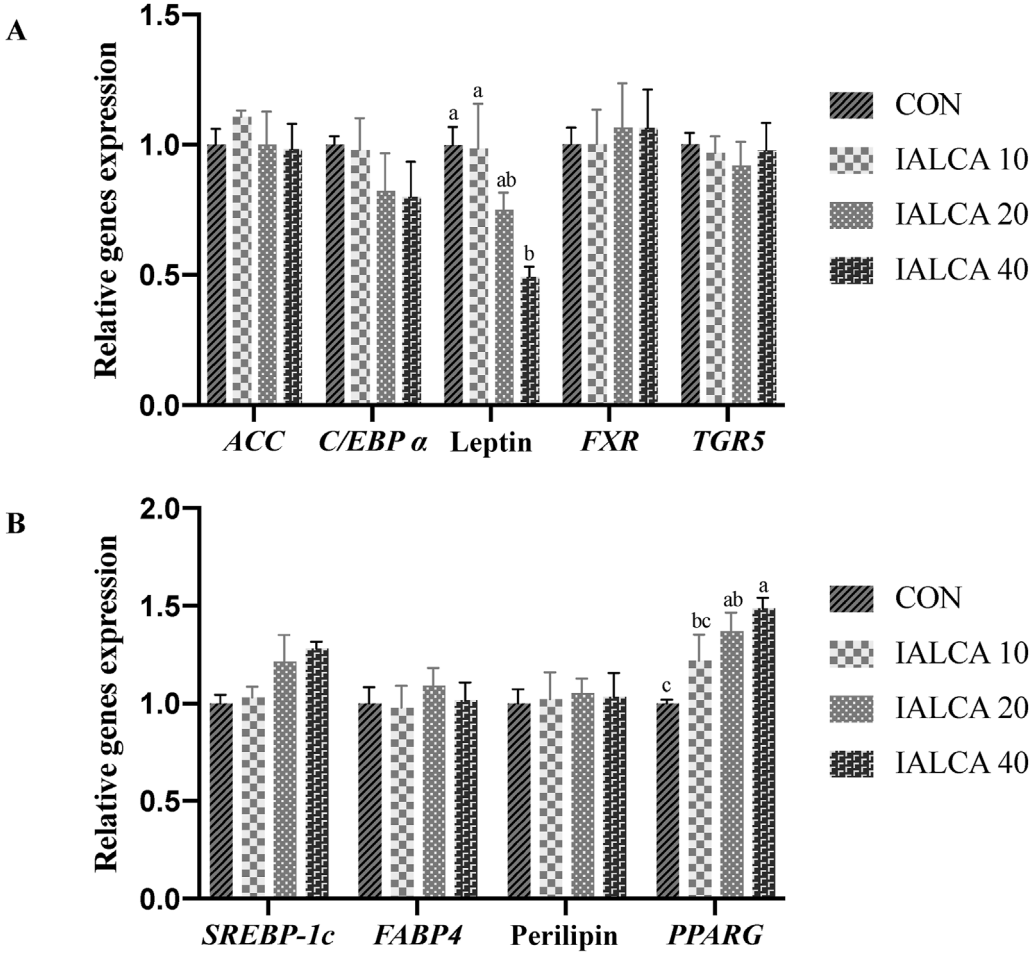

Fig. 6. Effects of IALCA (0, 10, 20, and 40 μmol/L) on lipid metabolism in adipocytes. *ACC* = acetyl-CoA-carboxylase; *C/EBPα* = enhancer binding proteins alpha; *FXR* = farnesoid X receptor; *TGR5* = Takeda G-protein-coupled receptor 5; *SREBP-1c* = sterol regulatory element-binding protein-1c; *FABP4* = fatty acid binding protein 4; *PPARG* = peroxisome proliferator-activated receptor gamma; IALCA = isoallolithocholic acid. [a,b,c]Bars without a common superscript differ at $P < 0.05$.

1c (SREBP-1c), and TGR5 activation has abilities to enhance energy expenditure (Fuchs et al., 2022; Zhang et al., 2016). In this study, the high levels of DCA and LCA derivatives and a low CDCA-3Gln level were observed in obese pigs. Bile acids are differentiated mainly by hydroxylation at the C positions, and the activation or suppression of FXR is determined by the number and position of hydroxyl groups on a BA molecule (Ahmad et al., 2019). Existing evidence supports that CDCA is able to activate FXR, whereas DCA becomes an inhibitor of FXR in the presence of CDCA (Ahmad et al., 2019; Jiao et al., 2018). Fu et al. (2019) observed that DCA antagonized FXR function, which can lead to uncontrolled proliferation of stem cells and DNA damage in intestinal stem cells. Jiao et al. (2018) noted that the occurrence of NAFLD was associated with an elevated level of serum DCA and the inhibited activity of hepatic FXR. Schmid et al. (2019) also found that DCA and CA had inhibitory effects on lipolysis in vitro . Interestingly, some studies showed that CDCA can promote adipocyte differentiation and lipogenesis by the FXR-PPARG pathway in 3T3-L1 cells (Shinohara et al., 2020). A contrary result was reported by Chen et al. (2017) and the researchers noted that CDCA inhibited the PPARG activity and lipid accumulation during adipocyte differentiation in 3T3-L1 cells by TGR5. In this study, obese pigs exhibited a high level of crude fat in liver and a relatively low expression of hepatic *FXR* and *ChREBP* genes, and a high gene expression of *PPARG* was observed in 3T3-L1 adipocytes treated with IALCA. These results demonstrate that the derivatives of DCA and LCA may regulate lipid accumulation via BA-related signalling pathways, but it is still unclear that how these BA regulate lipid storage in obese pigs via BA receptor and it needs further study.

5. Conclusion

In this study, compared with lean pigs, obese pigs had a higher lipid level within the body, and our results show that the gut microbiota and its derived secondary bile acids are related to body lipid metabolism. Specifically, the derivatives of lithocholic acid and deoxycholic acid in colonic digesta may contribute to the high level of body lipids in obese pigs.

Author contributions

Yaolian Hu: Investigation, Formal analysis, Writing – original draft. **Aimin Wu:** Resources. **Hui Yan:** Resources. **Junning Pu:** Resources. **Junqiu Luo:** Formal analysis. **Ping Zheng:** Formal analysis. **Yuheng Luo:** Formal analysis. **Jie Yu:** Formal analysis. **Jun He:** Formal analysis. **Bing Yu:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Daiwen Chen:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.019.

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