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Calorie restriction improves serum lipid metabolism, colon metabolites and microbiota in pigs

Jingliang Liu[a], Yusen Wei[a], Hanqiu Di[a], Yifan Zhong[a], Weidong Hua[b] and Haifeng Wang[a,]

[a] *College of Animal Science, Zhejiang University, The Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Hangzhou 310058, China*

[b] *Experimental Livestock Farm of Animal Husbandry and Veterinary Research Institute, Zhejiang Academy of Agricultural Sciences, Hangzhou, China*

Short title: Fasting on metabolism and gut microbiota of pigs

*Corresponding author.

Haifeng Wang,

College of Animal Science, Zhejiang University, The Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Hangzhou 310058, China

Email address: haifengwang@zju.edu.cn (HF. Wang)

Responses to the Comments by Editor and Reviewers

Dear Editor and Reviewers:

Thank you very much for your letter and for the reviewers' comments concerning our manuscript entitled "*Calorie restriction improves serum lipid metabolism, colon metabolites and microbiota in pigs*" (Manuscript ID: ANR-2024-0022). These comments are all valuable and helpful for improving our paper. We have studied comments carefully and made corrections and revisions in the new version. Our revised manuscript, in MS Word format, with the corrected sections marked in track change is attached.

We believe that the revised article has been improved substantially because of your constructive criticism. We hope all these emendations and clarifications will be satisfactory. We hope our manuscript could be sent for further review and accepted for publication.

Below is point-wise reply to the comments of the Reviewers. Should you need additional information, I shall be happy to provide.

Best regards,

Hai-Feng Wang

On behalf of all co-authors

List of Corrections for Comments of the Reviewers and Editors

To Editor' comments

ANR-2024-0022 entitled "Calorie restriction improves serum lipid metabolism, colon metabolites and microbiota in pigs" which you submitted to the Animal Nutriomics, has been reviewed. The comments of the reviewer(s) and editor are included at the bottom of this letter. The reviewer(s) and editor have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to their comments and revise your manuscript. To start your revision now, click the link below:

AU: Thank you for your valuable comments.

Reviewer: 1

In this manuscript, the objectives of this study are to determine the effects of Calorie restriction on the serum lipid metabolism, colon metabolites and microbiota in pigs. The results indicate that calorie restriction reduced the cumulative food intake, BW gain, serum total cholesterol, triglyceride, low density lipoproteins cholesterol, high density lipoproteins cholesterol, alanine aminotransferase and aspartate aminotransferase levels. The manuscript needs to make some revisions and provide more data to support the results. Details of comments are as follows:

AU: Thank you for your comments.

1.How did the author design the calorie restriction experiment? Please added the detailed illustration in abstract.

AU: Thank you for your suggestions. The pigs in calorie restriction experiment were fed 70% of the amount of feed in the control group, please see the description in the abstract (Page 2, Line 20-22).

2.The calorie restriction experiment should base on the NE to improve the accurate. Add the NE value in the Table 1 the ingredient and chemical composition.

AU: Thank you for your suggestions. We have supplemented the NE in the Table. At

the same time, we are sorry for making a mistaken in the calculating the DE and ME and we have corrected them in the Table 1. Please see the revised Table 1.

3.Add STTD P in Table 1 and delete “Total phosphorus”.

AU: Thank you for your suggestions. We have replaced total phosphorus with “Standard total tract digestible phosphorus” in Table 1.

4.Line 574: Crude protein, Lysine, Methionine and Methionine values are the calculated value or a measured value?

AU: Thank you for your suggestions. Crude protein was determined value, others including lysine, methionine and calcium were calculated values are the calculated value. We have mentioned as “Crude protein was determined value, and others were calculated values” in the revised Table 1.

5.Fig1A: due to the ADFI of CR group is designed and identical (70% of CON). In my mind the ADFI should not be analysis by T-test.

AU: Thank you for your valuable comments and suggestions. Although the ADFI of CR group is designed and identical (70% of CON), the statistical analysis was still carried out for making sure the difference between the CR and the CON.

6.Did the calorie restriction treatment affect meat quality of pigs?

AU: Thank you for your valuable comments and suggestions. We did not determine the meat quality of pigs. Certainly, calorie restriction treatment may have some effects on meat quality of pigs. We will pay attention to the meat quality in the future study.

Reviewer: 2

In this current manuscript, the authors investigated the effect of calorie restriction on serum lipid metabolism, colon metabolites and microbiota in pigs. Most of the results were positive and the discussion is very detailed, showed that calorie restriction may be a healthy diet treatment that can reduce obesity and improve metabolism. However, the innovation and significance of this research is not reflected, especially in the abstract. Additionally, some mistakes occur in figure legends, which needs to be

checked carefully. Overall, this is an interesting study that furthers the field of calorie restriction in pigs.

AU: Thank you for your valuable comments and suggestions. We have revised the abstract and figure legends according to your suggestions.

1. In the abstract, authors only described the backgrounds, methods, and results of this research, the conclusion and significance of this research should be added.

AU: Thank you for your valuable comments and suggestions. We have supplemented the conclusion and significance of this research in the abstract as "In conclusion, calorie restriction may affect metabolism, reduce obesity and improve intestinal microbiota, which may be a healthy diet treatment that can reduce obesity and improve metabolism" (Page 2, Line 33-35).

2. Are there any other studies investigated the effect of calorie restriction on pig metabolites and microbiota? If there is no other study, please point out in the introduction and make the innovation of this research obviously.

AU: Thank you for your valuable comments and suggestions. We have supplemented some explanation about the effect of calorie restriction on pig metabolites and microbiota such as "At present, some articles have studied the effect of starvation or fasting on intestinal microbiota of mini pig or piglets, however, to our knowledge, there is no studies about effect of specific degree of calorie restriction on intestinal microbiota and metabolites of finishing pigs [25.26]. The innovation of this article attributes to investigating the effect of calorie restriction at degree of 70% of normal feeding on intestinal microbiota, metabolites and the correlation between them" (Page 4, Line 79-85 in the revision).

3. In line 44, authors described that "The advantage of this nutritional model is that there are no side effects". In line 64-66, authors described that "feed restriction also has its defects". These two descriptions contradict each other, please check these carefully.

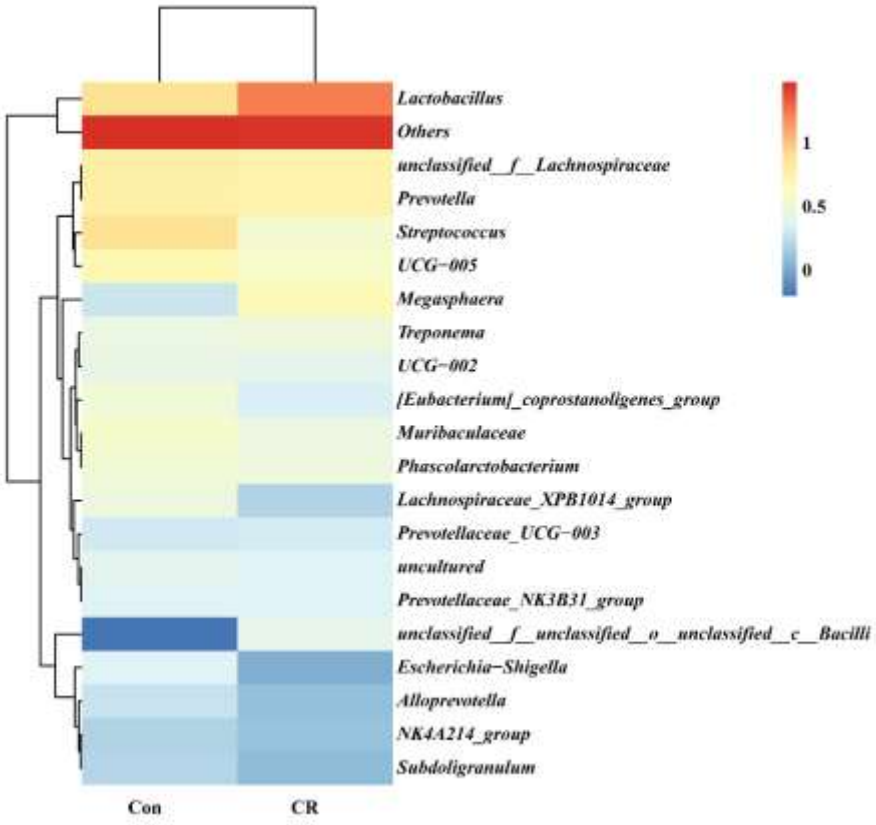
AU: Thank you for your careful reading and comments. We described that "The advantage of this nutritional model is that there are no side effects", which means there is no side effects on human health. We mentioned that "feed restriction also has its defects" in the article, in the following, we point out that this defect is on animal

growth performance. We have clarified and made revisions the article (Line 47; Line68 in the revised)

