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Research article

Impact of high-fat diet on ovarian epigenetics: Insights from altered intestinal butyric acid levels

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ABSTRACT

Objective: To investigate the effects of a high-fat diet (HFD) on the gut bacterium Roseburia intestinalis and butyric acid levels, and to assess their impact on ovarian function and epigenetic markers in mice.

Methods: A total of 20 female ICR mice aged 4 weeks were randomly assigned to two groups and fed either a control diet (CD) or an HFD for 36 weeks. Post-intervention, ileal contents were analyzed for the quantification of butyric acid using ELISA, while feces were obtained for Roseburia intestinalis expression assessment via qPCR. Histological evaluations of intestinal and ovarian tissues included H&E and Alcian Blue-Periodic Acid Schiff (AB-PAS) staining, alongside immunohistochemical analysis for F4/80, and immunofluorescent detection of Occludin, ZO-1, 5 mC, and H3K36me3. Ovarian health was assessed through follicle counts and morphological evaluations. Statistical analyses were performed using GraphPad Prism 8.0, with $P < 0.05$ considered significant.

Results: After 36 weeks, the HFD group showed significantly higher body weight compared to the CD group ($P < 0.01$). The HFD led to a decrease in Roseburia intestinalis and butyric acid levels, a reduction in intestinal goblet cells, and an increase in intestinal inflammation. Histological analyses revealed impaired ovarian follicular development and enhanced inflammation in the HFD mice, with immunofluorescent staining showing downregulation of the ovarian epigenetic markers 5 mC and H3K36me3.

Conclusion: Our study demonstrates that long-term HFD negatively impacts ovarian function and epigenetic regulation. We found decreased levels of the gut bacterium Roseburia intestinalis and its metabolite, butyric acid, which contribute to these adverse effects. Additionally, the associated intestinal inflammation and compromised mucosal barrier may contribute to these adverse outcomes on female reproductive health.

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1. Introduction

Obesity has become a major public health problem worldwide, affecting the quality of life and health status of many people. By 2038, it is estimated that over a third of the world's population will be obese (BMI $\geq$30 kg/m^2) [1]. Alarmingly, the rate of obesity in women of childbearing age has surged. Data from the U.S. National Health and Nutrition Examination Survey showed that obesity in women aged 20–39 years rose from 29.8 % in 2001–2002 to 39.7 % in 2017–2018 [2]. Similarly, in 2014, over half of European women in this age bracket were overweight or obese, imposing an economic burden of roughly 83 billion euros ($95 billion) annually [3]. China is not exempt, as the China Health and Nutrition Survey reported an increase in obesity rates among childbearing-age women from 2.5 % in 1991 to 12.6 % in 2015 (obesity is defined as BMI $\geq$27.5 kg/m^2 in China) [4].

The interplay between obesity and female reproductive health is well established. It takes longer for obese women to get pregnant than women of normal weight [5]. A published study from the United States indicated that for every 5 kg/m^2 increase in BMI, pregnancy and implantation rates decreased by 1 % [6]. Tortoriello et al. reported a 60 % reduction in natural pregnancy rates in diet-induced obese mice [7]. In addition, obesity not only causes menstrual disorders, endometrial diseases, and infertility in young women but also increases the probability of pregnancy complications such as postpartum death, macrosomia, premature delivery, and cesarean section [8–10]. Especially in assisted reproduction, obesity is negatively correlated with the number of oocytes obtained and the rate of embryo development [11]. Bo et al. demonstrated that the effect of HFD on precocious puberty is regulated by the interaction between gut microbiota and hormones [12]. HFD in women of childbearing age leads to disturbances in the gut flora and increases the risk of obesity in the offspring, which in turn can lead to precocious puberty in children [13]. Therefore, the regulation of gut flora has potential value in the treatment of female reproductive endocrine diseases.

