Supplemental Information for

Disrupted spermatogenesis in a metabolic syndrome model: the role of vitamin A metabolism in the gut-testis axis

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Materials and Methods

Animal models. All animal procedures were approved and conducted in accordance with the Inner Mongolia University Animal Care and Use Committee. ICR mice were obtained from Inner Mongolia University. Mice were kept at a constant temperature of 22 ± 2°C on a 12 h light/dark cycle and had unrestricted access to food and water. Mongolia male sheep experiments were accomplished at the Inner Mongolia University Experimental Station. Before the experiment, they were acclimatized to the environment with free access to food and water for at least 1 week. Sheep were randomly distributed and fed with a standard chow diet (ND) or a medium-energy (MED) or a high-energy diet (HED) (supplementary tables 1 and 2). The amount of essential vitamin supplementation was nearly the same.

Morphology and immunofluorescence. Testis tissues were quickly collected, fixed in 4% paraformaldehyde, placed in 70% ethanol, dehydrated and embedded in paraffin. The testis were serially sectioned into 5 μm sections. After deparaffinization (at 60 °C for 2 hours), the sections were prepared and stained with hematoxylin and eosin. Within the group, five testicular cross-sections which were at least 50 sections apart from each animal were examined.

For immunofluorescence, the sections were performed in 0.01 mol/L sodium citrate at 95 °C for 10 minutes. Samples were then blocked with BDT (3% BSA, 10% normal donkey serum in TBS) for 30 minutes. Antibody source and dilutions were as follows: MVH (abcam, ab27591, 1:200), STRA8 (abcam, ab49602, 1:300), SYCP3 (abcam, ab15093, 1:300), RDH10 (proteintech, 14644-1-AP, 1:200), RBP4

(proteintech, 11774-1-AP, 1:200), PLZF (abcam, ab189849, 1:200). Sections were examined under a Nikon fluorescence microscope (A1, Japan).

Single cell library preparation, sequencing and analysis. Single cell libraries were prepared using the 10×Genomics Chromium Single Cell 3′Library & Gel Bead Kit v2 (10×Genomics Inc, USA) following the manufacturer's instructions. Briefly, we obtained testicular cells from freshly isolated testicular samples. Testes were minced with sterilized scissors after washing three times with PBS. Mixed testes samples from each group were collected and digested in collagenase type IV (1 mg/ml) at 37°C for 15 min. The digestion process was stopped with DMEM (containing 10% FBS). The cell suspension was filtered with a 40μm cell strainer (BD Biosciences, USA). After centrifugation, the cells were suspended three times in PBS solution supplemented with 0.04% BSA (Sigma, USA).

The cell viability and cell concentration were evaluated before cDNA library construction. Trypan blue staining with a haemocytometer (Bio-Rad, USA) was applied to detect the cell viability (>80%). Then the concentration was 1000 cells/μl for loading to the single cell chip. Chromium 10×Single Cell System (10×Genomics, USA) was applied to form the Gel-Bead in Emulsions (GEMs) system. Then cells were barcoded and cDNA library was constructed. The sequencing was done by an Illumina NovaSeq 6000 (Illumina, USA) with pair end 150 bp (PE150) reads.

The raw sequencing data was processed using CellRanger v3.0.2 software according to 10×Genomics official pipeline (https://www.10xgenomics.com/). The 10×Genomics pre-built sheep genome for Oar_rambouillet_v1.0 was referenced. Then, the cell clustering and quality control were analyzed with scRNA-seq Seurat software (v3.1.5). After characterization of all cell clusters in testes samples, cells were further clustered based on their cell identity. After clustering, cluster-specific marker genes were identified using the "FindAllMarkers" function. The UMAP identified cell clusters were annotated with based on the previously reported canonical marker genes expression. Differential expression genes were used for enrichment analysis in Metascape (http://metascape.org). To interpret cell differentiation fate decisions, Monocle 2 (v2.16.0) was used to determine the single-cell pseudo-time trajectory according to the official tutorial (http://cole-trapnell-lab.github.io/monocle-release/docs/#constructing-single-cell-trajectories). To perform pseudo-time ordering to particular cell types, we firstly subclustered interested cell type from Seurat object.

Then, the Monocle object was constructed using the Monocle implemented "newCellDataSet" function from the Seurat object.

Metabolomics profiling of small intestine digesta samples. Small intestine digesta samples were collected and immediately stored at liquid nitrogen. 25 mg of samples was homogenized with 500 μL extract solution (acetonitrile: methanol: water = 2: 2: 1). The samples were homogenized at 35 Hz for 4 min and sonicated for 5 min in ice-water bath. All samples were incubated at -40°C for 1h and centrifuged at 12000 rpm for 15 min at 4°C. Then supernatant was transferred to a fresh tube and dried in a vacuum concentrator at 37°C. The dried samples were reconstituted in 200 μL of 50% acetonitrile by sonication on ice for 10 min. The samples were subsequently centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were subjected to metabolomics profiling by UHPLC-QTOF-MS.