4. Why H&E staining was performed only use ileal samples? The H&E staining of duodenum and jejunum should be added if these samples were collected.
AU: Thank you for your suggestions. The H&E staining of duodenum and jejunum were non carried out. We will pay attention to these intestinal sections in the future study.

5. In line 137, the methods about 16S rRNA gene analysis is too short and unbusinesslike. Authors analyzed the composition of gut microbiota at the genus level, not the phylum level. Both α diversity and β diversity are analyzed, please add the methods. Furthermore, PCA and PCoA are different.
AU: Thank you for your valuable comments and suggestions. We have supplemented the methods about 16S rRNA gene analysis. And we have added the methods about α diversity and β diversity (Page 6-7, Line 145-151 in the revision). We also analyzed from the phylum level, there was no significant difference in the phylum, genus level analysis in order to find significant differences in the genus, to facilitate further screening and verification. This article uses PCoA, and the PCA in the article has been changed to PCoA (Page 6, Line 147 in the revision).

6. It will be better if authors add the functional capacity of the intestinal bacterial community using PICRUSt.
AU: Thank you for your valuable comments and suggestions. We analyzed the intestinal microbiota using PICRUSt, and the heatmap of relative content at the genus level is shown in the following Figure. From the figure, it can be seen that the graph obtained by this analysis method is similar to the result in Figure 4C in the text. To avoid duplication, PICRUSt analysis graphs will not be included.



PICRUSt analysis graph

7. In the figure 1 legend，authors only showed that "* P < 0.05", please explain "**" .

AU: Thank you for your valuable comments and suggestions. We have supplemented the "**" as "*P* < 0.01" in the Figure 1 legend (Page 17, Line 525 in the revision).

8. In the figure 3 legend，scale bar is 100 μm, not 50 μm. Figure 3 didn't show that there were no significant differences in heart, liver and spleen indices.

AU: Thank you for your carefully check and suggestions. We have corrected the scale bar as 100 μm. We deleted the sentence about heart, liver and spleen indices in the figure legends.

9. Figure 5 didn't show that the relative abundance of Lactobacillus in CR group was increased.

AU: Thank you for your carefully check and suggestions. We have deleted the sentence about the relative abundance of Lactobacillus in the figure legends.

10. The authors need to be more careful to avoid small mistakes when writing.

AU: Thank you for your valuable comments and suggestions. We have corrected the writing and grammar mistaken in the revision.

ACalorie restriction improves serum lipid metabolism, colon metabolites and microbiota in pigs

Jingliang Liu[a], Yusen Wei[a], Hanqiu Di[a], Yifan Zhong[a], Weidong Hua[b] and Haifeng Wang[a, 1]

[a] *College of Animal Science, Zhejiang University, The Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Hangzhou 310058, China*

[b] *Experimental Livestock Farm of Animal Husbandry and Veterinary Research Institute, Zhejiang Academy of Agricultural Sciences, Hangzhou, China*

Short title: Fasting on metabolism and gut microbiota of pigs

*Corresponding author.

Email address: haifengwang@zju.edu.cn (HF. Wang)

Number of figures: 7; Tables, 3; Supplement tables, 2; Supplement figures, 2.

Address correspondence to Dr. Hai-Feng Wang (e-mail: haifengwang@zju.edu.cn).

Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; CR, calorie restriction; FC, fold change; HDL-C, high density lipoproteins cholesterol; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDL-C, low density lipoproteins cholesterol; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; OTUs, operating taxonomic units; PCoA, principal component analysis; SCFAs, short-chain fatty acid; TC, total cholesterol; TG, total triglyceride.

ABSTRACT

Calorie restriction plays roles in reducing food intake and weight gain, and improving health and lifespan. We hypothesized that calorie restriction would affect body weight, serum indices, gut microbiota, metabolites and short-chain fatty acid (SCFAs) of finishing pigs. Castrated male (Landrace×Yorkshire) pigs (86.13 ± 3.50 kg) were randomly assigned into two groups indicated as control (Con) and calorie restriction (CR) (8 pigs/group), respectively. Pigs in the Con group consumed feed *ad libitum*, whereas pigs in the CR group were fed 70% of the amount of feed in the Con group. The trial lasted for 38 days. Blood and colonic contents were collected for serum parameters, and microbiota and metabolome analysis, respectively. Main effects were tested by Student's *t*-test. We found that for finishing pigs, calorie restriction reduced the cumulative food intake, BW gain, serum total cholesterol, triglyceride, low density lipoproteins cholesterol, high density lipoproteins cholesterol, alanine aminotransferase and aspartate aminotransferase levels. Calorie restriction did not change the α and β diversity of intestinal microbiota. However, calorie restriction significantly increased the abundance of *Romboutsia* and *unclassified_c_Bacilli,* and significantly reduced the abundance of *Lachnospiraceae_XPB1014_group*, *Candidatus_Saccharimonas*, *Escherichia-Shigella* and *Gastranaerophilales.* Calorie restriction also simultaneously changed the structure of intestinal metabolites and increased the concentration of isobutyric acid, isovaleric acid and valeric acid. In conclusion, calorie restriction may affect metabolism, reduce obesity and improve intestinal microbiota, which may be a healthy diet treatment that can reduce obesity and improve metabolism.

Keywords: calorie restriction, body weight, gut microbiota, metabolites, pig

1. Introduction

Calorie restriction refers to reducing 10–40% reduction in calorie intake while ensuring that the nutritional level of the food remains the same. Calorie restriction is the most reported dietary intervention with multiple health benefits [1]. It prevents cancer, hypertension, diabetes, and other age-related diseases for primates [2, 3]. Studies have shown that under the condition of ensuring nutritional supply, calorie restriction prolongs the lifespan of experimental animals and reduces or delays the occurrence of age-related diseases in short-lived organisms such as unicellular organisms and mice [4]. At present, it is generally believed that calorie restriction is an effective nutritional intervention, and it has been proved that calorie restriction can improve body health, prolong life, and enhance the ability to cope with stress. The advantage of this nutritional model is that there are no side effects of human health [5-7].

At present, there are many studies on the 30% calorie restriction in animals showing a significant effect on health. For example, the onset of aging-related diseases in rhesus monkeys was delayed and their life expectancy was prolonged after a 30% calorie restriction [2], which suggests that calorie restriction can improve the health and survival of rhesus monkeys [4, 8]. Similarly, a 30% calorie restriction diet can prolong the lifespan of mouse lemurs by 50% and reduce the incidence of aging-related diseases [9].