There are more than 250 types of microorganisms in the human gut, including bacteria, fungi, viruses, and archaea, collectively known as "gut microbiota" or "microbiome" [14]. HFD can damage the gut barrier by altering the composition of the microbial community, inducing mitochondrial dysfunction, decreasing the expression of tight junction protein, and accelerating the apoptosis of intestinal epithelial cells [15]. The breakdown of this barrier may allow endotoxins and bacteria to enter the circulatory system, further triggering systemic inflammation. Notably, this low-grade inflammation is closely associated with insulin resistance, fat accumulation, and other pathophysiological changes associated with obesity. In females, the intestinal and reproductive tract microbiota form two highly complex biological ecosystems, both of which exhibit the same five dominant bacterial groups [16]. In healthy conditions, Lactobacillus, which dominates vaginal microbes, is widely believed to originate from the gut [16]. Any disruption in the two ecosystems can lead to fertility issues. Short-chain fatty acids (SCFAs), as an important metabolite of intestinal microorganisms, not only provide the main energy for intestinal epithelial cells but also participate in the regulation of the intestinal inflammatory response [17]. In particular, butyrate, a SCFA, has positive effects on the regulation of intestinal hormones, inhibition of proinflammatory factor production, and enhancement of gastrointestinal barrier function [18]. The interactions between gut microbiota, butyrate concentration, and host energy metabolism are quite complex. More research is needed to better understand the relationship between HFD-induced reduced butyric acid levels and female reproduction.

Gut flora and their metabolites have extensive control over epigenetic processes, including DNA methylation, histone modifications, and the regulation of non-coding RNAs [19]. The epigenetic modification networks play a pivotal role in the development of mammalian oocytes [20]. The epigenetic regulation of oocyte development primarily focuses on DNA methylation, chromatin remodeling, histone modifications, and other epigenetic mechanisms [21,22]. Any imbalance in these modifications can have adverse effects on multiple physiological processes, particularly when combined with HFD.

Initially, we investigated the association between HFD and ovarian epigenetic changes in mice, primarily concentrating on the epigenetic markers 5 mC and H3K36me3. Furthermore, we investigated the possible mediating function of gut microbiota metabolites, particularly butyric acid, in HFD-induced epigenetic alterations.

2. Materials and methods

2.1. Animals and diets

Female ICR mice purchased from the Laboratory Animal Center of Xi'an Jiaotong University Health Science Center (Xi'an, China) were kept in a controlled environment (23 $\pm$ 1 °C, 55 $\pm$ 10 % humidity, 12-h light-dark cycle). Upon arrival, the 3-week-old mice were given 7 days for acclimatization, during which they had free access to water and standard food. Subsequently, the mice were randomly divided into two dietary groups (n = 10/group, 5 mice per cage). Mice in the CD group were fed a chow diet (70 % carbohydrate, 20 % protein, 10 % fat; #D12450B, Research Diets, New Brunswick, NJ, USA), whereas those in the HFD group were provided a high-fat diet (20 % carbohydrate, 20 % protein, 60 % fat; #D12492, Research Diets, New Brunswick, NJ, USA). After 36 weeks, tissue and blood samples were collected before euthanizing the mice. All animal experiments were approved by the Xi'an Jiaotong University's Animal Experimental Ethics Committee and were conducted in compliance with the National Research Council's Guidelines for the Care and Use of Experimental Animals.

2.2. Immunohistochemistry (IHC)

Ovarian and intestinal tissues (jejunum, ileum, and colon) were fixed in 4 % paraformaldehyde for 24 h and then dehydrated using an ethanol gradient dehydrator (KD-TS3B, KEDEE, Jinhua, China). Tissues were embedded in paraffin and sectioned to a thickness of 5

μm. Following this, sections were dewaxed using xylene and a series of alcohols. Endogenous peroxidase activity was blocked using an endogenous peroxidase-blocking buffer (SP KIT-A3, MXB, Fuzhou, China). Antigen retrieval was performed using a sodium citrate solution (pH 6.0) (MVS-0101, MXB, Fuzhou, China) under specific microwave settings (high for 3 min, a 30-s pause, then medium-low for 8 min). Sections were incubated at 37 °C for 50 min with goat serum from the same source as the secondary antibody (Boster, Pleasanton, CA, USA). Following this, the samples were incubated overnight at 4 °C with the primary antibody anti-F4/80 (Cat# ab16911, Abcam, Cambridge, UK), which targets the F4/80 antigen, a specific marker for mouse macrophages. Sections were then washed three times with PBS and incubated with a diluted secondary antibody at room temperature for 45 min. Visualization was achieved using DAB for 30 s, stopped with distilled water, and followed by hematoxylin counterstaining. After dehydration, slides were mounted with neutral gum (ZLI-9018, ZSJB-BIO, Beijing, China) and examined under a microscope. Image analysis was conducted using ImageJ software to accurately quantify the F4/80-positive area, blank area, and total area. The net tissue area was determined by subtracting the blank area from the total area, and the positivity percentage was calculated by dividing the positive area by the net tissue area.