Liquid chromatographic separation for processed small intestine digesta samples was achieved on a UPLC BEH Amide column (100 mm × 2.1 mm, 1.7 μm, Waters) using a 1290 Infinity series UHPLC System (Agilent Technologies). The mobile phase was composed of 25 mM ammonium acetate and 25 mM ammonia hydroxide in water (A) and acetonitrile (B). The flow rate was set at 0.5 ml/min with the following optimal gradient elution condition: 0 to 0.5 min, 95% B; 0.5 to 7.0 min, 95%~65% B; 7.0 to 8.0 min, 65%~40% B; 8.0 to 9.0 min, 40% B; 9.0 to 9.1 min, 40%~95% B; 9.1 to 12.0 min, 95% B. The column temperature was 25°C. The auto-sampler temperature was 4°C. The injection volume was 2 μl (pos) or 2 μl (neg), respectively.

For LC/MS experiment, the TripleTOF 6600 mass spectrometry was used for its ability to acquire MS/MS spectra on an information-dependent bass (IDA). The cycle time was set at 0.56s. In each cycle, the most intensive 12 precursor ions with intensity above 100 were chosen for MS/MS at collision energy of 30 eV. The operation conditions were set as following: Gas 1, 60 psi; Gas 2, 60 psi; Curtain Gas, 35 psi; Source Temperature, 600°C; Declustering potential, 60 V; Ion Spray Voltage Floating, 5000 V (pos) or -4000 V (neg), respectively. In this mode, data were monitored by acquisition software (Analyst TF 1.7, AB Sciex). MS raw data files were converted to the mzXML format by ProteoWizard, and processed by R package XCMS (version 3.2).

16S rDNA amplicon sequencing and bioinformatics analysis. Genomic DNA of small intestine digesta samples was extracted using a QIAGEN kit, and quantified using a Qubit 2.0 fluorometer (Invitrogen, USA). The V3, V4 hypervariable regions of the 16S rDNA gene was selected for generating amplicons using universal primers with barcode. DNA libraries were recovered using a GeneJET Gel Extraction Kit (Thermo Scientific, USA), and quantified using a Qubit 2.0 fluorometer. The purified DNA libraries were constructed with NEB NextR UltraTM DNA Library Prep Kit for Illumina according to the manufacturer's instructions and index codes were added (NEB, United States). Then, the DNA libraries were sequenced using the 250 bp paired-end strategy on the Illumina platform according to the standard protocol (Illumina, USA). To get the raw tags, paired-end reads were merged by FLASH (V1.2.7). Then raw tags were filtered and clustered by FASTX-Toolkit. We identified possible chimeras by employing UCHIME. The denoised sequences were clustered using USEARCH (version 10.0) and tags with similarity≥97% were regarded as an operational taxonomic units (OTUs) using MOTHUR pipeline. Taxonomy was assigned to all OTUs by searching against the Silva databases using the uclust within QIIME. The Principal coordinate analysis (PCoA), taxonomy, and heatmap of differential abundance were calculated and visualized using R software.

Sperm smear and sperm count measurement. The cauda epididymis was dissected from adult sheep, and incubated for 30 min at 37℃ to release spermatozoa. Spermatozoa were suspended in 1 ml phosphate-buffered saline, washed three times, spread onto slides, and fixed for staining. The viewing areas were captured using a CCD camera. The sperm count was quantified by using a haemocytometer under a Nikon light microscope.

PCR and Real-time quantitative PCR (qPCR) analyses. Genomic DNA of small intestine digesta samples and fecal were extracted using a EasyPure® Stool Genomic DNA Kit, and quantified using a Qubit 2.0 fluorometer (Invitrogen, USA). The primer for *Ruminococcaceae_NK4A214_group* (forward:5'-AACTCATAAACTGCATTTGAAACTGTACT-3' and reverse: 5'-AGCGTCAGTTGCTGTCCAGTAGAC-3') and total bacterium (forward:5'-ACTCCTACGGGAGGCAGCAG-3' and reverse: 5'-ATTACCGCGGCTGCTGG-3') were used. Real-time PCR was carried out with the Roche Light Cycler 480II System.

Western blot analysis. For western blot analyses, testis were extracted in cold RIPA buffer on ice, which supplemented with 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche, USA). The protein lysates were collected and resolved by SDS-PAGE, then transferred onto a polyvinylidene fluoride membrane (Millipore, IPVH00010, Germany) and probed with the primary antibodies. Antibodies were diluted as follows: STRA8 (abcam, ab49602, 1:1000), RDH10 (proteintech, 14644-1-AP, 1:1000), PLZF (abcam, ab189849, 1:1000), GAPDH (proteintech, 60004-1-IG, 1:5000), β-ACTIN (sigma, A1978, 1:10000).

Targeted vitamin A and total bile acid quantification. Vitamin A was quantified by a UPLC I Class system (Agilent 1290 infinity UHPLC) on a C-18 column (Waters, BEH C18 1.7μm, 2.1 mm×50 mm column). Testes samples (100mg) were transferred to a clean centrifuge tube after being thawed on ice. Internal standard solution (10μL), water-acetonitrile-methanol (1:2:2, v/v/v, 0.5 mL) and several 1.0 mm ceramic beads were added, and then homogenized using Biospec MiniBeadbeater before centrifuged at 14,000g for 10min at 4°C. Then, the supernatant was analyzed using HPLC-MS/MS. Mass spectrometry analysis was performed using 5500 QTRAP (AB SCIEX). The 5500 QTRAP APCI source conditions were as follows: source temperature: 550°C; Gas1: 55; Gas2: 55; Curtain gas: 40; IonSapary Voltage Floating: +5500V. MRM method was used for MS quantitative data acquisition.