Previous studies have shown that energy intake is affected by intestinal microbiota [10, 11]. It has been found that calorie restriction can significantly change the intestinal microbiota in mice[12]. As popular dietary intervening patterns, both calorie restriction and intermittent fasting have a great impact on body metabolism. The effect of diet on metabolism depends on gut microbiota [13]. Current studies have confirmed that food intake, food composition and diet improve metabolism by changing the gut microbiota [14]. Complex carbohydrates are taken up by intestinal microbiota and fermented into short-chain fatty acids (SCFAs) in the colon [15-17]. SCFAs are absorbed by the colon and play a role in its health [18, 19].

Calorie restriction has great influence on body composition and metabolic function. Studies on rhesus monkeys showed that the body fat of the calorie-restricted rhesus monkeys

decreased and their body weight was significantly lower than that of the control group [20]. Calorie restriction can significantly improve metabolic function, especially insulin sensitivity [21, 22]. Studies have found that calorie restriction can significantly improve glucose homeostasis and effectively prevent the occurrence of diabetes [2]. Of course, feed restriction also has its defects in animal production. The defect of feed restriction lies in the slow growth of animal weight, which is easy to cause animal restlessness. However, many physiological and metabolic studies of human body need animal models, pigs act as an excellent animal model for studying human nutrition and metabolism for they are closely related to humans in terms of genetics, anatomy, and physiology [23, 24].

We hypothesized that calorie restriction would affect body weight, serum indices, gut microbiota, metabolites and short-chain fatty acid (SCFAs) of pigs as a model for human. The purpose of selecting fattening pigs as experimental animals is that the growth and development of pigs in the fattening stage is nearly complete, and the nutrition intake in the later stage is mainly used to deposit fat. In terms of this characteristic, fattening pigs are just similar to people at the obesity stage, so they can be used as experimental animals to study the effects of calorie restriction on obese people. At present, some articles have studied the effect of starvation or fasting on intestinal microbiota of mini pig or piglets, however, to our knowledge, there is no studies about effect of specific degree of calorie restriction on intestinal microbiota and metabolites of finishing pigs[25, 26]. The innovation of this article attributes to investigating the effect of calorie restriction at degree of 70% of normal feeding on intestinal microbiota, metabolites and the correlation between them.

2. Materials and methods

2.1. Animals and experiment design

A total of 16 castrated male pigs (Landrace × Yorkshire) (86.13 ± 3.50 kg) were randomly assigned into two groups, the control (Con) and calorie restriction (CR) groups (8 pigs/group). All animal procedures were performed fully according to the "Regulation for the Use of Experimental Animals" of Zhejiang Province, China. This study was specifically approved by

the Animal Care and Use Committee of Zhejiang University (ETHICS CODE Permit no. ZJU20170529). Pigs were housed individually throughout the experiment. Pigs in the Con group were provided with *ad libitum* access to basal diet, whereas pigs in the CR group were fed twice a day, at 09:00 and 15:00, with a total of 70% of the amount of feed that consumed by pigs in the Con group. In more detail, daily feed allowance was recalculated every three days to take into account the increase in BW and feed intake of the Con group. Therefore, the feed intake of CR group consists of two parts, one is 70% of the average daily feed intake of Con group in the first three days, and the other is 70% of the average daily feed intake gain of Con group in the first three days.

The basal diets were formulated to meet National Research Council (2012) recommendation (**Table 1)**. The trial lasted for 38 days. All animals were maintained under standard conditions (25±1°C) and were free to water.

After binding pigs, the body weight was measured at 0, 10, 20, 30 and 38 days at 09:00. And the blood was collected from anterior vena cava on the 1st, 20th and 38th day of the experiment at 16:00, then provide feed to pigs after blood collection. The serum was collected by centrifugation at 3000 × g for 10 min at 4 ℃ and stored in refrigerator at -20 ℃ for the determination of serum biochemical indices. After the 38th day of the trial, all pigs fasted overnight and were euthanized to measure the weight of internal organs (heart, liver and spleen) and collect the ileum tissue and the contents of the colon.

2.2. Serum biochemical and free amino acid analysis

The levels of triglyceride (TG), serum glucose, total cholesterol (TC), low density lipoproteins cholesterol (LDL-C), and high density lipoproteins cholesterol (HDL-C) were quantified using the corresponding ELISA kits (nos. A110-1, F006, A111-1, A113-1 and A112-1, respectively; Nanjing Jiancheng Bioengineering Institution, Nanjing, China). Serum levels of alanine aminotransferase (ALT) (no. C009) and aspartate aminotransferase (AST) (no. C010) were measured using kinetics-based assays with commercially available kits (Nanjing Jiancheng Bioengineering Institution, Nanjing, China) using an automatic biochemistry analyzer (Selecta XL; Vital Scientific, Newton, MA, USA) according to the

manufacturers' protocols.

The sample (serum on the 38th day) was precipitated with 5% sulfosalicylic acid (1:4), ultrasonically extracted for 30 min, and then further centrifuged at 18,000 r/min for 30 min. The supernatant was taken and filtered with 0.22 μm filter membrane before analyzed on the machine. As mentioned earlier [27], free amino acid content was detected using a High-Speed Amino Acid Analyzer (L-8900, Hitachi) with Na^+ cation exchange column (4.6 mm × 60 mm, 3 μm particles). The chromogenic agent is ninhydrin/sodium acetate buffer and the buffer system is citric acid buffer B1 (pH=3.2), B2 (pH=3.0), B3 (pH= 4.0), B4 (pH=4.9). The flow rates were 0.4 mL/min for the mobile phase. Other parameters: column temperature (55 °C), the post-column reaction equipment (135 °C). The detector was UV-Vis at 570 and 440 nm.

2.3. Hematoxylin-eosin (H&E) staining

H&E staining was performed as previously described [28]. Ileal samples were fixed, dehydrated, and embedded in paraffin. The sections were prepared and subsequently stained with H&E. Photomicrographs were obtained using an optical microscopy system (Olympus Corporation, Tokyo, Japan). Quantitative measurement of ileal villi height and crypt depth were conducted with Image J (National Institutes of Health, Bethesda, MD, USA).

2.4. 16S rRNA gene analysis

The colonic contents were collected for the bacterial 16S rRNA sequencing. High-resolution 16S rRNA gene analysis was performed according to the methods [29]. DNA was extracted from the colonic contents using the Qiagen DNA Kit (51640, Germany) according to the instructions by manufacturer. The selected region of 16S rRNA amplification was V3-V4 region, and the common primers used were 341F and 806R. The on-board sequencing was carried out using an Illumina NovaSeq PE250 (Illumina, San Diego, USA), followed by bioinformatics analysis. Chimeric sequence detection and *de novo* operational taxonomic units (OTU) picked up with 0.97 identities were implemented using Usearch (version 7.0) and UPARSE (http://drive5.com/uparse/), respectively [30]. OTU abundance tables were obtained, and QIIME1 (v1.9.1) was implemented for OTU profiling, alpha/beta diversity (principal coordinate analysis, PCoA), and rank abundance curve analyses. Linear

discriminant analysis (LDA) effect size (LEfSe) and rank sum test (R version 3.5.1) were used to screen differential bacteria between the two groups.

2.5. Metabolomic analysis

The whole intestinal contents were extracted and centrifuged at 13300 × g for 15 min at 4℃. About 25 mg of sample was transferred into an EP tube using 500 μL extraction solvent (methanol: acetonitrile: water = 2:2:1, v/v, with isotopically-labelled internal standard mixture). The solution was homogenized at the frequency of 35Hz for 4 min, followed by ultrasonic for 5 min. Repeat the previous step for three times. The solution was incubated at -40℃ for 1 h and centrifuged at 12000 rpm for 15 min (4℃). Absorb the supernatant for follow-up analysis.

LC-MS/MS analyses were conducted using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo) [31]. The mobile phase was 25 mmoL/L ammonium acetate-25 mmoL/L ammonia hydroxide-water (pH=9.75) (A) and acetonitrile (B). The parameters of the auto-sampler were 4 ℃, 3 μL. Colonic samples were measured in positive ionization modes. Finally, it was annotated with MS2 database (Biotree DB), with a cutoff value of 0.3 [32].

2.6. Short-chain fatty acid analysis

Colonic contents (0.4 g) were mixed and vortexed with 1.5 mL of phosphate-buffered saline. The sample was centrifuged at 15,000×g for 15 min at 4℃. The supernatant was then taken into the new test tube and 25% metaphosphoric acid was added at 9:1 (v/v). Finally, the supernatant was passed through a 0.22 μ m filter membrane, and the samples were used for SCFAs analysis. The concentration of VFAs in the supernatant was analyzed by gas chromatograph (GC-2010; Shimadzu Corp, Kyoto, Japan) equipped with a column (HP-INNOWAX 19091N-133, 30 m×0.25 mm×0.25μm) [33].

2.7. Statistical analysis

Data were analyzed using SAS software (SAS9.04, Cary NC, USA). The method of analysis is Student's *t*-test, and the data were presented as means ± SEMs. $P < 0.05$ was

considered to be statistically significance difference. The abundances of microbiota among the two groups were compared using the Kruskal–Wallis H-test. Spearman was used to analyze the correlations of intestinal microbiota, metabolites and SCFAs.

3. Results

3.1. Growth performance

As expected, the calorie restriction significantly decreased the daily feed intake (**Fig. 1A)** and average daily gain (**Fig. 1B)** in the CR group than in the Con group ($P < 0.05$), while there was no significantly difference in the feed to gain ratio ($P > 0.05$, **Fig. 1C**). Compared with the Con group, the cumulative food intake of the CR group decreased significantly since the third day of the experiment (**Fig. 2A**). Accordingly, the body weight was significantly decreased in the CR group versus the Con group at 10, 20, 30 and 38 days at 09:00 (**Fig. 2B**).

3.2. Serum biochemical indices and free amino acid

No significant difference was observed in serum biochemical indices between the two groups on the first day of the experiment ($P > 0.05$, **Table 2**). On the 20th day of the experiment, the contents of TC (16% lower, $P < 0.05$), TG (33% lower, $P < 0.05$), ALT (46% lower, $P < 0.05$) and AST (56% lower, $P < 0.05$) in serum were significantly lower in CR group than in the Con group. On the 38th day of the experiment, the CR group had significantly lower contents of TC (24% lower, $P < 0.05$), TG (34% lower, $P < 0.05$), LDL-C (20% lower, $P < 0.05$), HDL-C (19% lower, $P < 0.05$), ALT (23% lower, $P < 0.05$) and AST (20% lower, $P < 0.05$) in serum than the Con group (**Table 2**).

There were no significant differences in the contents of total free amino acid, alanine, aspartic acid, cystine, threonine, serine, glutamic acid, glycine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine, proline in serum between the Con and CR groups on the 38th day ($P > 0.05$, **Table S1**).

3.3. Visceral organ indices and intestinal morphology

After calorie restriction, the CR group had a significantly lower heart weight (13% lower, $P < 0.05$) than the Con group (**Table S2**). There were no significant differences in heart, liver and spleen indices between the Con and CR groups ($P > 0.05$). There were no significant

differences in ilea villus height, crypt depth and villus height/crypt depth ratio ($P > 0.05$, **Fig. 3**).

3.4. Intestinal bacterial community

The results of gut microbiota sequencing showed that a total of 562,022 high-quality reads were acquired, and the average numbers of high-quality reads were 35,126. All the sequences were clustered into 1045 bacterial OTUs according to the threshold of 97% similarity. The rarefaction curve of observed species (**Fig. S1A)** and Chao 1 index (**Fig. S1B)** of gut microbiota plateaued with the increase of reads. The venn diagram showed that the OTUs increased in the CR group than in the Con group (**Fig. S1C**).

Then, the α diversity and β diversity between the two groups were evaluated. In Chao 1 index, there was no significant difference between the two groups ($P > 0.05$, **Fig. 4A**). When shown by the PCoA diagram of weighted Unifrac distance, there was no significant difference in microbial structure between the two groups ($P > 0.05$, **Fig. 4B**).

At the genus level, the composition of gut microbiota (top 20) is shown in **Fig. 4C**. The differential genus is shown in **Fig. 5**. Compared with the Con group, the relative abundance of *Lactobacillus* in CR group was increased, although there was no significant difference ($P > 0.05$). Compared to the Con group, the CR group had significantly lower abundance of *Lachnospiraceae*_XPB1014_group (FC=0.53, $P < 0.05$ **Fig. 5A**), *Candidatus_Saccharimonas* (FC=0.33, $P < 0.05$ **Fig. 5B**), *Escherichia-Shigella* (FC = 0.46, **Fig. 5C**) and *Gastranaerophilales* (FC = 0.24, $P < 0.05$ **Fig. 5D**), whereas exhibited a significant increase in the abundance of *Romboutsia* (FC = 1.41, $P < 0.05$ **Fig. 5E**) and *unclassified_c_Bacilli* (FC = 4.29, $P < 0.05$ **Fig. 5F**).

3.5. Metabolomic profile of the colonic contents

In the positive mode, we observed a significant separation between the two groups (OPLS-DA score plots, **Fig. 6A**). Hotelling's T-squared ellipse showed that there were significant differences between the two groups, and both were within 95% confidence interval (**Fig. 6B**).

We further analyzed the contents of the two groups of metabolites and showed the 20 metabolites with the most significant differences (**Fig. 7A**). The CR group was enriched with

16 metabolites (Pi-Methylimidazoleacetic acid, m-Aminobenzoic acid, 3-Methylguanine, Tyramine, Pyrrolidonecarboxylic acid, Adenine, Pantothenic acid, L-Valine, L-Phenylalanine, L-Methionine, N-Acetylhistamine, Alanyl-Leucine, N-Alpha-acetyllysine, Asymmetric dimethylarginine, 9-HODE, 6,10,14-Trimethyl-5,9,13-pentadecatrien-2-one) ($P < 0.05$) whereas had 4 lower metabolites (1H-Indole-3-carboxaldehyde, Pyricdoxine, Duryl aldehyde, 28-Norcyclomusalenone) ($P < 0.05$) compared with the Con group.

In order to determine the metabolic pathway of differential metabolites enrichment in colon, KEGG pathway database was used to analyze it. The results of metabolic pathway analysis were shown by bubble chart.

The different metabolites were enriched in several biochemical pathways. Among them, the phenylalanine metabolism, linoleic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis pathway were most significantly affected by CR group compared with the Con group ($P < 0.05$, **Fig. 7B**).

3.6. Short-chain fatty acid in colonic content

The CR group had no significant difference in the content of total SCFAs compared with the Con group ($P > 0.05$, **Table 3**). The CR group had a significantly higher isobutyric acid (21% increase, $P < 0.05$), isovaleric acid (28% increase, $P < 0.05$) and valeric acid (29% increase, $P < 0.05$) content than the Con group (**Table 3**).

The Spearman correlation between differential bacteria (genus) and metabolites was explored in 16 individuals in two groups (**Fig. S2A**). It was found that the concentrations of L-Glutamic acid and gamma-aminobutyric acid were significantly negatively correlated with *Lachnospiraceae_XPB1014_group*. The concentrations of xanthine and 9,10-epoxyoctadecanoic acid were significantly negatively correlated with *Romboutsia*. We also analyzed the Spearman correlations between differential bacteria (genus) and SCFAs (**Fig. S2B**). Bacteria from genera *Escherichia-Shigella, Romboutsia* and *Candidatus_Saccharimonas* were positively correlated with the body weight, whereas were negatively correlated with the valeric acid.