For serum and testicular vitamin A measurements, serum and testes samples were processed for vitamin A concentration assays by a ELISA kit (Novus, NBP2-60192). For retinene measurements, transplanted material samples were processed for retinene concentration assays by a kit (Dogesce, DG96139Q). For total bile acid measurements, transplanted material samples were processed for TBA concentration assays by a kit (Nanjing Jiancheng Bioengineering Institute, E003-2-1).

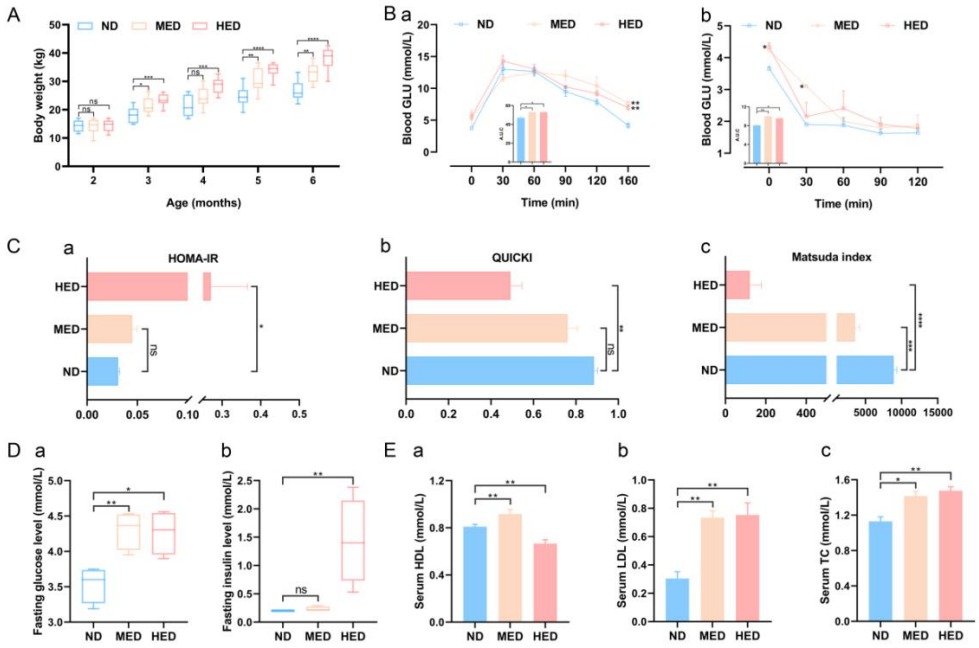
Blood glucose examination during Glucose Tolerance Tests (GTT) and Insulin Tolerance Tests (ITT). During GTT, an intravenous injection of glucose with a single dose of 2g/kg body weight was performed in 12h-fasted sheep. Blood samples were collected before glucose injection (0 min) and at 30, 60, 90, 120 and 160 min afterward. During ITT, an intravenous injection of insulin with a single dose of 0.75

IU/kg body weight was performed in 12h-fasted sheep. Blood samples were collected before insulin injection (0 min) and at 30, 60, 90 and 120 min afterward. Blood glucose concentration were immediately measured by gas chromatograph-mass spectrometer (GCMS-QP2010ultra).

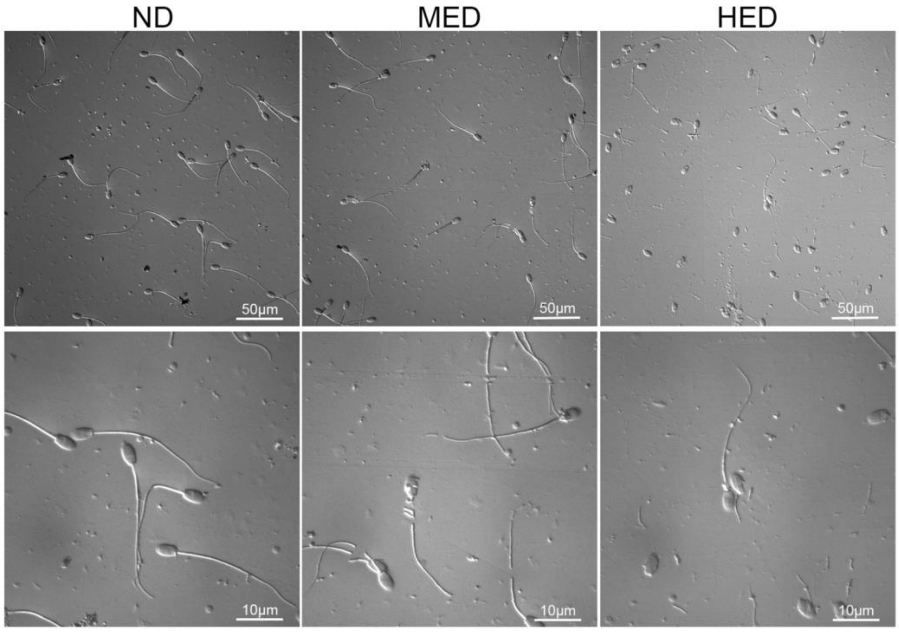
Fecal microbiota transplantation. The small intestine luminal content (microbiota) from each group was collected from donor sheep and pooled. The 1g fecal samples were diluted with 1ml 20% sterile glycerol (saline), homogenized and frozen. Before inoculation, fecal samples were diluted in sterile saline to a working concentration of 0.05 g/ml and filtered through a 70-μm cell strainer. Fresh transplanted material was prepared within 10 min before oral gavage to prevent changes in bacterial composition. Three-week-old male ICR mice were used in current investigation. The recipient mice were treated with fresh transplant material from either ND-treated sheep or HED-treated sheep. Oral gavage with fecal transplant material was conducted daily through the 8-week experiment.