4. Discussion

Our hypothesis was confirmed that calorie restriction with 30% low intake significantly reduced BW gain, serum TC, TG, ALT and AST levels in pigs, and changed the abundance of genus such as *Romboutsia*, and also increased the concentration of isobutyric acid, isovaleric acid and valeric acid. It is suggested that calorie restriction may be a healthy diet treatment for improving metabolism and reducing obesity. Several studies on calorie restriction in pigs yielded the similar results [34-36]. Weight loss was also observed in calorie restriction tests in mice [37]. A study of 40% calorie restriction in mice also resulting in a 25% weight loss [38].

Studies have shown that moderate calorie restriction is associated with reducing cardiovascular disease (CVD) mortality [39] and improving metabolic risk factors [40]. And the level of serum cholesterol is related to the occurrence of CVD, lowering the level of cholesterol has been proved to reduce the mortality of CVD [41]. In this study, the level of serum TC in the CR group decreased significantly, indicating that the calorie restriction reduced the risk of metabolic diseases. In the blood, LDL-C is the main carrier of cholesterol, and when LDL-C decreases, so does the total cholesterol in the blood [42]. It is reported that there is a significant correlation between the changes of LDL-C and body weight [43]. In addition, studies have shown that HDL decreases after calorie restriction, which is consistent with the results of this experiment [44].

It is well known that dietary changes can affect the composition and function of intestinal microbial community [45]. A large number of studies have shown that intestinal microbiota can be affected by food intake [46-48]. The study of calorie restriction in obese people found that calorie restriction combined with physical exercise can significantly change the gut microbiota [47]. In this study, the structure of intestinal microbiota (α and β diversity) did not change significantly by a 38-days calorie restriction for finishing pigs.

However, calorie restriction significantly changed the relative abundance of *Lachnospiraceae_XPB1014_group, Candidatus_Saccharimonas, Romboutsia, Escherichia-Shigella, Gastranaerophilales* and *unclassified_c_Bacilli*. Previous studies have shown that *Lachnospiraceae_XPB1014_group* is negatively correlated with body fat weight [49]. In this study, the relative abundance of *Lachnospiraceae_XPB1014_group* decreased significantly in

the CR group, which is corresponding with the low body weight. In addition, the abundance of intestinal harmful bacteria decreased after calorie restriction in this study.

Research proves that the intestinal *Escherichia-Shigella* of rats fed with HFD was significantly higher than that of rats fed with normal food, which may impair the gut barrier [50]. Studies have found that the abundance of *Escherichia-Shigella* is closely related to the overgrowth of bacteria in the small intestine [51]. In addition, low levels of *Escherichia-Shigella* may represent lower levels of antigens and improved inflammatory status [52]. Moreover, *Escherichia-Shigella* is reported to be positively correlated with nonalcoholic fatty liver disease (NAFLD) and involved in the conversion of primary bile acid to secondary bile acid[53]. In this study, the abundance of *Escherichia-Shigella* decreased significantly in the CR group, indicating that the intestinal barrier function may be enhanced and inflammation decreased.

It was reported that *Romboutsia* is negatively correlated with obesity-related indicators [54]. Similarly, in this study, the abundance of *Romboutsia* increased and body weight gain decreased in CR group. *Romboutsia sedimentorum* can use glucose to produce acetic acid and isobutyric acid, which is beneficial for reducing obesity [55]. *Romboutsia ilealis* is abundant in the small intestine of animals and has the ability to break down carbohydrates [56]. Thus, *Romboutsia* may be used to predict and treat obesity.

Gut microbiota can produce SCFAs from food ingredients that have not been absorbed/digested by host [57, 58]. The most abundant SCFAs in the colon are acetic acid, propionic acid and butyric acid, accounting for 90% of the total SCFAs [59]. SCFAs can activate the oxidation of fatty acids, inhibit the lipolysis, and eventually lead to the reduction of free fatty acids in plasma and the decrease in body weight [60, 61]. Isobutyric acid and isovaleric acid are branched short-chain fatty acids (BSCFAs), which can be produced by valine and leucine fermentation, accounting for only 5% of the total SCFAs production [62]. In this study, the 30% calorie restriction had no significant effect on total SCFAs, but significantly increased the concentrations of isobutyric acid, isovaleric acid and valeric acid. According to the correlation analysis, the concentrations of isobutyric acid, valeric acid and

isovaleric acid were significantly negatively correlated with *Lachnospiraceae_XPB1014_group* and *Romboutsia*.

In conclusion, calorie restriction reduced BW gain, serum total cholesterol, triglyceride, low density lipoproteins cholesterol and high density lipoproteins cholesterol levels, simultaneously changed the structure of intestinal metabolites and increased the concentration of isobutyric acid, isovaleric acid and valeric acid, but not the α and β diversity of microbiota. These results indicated that calorie restriction may affect metabolism, reduce obesity and improve intestinal microbiota. The above shows that calorie restriction may be a healthy diet treatment that can reduce obesity and improve metabolism.

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Author Contributions

H.F.W. designed the experiments. J.L.L, Y.S.W., H.Q.D. and W.D.H. performed the experiments. J.L.L, Y.F.Z. and H.F.W analyzed the data. H.F.W. and J.L.L wrote and revised the main manuscript. All authors read and approved the final manuscript.

Author Declarations

The authors declare no competing interests.

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Figures legends

Fig. 1. Effects of calorie restriction on performance of pigs. (A) Average daily feed intake. (B) Average daily gain. (C) Feed to gain ratio. Calorie restriction significantly decreased the daily feed intake and average daily gain in the CR group than in the control group ($P < 0.05$), while there was no significantly difference in the feed to gain ratio ($P > 0.05$). Data are presented as mean ± SEM; ** $P < 0.01$, n = 8. Con, control; CR, calorie restriction. The Con pigs were provided with *ad libitum* access to basal diet, and the amount of feed in the CR group was 70% of that in the Con group.

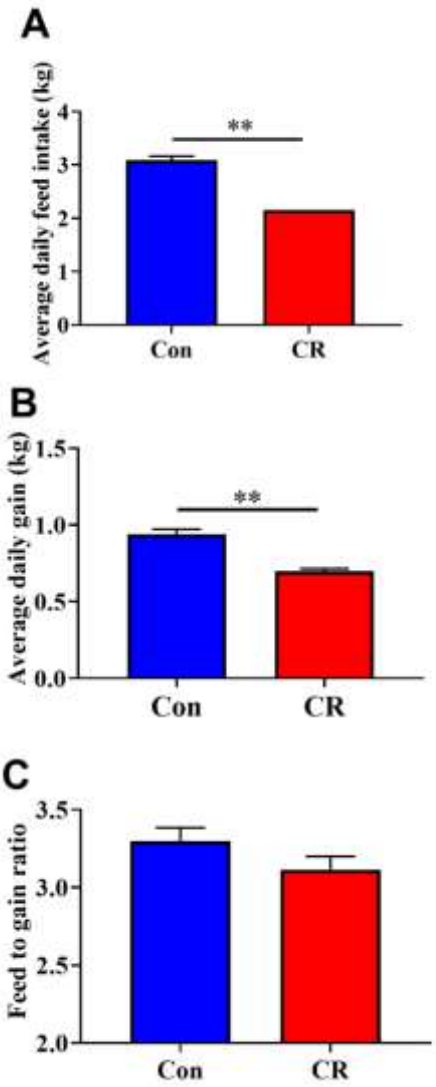

Fig. 2. Change of cumulative feed intake and body weight of pigs with or without calorie restriction. (A) The cumulative food intake of average per pig. (B) The body weight of pig. Compared with the Con group, the cumulative food intake of the CR group decreased significantly since the third day of the experiment. Accordingly, the body weight was significantly decreased in the CR group versus the Con group at 10, 20, 30 and 38 days. Data are presented as mean ± SEM; * $P < 0.05$, n = 8. Con, control; CR, calorie restriction. The Con pigs were provided with *ad libitum* access to basal diet, and the amount of feed in the CR group was 70% of that in the Con group.