Statistics. GraphPad Prism（USA） was used for statistical analysis. The results of biological assay are presented as means ± SEM. All results were considered statistically significant at $P < 0.05$. Statistical significance between two groups was determined by Student's t test. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison's test was used to assess the statistical significance of differences among three or more groups. Differential metabolites were defined as those with variable importance in the projection (VIP) >1.0 and adjusted *P* values less than 0.05.

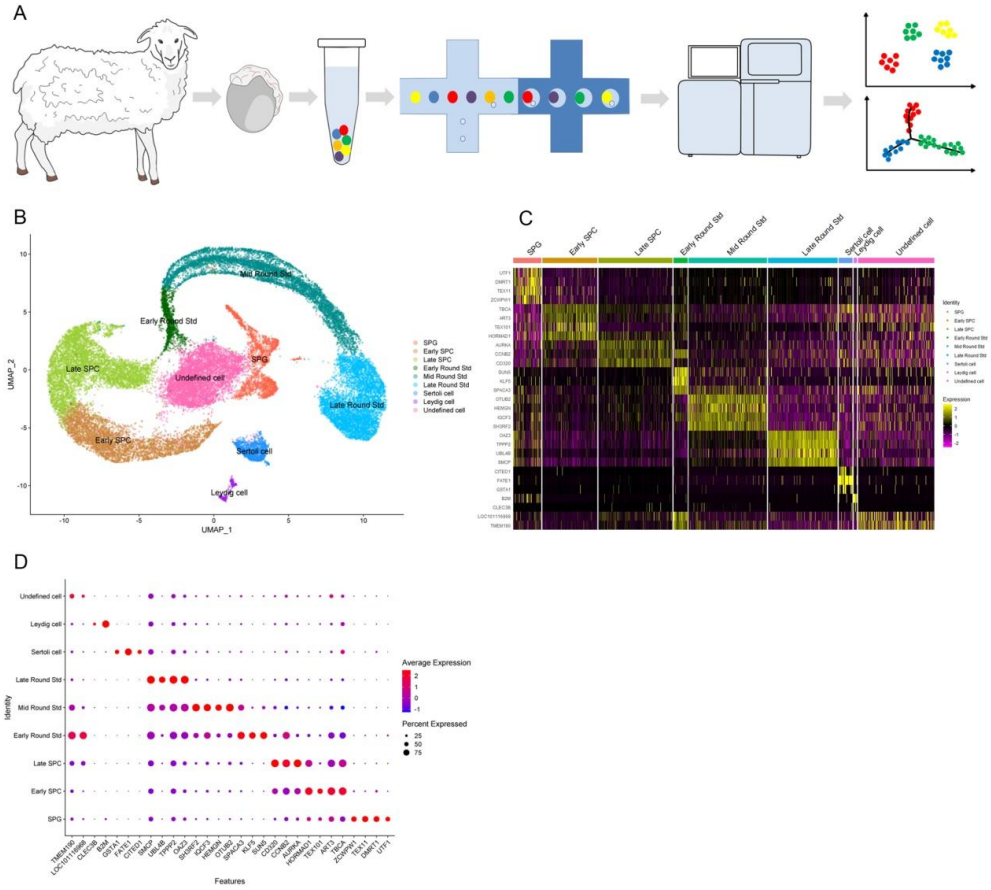
Supplementary figures and figure legends



Supplementary figure 1 Consumption of an excessive-energy diet caused symptoms of metabolic disorder in the sheep model. Sheep were randomly divided into three groups, which involved continuous feeding with a long-term high-calorie diet for six months beginning at two months of age. The experiment included three treatments: a normal diet (ND), a medium-energy diet (MED), and a high-energy diet (HED). (A) Body weights of the ND, MED, and HED-fed groups over a period of 6 months. (B) (a) Glucose tolerance test (GTT) and (b) insulin tolerance test (ITT) with area under the curve (AUC) in the ND, MED, and HED groups. (C) (a) HOMA-IR, (b) QUICKI, and (c) Matsuda indexes were calculated for the three groups. (D) Fasting (a) glucose and (b) insulin levels in the three groups. (E) (a) HDL, (b) LDL, and (c) total TC levels in blood serum from the three groups. **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001. ns, no significant difference.



Supplementary figure 2 Morphology of the spermatozoa from ND, MED, and HED groups. The representative sperm smear for morphology with multiple magnifications. Scale bar = 50 μm and 10 μm.



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235 **Supplementary figure 3 10X Genomics profiling of sheep spermatogenic cells.** (A)
236 Schematic diagram of the scRNA-seq analysis procedure. (B) UMAP plot of testes
237 clusters defined by scRNA-seq analysis. (C) Heatmap of the most common
238 differentially expressed genes (DEGs) from nine clusters. (D) Dot plot of marker
239 genes expressed in the nine clusters.

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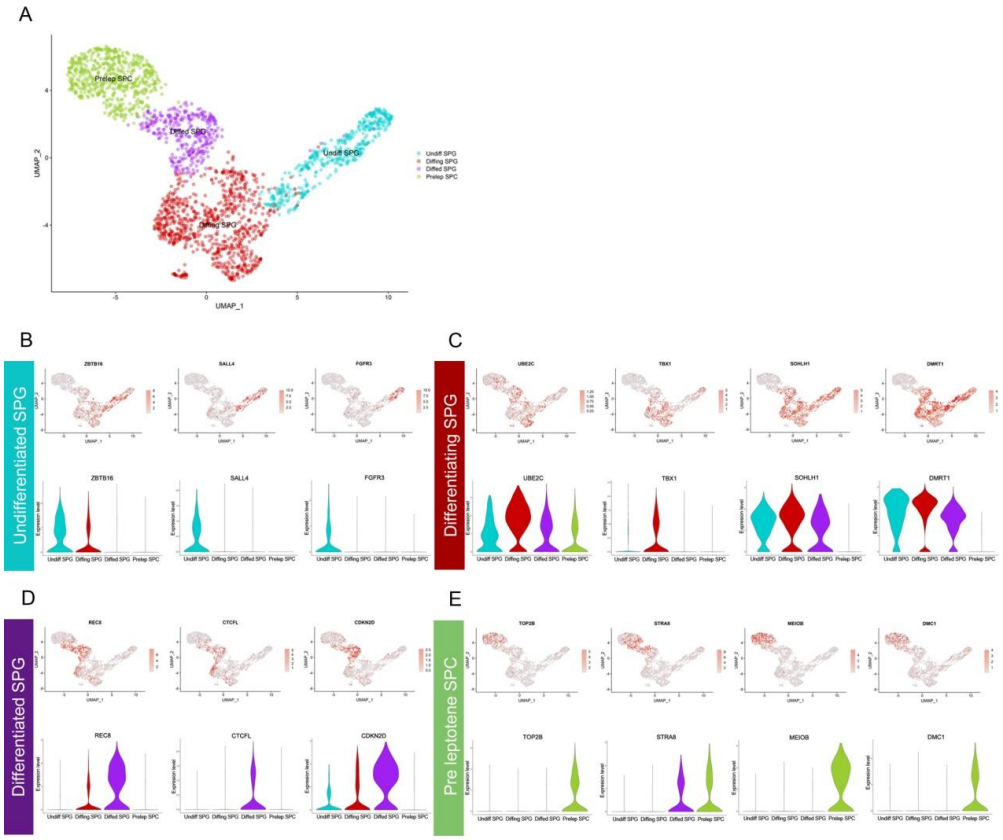
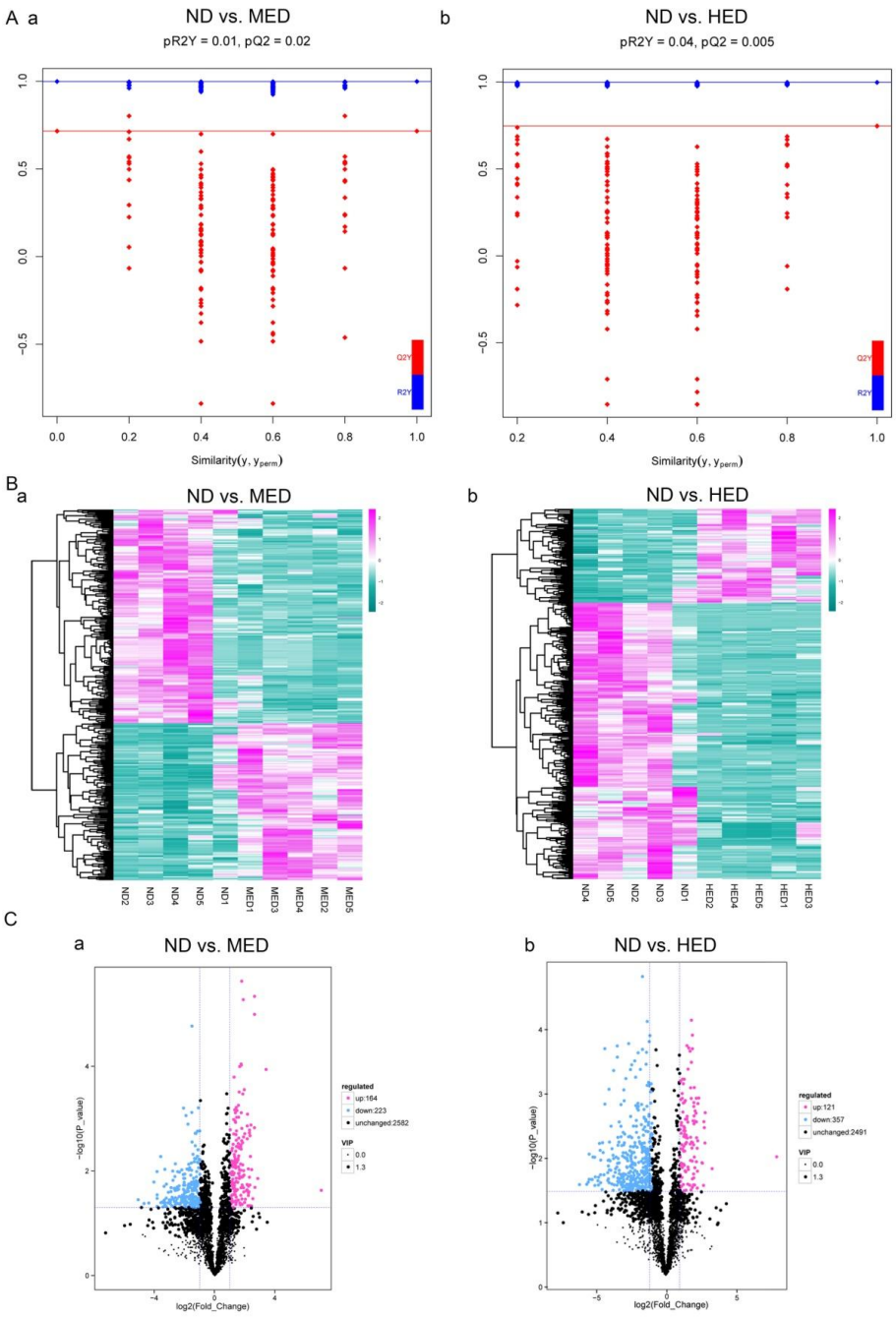
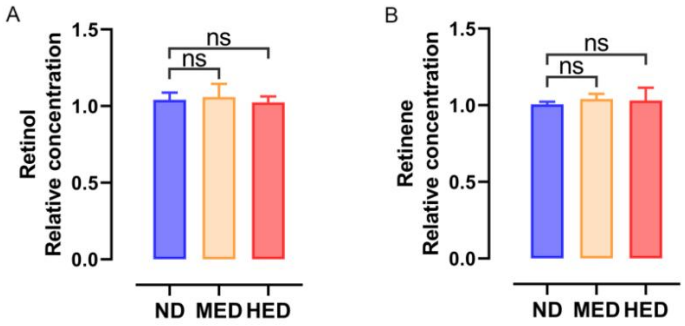