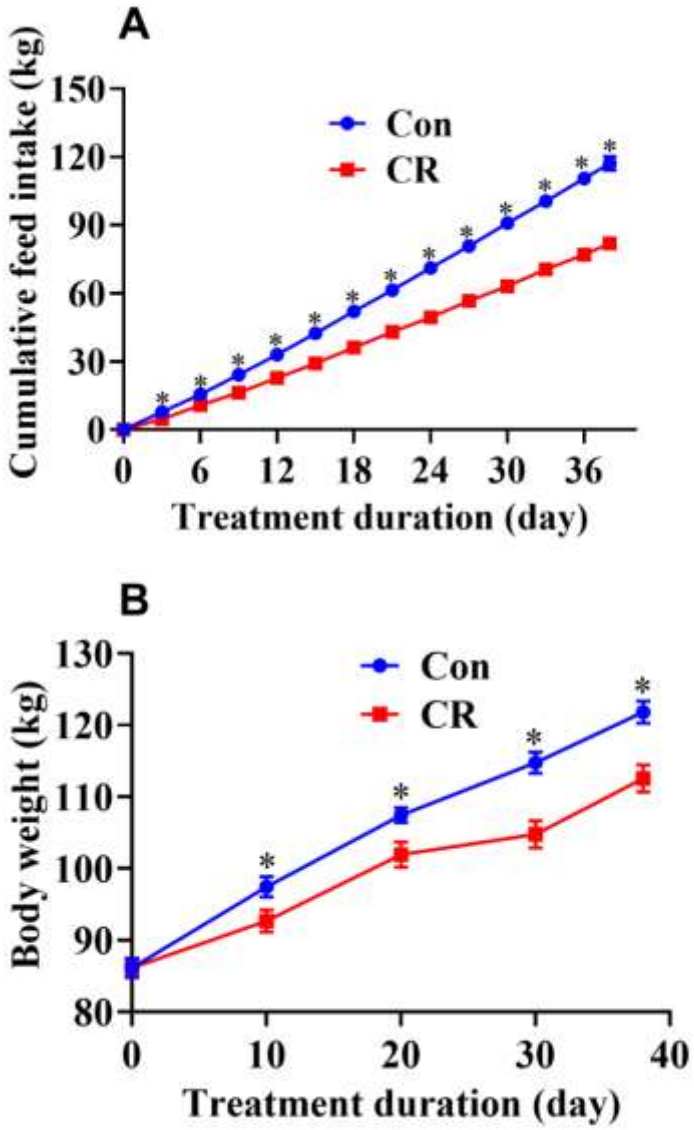

Fig. 3. Villus height and crypt depth of the ilea in the Con group and CR group. (A) Representative H&E staining of ileum sections. Scale bar: 100 μm; (B) Villus height, crypt depth and villus/crypt ratio of ileum between the two groups. There were no significant differences in ilea villus height, crypt depth and villus height/crypt depth ratio between the Con and CR groups ($P > 0.05$). Data are presented as mean ± SEM; * $P < 0.05$, n = 8. Con, control; CR, calorie restriction. The Con pigs were provided with ad libitum access to basal diet, and the amount of feed in the CR group was 70% of that in the Con group.

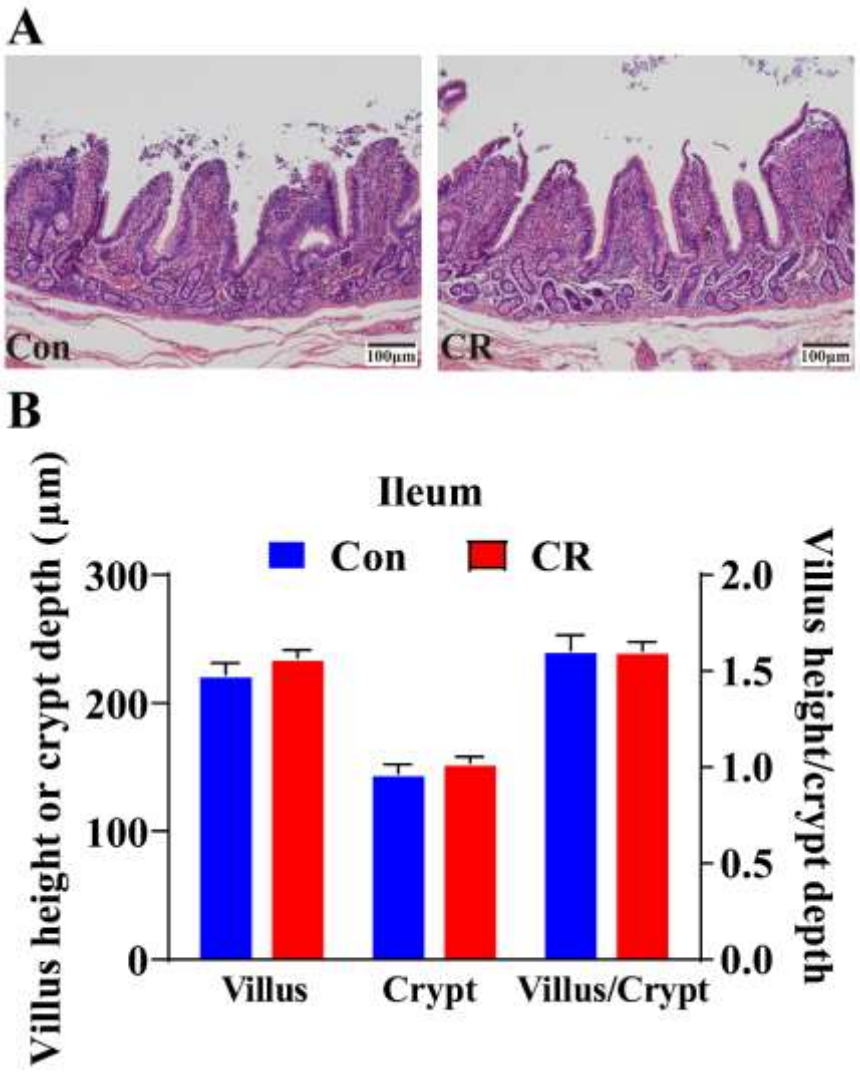

Fig. 4. Alpha diversity of (A) Chao 1 index and beta diversity of (B) weighted Unifrac between the Con and CR groups. The relative abundance in colonic microbiota at genus level (C) between the two groups. The α diversity and β diversity between the two groups were evaluated. In Chao 1 index, there was no significant difference between the two groups ($P > 0.05$). When shown by the PCoA diagram of weighted Unifrac distance, there was no significant difference in microbial structure between the two groups ($P > 0.05$). The top 20 genus are shown in the composition of gut microbiota. Con, control; CR, calorie restriction. The Con pigs were provided with ad libitum access to basal diet, and the amount of feed in the CR group was 70% of that in the Con group.