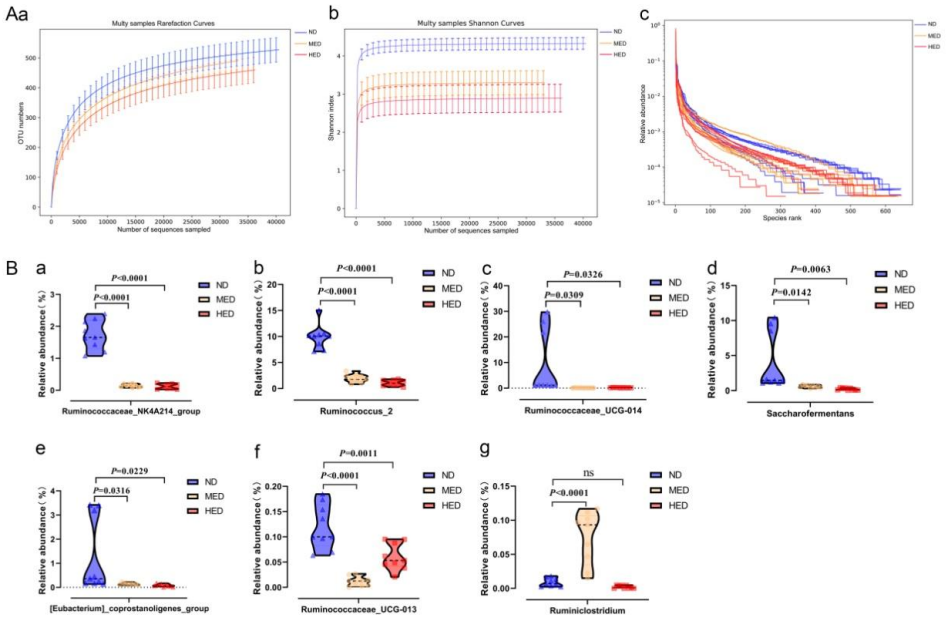
Supplementary figure 4 Identification and characterization of sheep SPG subsets. (A) UMAP plot of SPG subsets. (B-E) UMAP plot, violin plot of undifferentiated SPG (undiffed SPG), differentiating SPG (diffing SPG), differentiated SPG (diffed SPG), and pre-leptotene SPC (pre-L-SPC) marker genes.



Supplementary figure 5 Metabolomics profiling was altered in a MetS model. (A) PLS-DA validation diagram in (a) ND vs. MED comparison and (b) ND vs. HED comparison. (B) Heatmaps of general differential metabolites in (a) ND vs. MED comparison and (b) ND vs. HED comparison. (C) Volcano plots of general differential metabolites in (a) ND vs. MED comparison and (b) ND vs. HED comparison.



Supplementary figure 6 Concentration analysis of retinol and retinene in the diet from ND, MED and HED groups. Analysis of (A) retinol and (B) retinene levels in each indicated group. ns, no significant difference.



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283 **Supplementary figure 7 The α-diversity of gut microbiota and the abundance of**
284 **genus-level gut microbiota belonging to *Ruminococcaceae* in a MetS model.** (A)
285 (a) rarefaction curves, (b) Shannon curves, and (c) OTU rank curves of gut microbiota
286 for ND, MED, and HED samples. (B) Comparison of the relative abundance of genus-
287 level bacteria (a) *Ruminococcaceae_NK4A214_group*, (b) *Ruminococcus_2*, (c)
288 *Ruminococcaceae_UCG-014*, (d) *Saccharofermentans*, (e)
289 *[Eubacterium]_coprostanoligenes_group,* (f) *Ruminococcaceae_UCG-013*, (g)
290 *Ruminiclostridium* belonging to *Firmicutes Ruminococcaceae* in the indicated groups.
291 ns, no significant difference.
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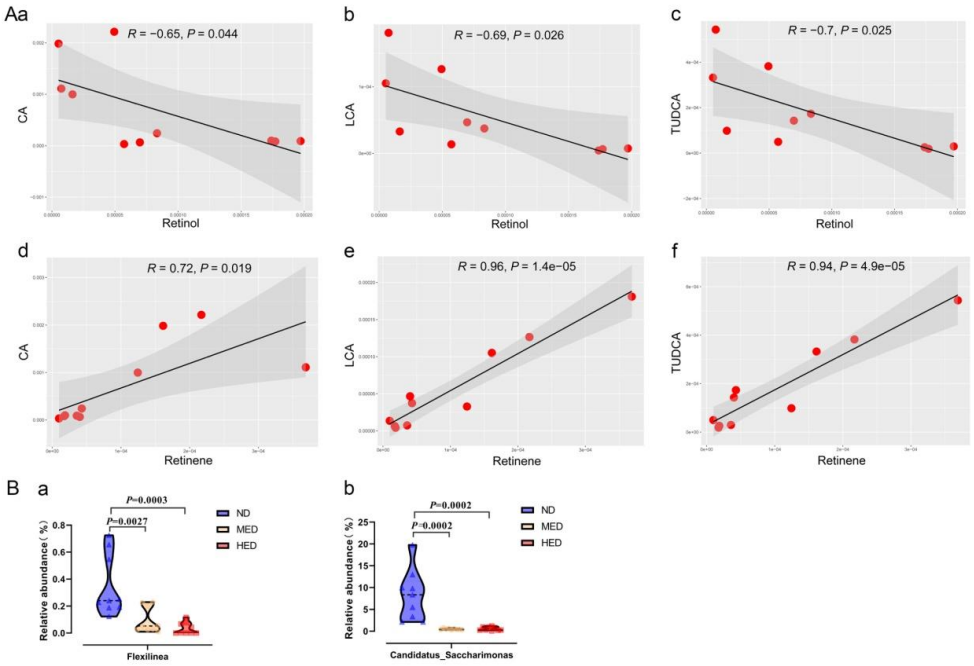
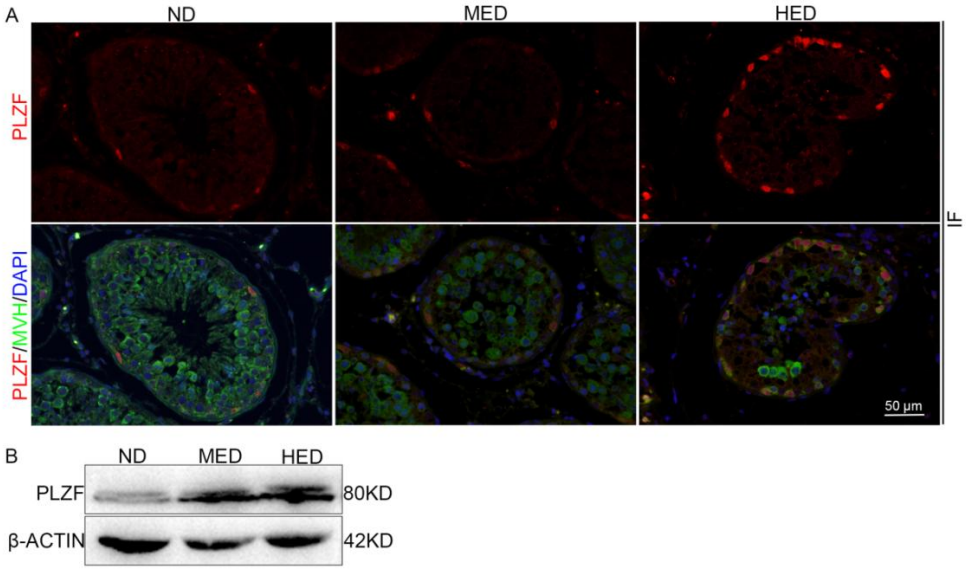
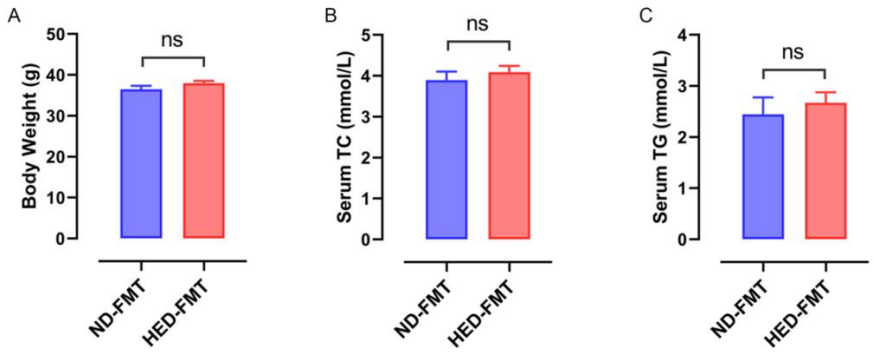