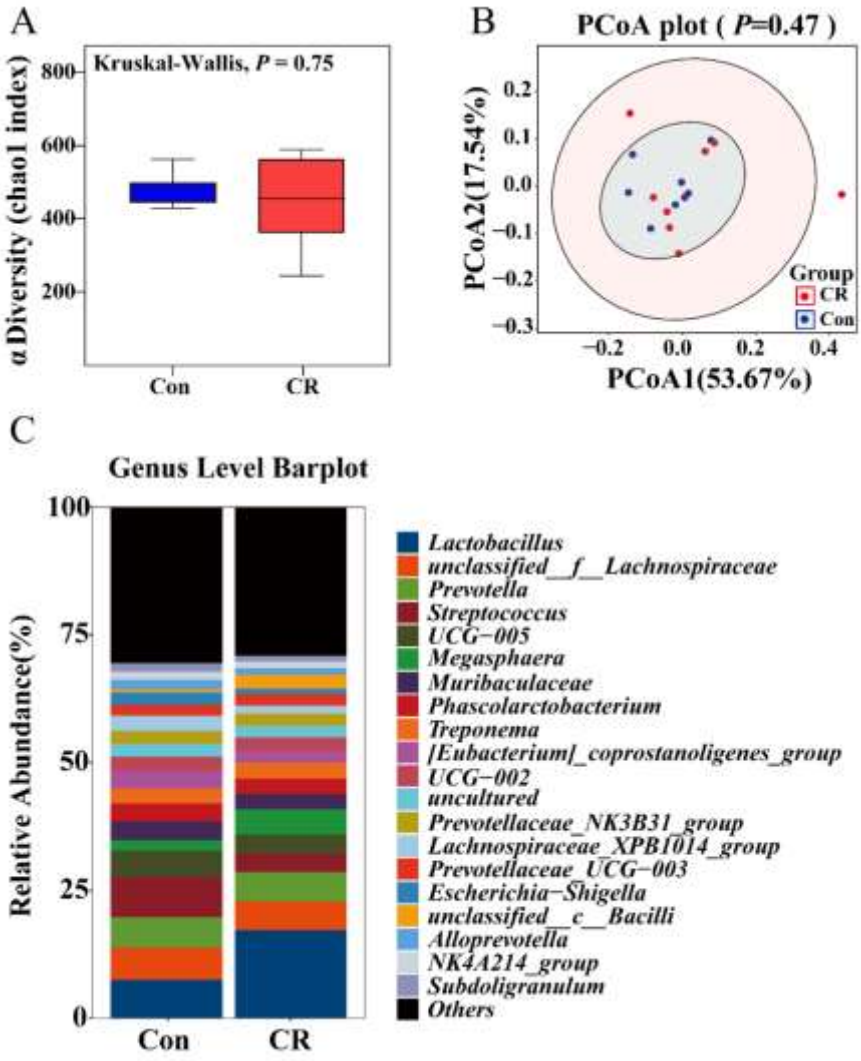

Fig. 5. The differences in colonic microbiota at genus level between the two groups. Compared to the Con group, the CR group had significantly lower abundance of *Lachnospiraceae*_XPB1014_group (FC=0.53, $P < 0.05$), *Candidatus_Saccharimonas* (FC=0.33, $P < 0.05$), *Escherichia-Shigella* (FC = 0.46) and *Gastranaerophilales* (FC = 0.24, $P < 0.05$), whereas exhibited a significant increase in the abundance of *Romboutsia* (FC = 1.41, $P < 0.05$) and *unclassified_c_Bacilli* (FC = 4.29, $P < 0.05$). Data are presented as mean ± SEM; * $P < 0.05$, n = 8. Con, control; CR, calorie restriction. The Con pigs were provided with *ad libitum* access to basal diet, and the amount of feed in the CR group was 70% of that in the Con group.

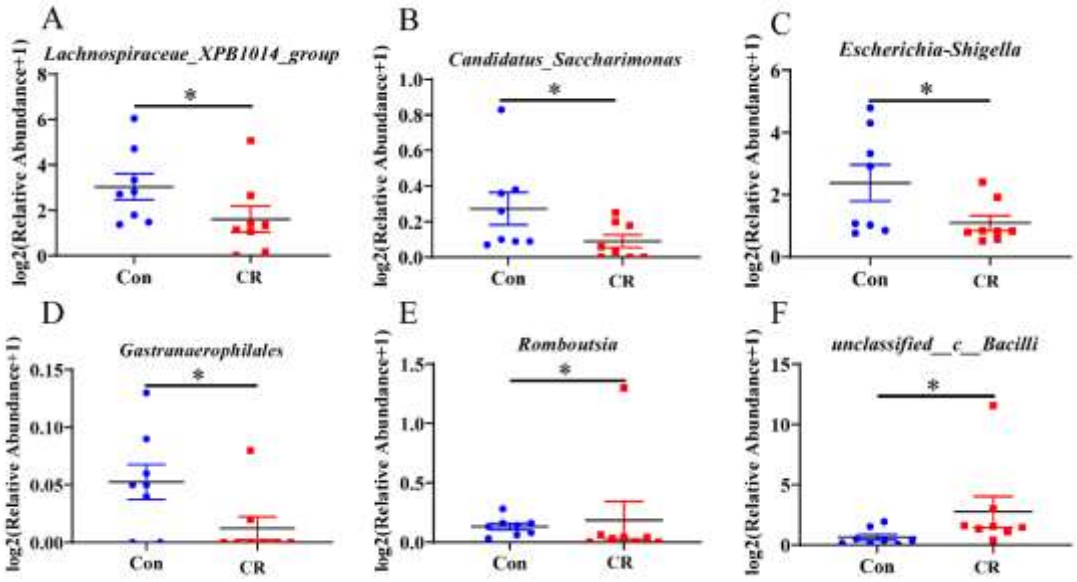

Fig. 6. The OPLS-DA score plots comparing Con and CR pigs in (A) positive electrospray ionization mode metabolomics profiles of colonic contents. (B) The permutation test was evaluated based on the corresponding OPLS-DA model. A significant separation was found between the two groups (OPLS-DA score plots). Hotelling's T-squared ellipse showed that there were significant differences between the two groups, and both were within 95% confidence interval. n = 8. Con, control; CR, calorie restriction. The Con pigs were provided with *ad libitum* access to basal diet, and the amount of feed in the CR group was 70% of that in the Con group.