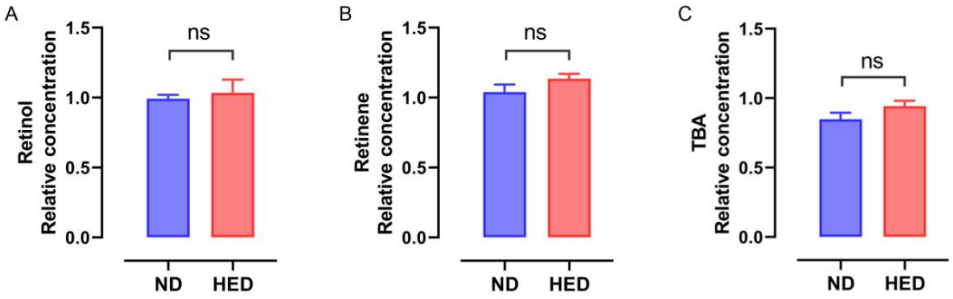
Supplementary figure 8 Correlation analysis of differential metabolites and related genus-level gut microbiota was altered in a MetS model. (A) Pearson's correlation analysis of (a-c) retinol, (d-f) retinene levels with cholic acid (CA), lithocholic acid (LCA), and tauroursodeoxycholic acid (TUDCA) levels. (B) Comparison of the relative abundance of (a) *Flexilinea*, (b) *Candidatus_Saccharimonas* in the indicated groups.



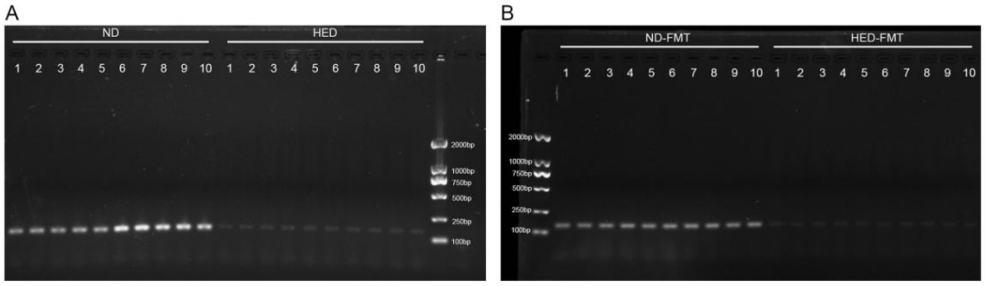
Supplementary figure 9 The expression analysis of undifferentiated spermatogonia marker gene PLZF. (A) The representative images of PLZF immunofluorescence (IF) staining in sections of testicular tissue. Scale bar = 50 μm. (B) Western blotting of PLZF and β-ACTIN in each indicated group.



Supplementary figure 10 Analysis of body weight, serum TC levels, and serum TG levels between ND-FMT and HED-FMT groups. Analysis of (A) body weight, (B) serum TC levels, and (C) serum TG levels in each indicated group. ns, no significant difference.



Supplementary figure 11 Analysis of transmitted material from the donors ND and HED groups. (A) Analysis of retinol levels in each indicated group. (B) Analysis of retinene levels in each indicated group. (C) Analysis of total bile acid (TBA) levels in each indicated group. ns, no significant difference.



Supplementary figure 12 Comparison of the relative abundance of *Ruminococcaceae_NK4A214_group*. (A) The PCR analysis of *Ruminococcaceae_NK4A214_group* abundance in the transmitted material from the donors ND and HED groups. (B) The PCR analysis of *Ruminococcaceae_NK4A214_group* abundance in faecal samples from ND-FMT and HED-FMT groups.

Supplementary table 1 Effective energy per kg of diet (Unit: Mcal)

Groups	ND	MED	HED
Effective Energy	1.05	1.46	2.01

Supplementary table 2 The percentage of nutrient composition

Nutrients/Groups	ND (%)	MED (%)	HED (%)
Protein	16.61	20.99	25.31
Fat	3.24	3.54	3.83
Carbohydrates	80.15	75.47	70.86

Supplementary table 3 The rate of cell viability and cell aggregation

Groups	Cell Viability (%)	Cell Aggregation Ratio (%)
ND	88.00	4.84
MED	87.50	2.77
HED	89.42	1.89