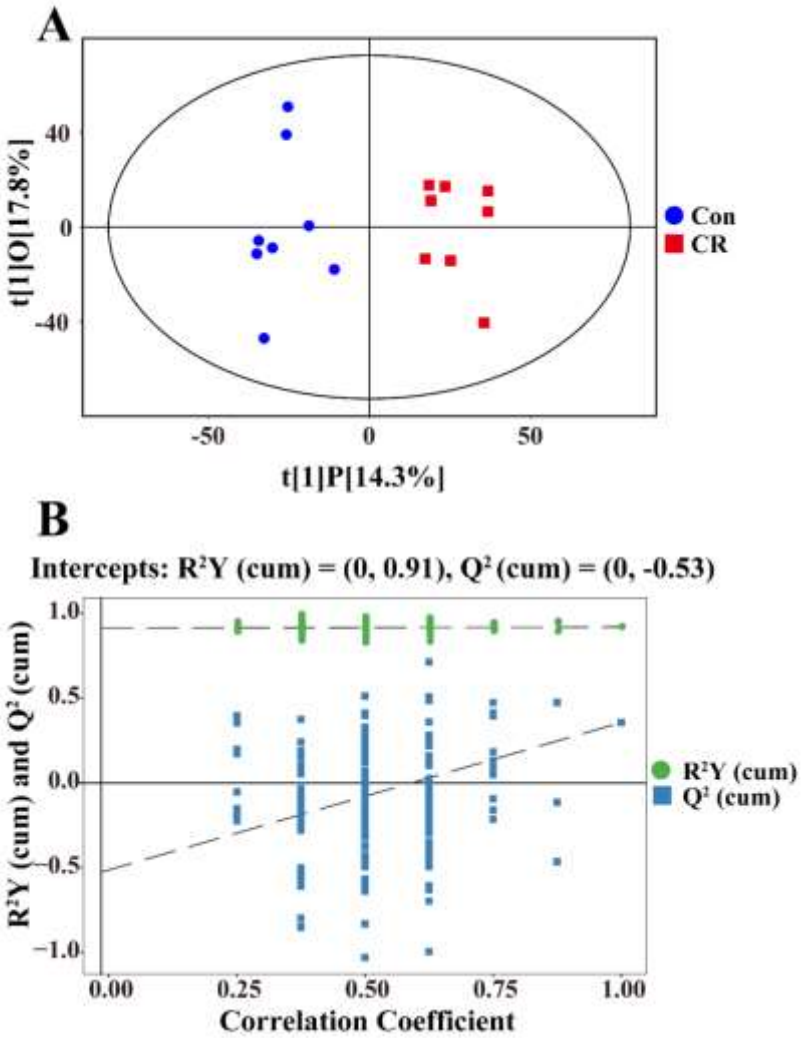

Fig. 7. Hierarchical clustering heat map (A) and topology analysis of metabolic pathways (B) of significantly differential metabolites from colonic contents of pigs from CR and Con groups. The CR group was enriched with 16 metabolites (Pi-Methylimidazoleacetic acid, m-Aminobenzoic acid, 3-Methylguanine, Tyramine, Pyrrolidonecarboxylic acid, Adenine, Pantothenic acid, L-Valine, L-Phenylalanine, L-Methionine, N-Acetylhistamine, Alanyl-Leucine, N-Alpha-acetyllysine, Asymmetric dimethylarginine, 9-HODE, 6,10,14-Trimethyl-5,9,13-pentadecatrien-2-one) ($P < 0.05$) whereas had 4 lower metabolites (1H-Indole-3-carboxaldehyde, Pyricdoxine, Duryl aldehyde, 28-Norcyclomusalenone) ($P < 0.05$) compared with the Con group. The phenylalanine metabolism, linoleic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis pathway were most significantly affected by CR compared with the Con ($P < 0.05$). n = 8. Con, control; CR, calorie restriction. The Con pigs were provided with *ad libitum* access to basal diet, and the amount of feed in the CR group was 70% of that in the Con group.

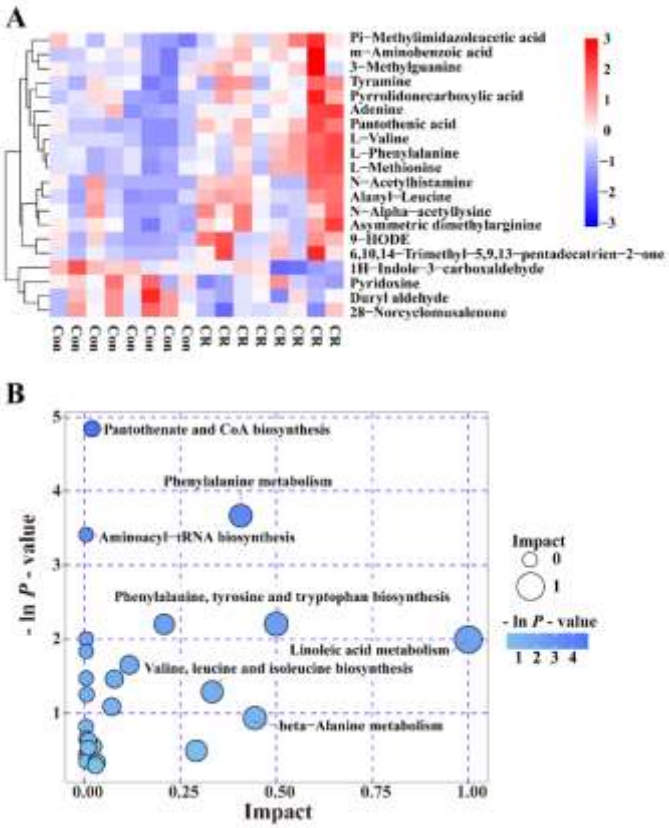

Table 1. Ingredient and chemical composition of the basal diet as fed basis

Items,	g/kg
Ingredients	
Corn grain	647
Soybean meal	160
Wheat bran	160
$CaHPO_4$	2
Calcium powder	10
NaCl	3.5
Phytase	0.1
L-lysine hydrochloride	2.4
Premix[1]	12
K_2SO_4 ($MgSO_4$)	3
Total	1000
Nutrient levels[2]	
Digestible energy (MJ/kg)	13.50
Metabolizable Energy (MJ/kg)	12.91
Net Energy (MJ/kg)	8.72
Crud protein	146
Lysine	8.2
Methionine	2.3
Calcium	4.7
Standard total tract digestible phosphorus	2.2

[1]Premix provided per kg of diets: retinyl acetate, 7420 IU; cholecalciferol, 1000 IU; rac-α-tocopheryl acetate, 25 IU; menadione, 2.5 mg; thiamin, 1.2 mg; riboflavin, 4.5 mg; pyridoxol, 3.0 mg; cobaltamine, 20 μg; nicotinic acid, 25 mg; pantothenic acid 15 mg; folic acid, 1.1 mg; biotin 0.425 mg； Fe, 130mg; Mn, 42mg; Zn, 100mg; Cu, 24.8mg; I, 0.4mg; Se, 0.3mg.

[2] Crude protein was determined value, others were calculated values.

Table 2. Effects of calorie restriction on serum biochemical indices of pigs.

Items	Day	Con	CR	SEM	*P*-value
Glucose (mg/dL)	1d	110±3.18	102±4.66	2.91	0.18
	20d	102±5.43	113±4.68	3.75	0.14
	38d	116±5.83	116±5.79	3.97	0.95
TC (mmol/L)	1d	2.98±0.21	2.90±0.20	0.14	0.79
	20d	3.15±0.14[a]	2.66±0.09[b]	0.1	<0.01
	38d	3.55±0.34[a]	2.71±0.13[b]	0.21	0.04
TG (mmol/L)	1d	0.53±0.04	0.50±0.06	0.04	0.78
	20d	0.74±0.07[a]	0.50±0.04[b]	0.05	<0.01
	38d	0.74±0.06[a]	0.49±0.03[b]	0.05	<0.01
LDL-C (mmol/L)	1d	0.45±0.02	0.48±0.07	0.04	0.7
	20d	0.58±0.02	0.52±0.03	0.02	0.13
	38d	0.62±0.04[a]	0.50±0.04[b]	0.03	0.048
HDL-C (mmol/L)	1d	2.21±0.11	2.13±0.04	0.06	0.45
	20d	2.11±0.13	1.88±0.19	0.12	0.33
	38d	2.21±0.13[a]	1.78±0.12[b]	0.1	0.03
ALT (IU/L)	1d	26.6±1.56	26.4±1.27	0.97	0.9
	20d	39.5±1.06[a]	21.3±1.96[b]	2.59	<0.01
	38d	34.1±1.41[a]	26.2±2.35[b]	1.68	<0.01
AST (IU/L)	1d	6.32±0.31	6.43±0.91	0.46	0.91
	20d	10.2±0.91[a]	4.50±0.36[b]	0.87	<0.01
	38d	5.58±0.35[a]	4.46±0.36[b]	0.28	0.04

Con: control; CR: calorie restriction. The amount of feed in the CR group was 70% of that in the Con group. TC, total cholesterol; TG, total triglyceride; LDL-C, low density lipoproteins cholesterol; HDL-C, high density lipoproteins cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Values are means ± SEMs, n = 8/group. In the same row, values with no letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).

Table 3. Effects of calorie restriction on short chain fatty acids in colon of pigs

Items	Con	CR	SEM	*P*-value
Acetic acid (mg/g)	11.2±0.54	10.8±0.30	0.30	0.48
Propionic acid (mg/g)	1.06±0.06	1.06±0.05	0.04	1.00
Isobutyric acid (mg/g)	0.14±0.01^{b}	0.17±0.01^{a}	0.01	0.04
Butyric acid (mg/g)	0.76±0.04	0.76±0.06	0.03	0.97
Isovaleric acid (mg/g)	0.25±0.01^{b}	0.32±0.03^{a}	0.02	0.048
Valeric acid (mg/g)	0.26±0.02^{b}	0.34±0.03^{a}	0.02	0.03
Total SCFAs (mg/g)	13.7±0.63	13.4±0.40	0.36	0.72

Con: control; CR: calorie restriction. The amount of feed in the CR group was 70% of that in the Con group. Values are means ± SEMs, n = 8/group. In the same row, values with no letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).