2.3. Immunofluorescence (IF)

Paraffin sections of 5 μm thickness were deparaffinized and subjected to antigen retrieval following the procedure detailed in Section 2.2 for IHC. The sections were then incubated with primary antibodies, including Occludin Rabbit pAb (Cat# A2601, ABclonal, Wuhan, China), ZO-1 (Cat# A0659, ABclonal, Wuhan, China), and TriMethyl-Histone H3–K36 (Cat# A2366, ABclonal, Wuhan, China), overnight at 4 °C. They were then treated with a 1:200 dilution of fluorescein and incubated at room temperature for 45 min. Subsequently, the nuclei were stained with 4′, 6-diamino-2-phenylindole (DAPI, C1005, Beyotime). The slides were washed in PBST for 5 min; this step was repeated three times, and the sections were then observed under a fluorescence microscope (CKX53 and URFLT, Olympus, Tokyo, Japan).

For 5 mC staining, after dewaxing and antigen retrieval, the sections were treated with 0.5 % Triton X-100 for 15 min for permeabilization. Following this, the sections were incubated in 2 N HCl at 37 °C for 1 h to denature the genomic DNA and washed three times with PBS. They were then neutralized with 100 mM TRIS-HCl (pH 8.5) for 10 min, blocked with goat serum, and incubated with the primary antibody Anti-5-Methylcytidine (Cat# 33D3, Eurogentec, Liege, Belgium) overnight at 4 °C. Standard immunofluorescence staining procedures were subsequently employed.

2.4. Hematoxylin and Eosin (H & E) staining and follicle counting

Ovarian, jejunum, ileum, and colon tissues were fixed in 4 % paraformaldehyde at room temperature for 24 h. Subsequently, the tissues were dehydrated, embedded in paraffin, and sectioned into 5 μm-thick slices. These sections were then dewaxed with xylene and rehydrated through a gradient alcohol series, followed by staining with H&E dye. After staining, the sections were further dehydrated and sealed. Under a microscope, we observed the morphological changes of the ovary and intestinal tissues in each experimental group. We conducted follicle counts for different stages on the right ovary and calculated the ratio of primary to growing follicles for each ovary. Germ cells surrounded by a single layer of flat granulosa cells were identified as primordial follicles, while those surrounded by one or more layers of cuboidal granulosa cells were categorized as growing follicles [23].

2.5. Intestinal score and Alcian Blue-Periodic Acid Schiff (AB-PAS) staining

The grading of mucosal damage in the small intestine was assessed according to the procedure described by Chiu and colleagues [24]. For the colonic pathology score, we referred to previously published methods by Sun et al. [25]. The score included assessments of inflammatory cell infiltration (0. no infiltration; 1. increased inflammatory cells in lamina propria; 2. inflammatory cells extend to the submucosa; 3. transmural inflammatory cell infiltration) and tissue injury (0. no mucosal injury; 1. discrete epithelial lesions; 2. erosion or focal ulcer; 3. severe mucosal damage, extensive ulcers extending to the intestinal wall). The combined scores of inflammatory cell infiltration and epithelial damage yielded a total score that ranged from 0 to 6. Furthermore, the AB-PAS staining kit (Maixin Biotech, Fuzhou, China) was employed to stain the goblet cells of the jejunum, ileum, and colon as per the manufacturer's instructions. Subsequently, we quantified the number of goblet cells in each villus in the jejunum and ileum and each crypt in the colon.

2.6. Assay for butyric acid (BA)

Upon euthanizing the mice, the ileum contents were immediately collected. A specific amount of intestinal contents was transferred into a centrifuge tube containing 800 μL of ultrapure water. The mixture was then thoroughly combined and centrifuged at 4 °C for 5 min at 10,000 r. Subsequently, 50 μL of the supernatant was collected for future experimental procedures. ELISA Kit for Butyric Acid (Cloud-Clone Corp, Houston, America) was used to detect butyric acid following the manufacturer's instructions. After designating specific wells for standards and blank controls, we added the samples to their respective wells, followed by the addition of Detection Reagent A. After cleaning, we introduced Detection Reagent B and repeated the cleaning step. The color developed over time, and we terminated the reaction to prevent further color development. Finally, we took measurements immediately using a microplate reader at a wavelength of 450 nm.

2.7. Relative quantification of Roseburia intestinalis

The DNA from the mouse colon contents was extracted following the manufacturer's instructions (DNeasy PowerSoil Pro Kits, QIAGEN, Germany) and subsequently subjected to Quantitative Real-time PCR. The SYBR®Green Premix Pro Taq HS qPCR kit (AG11701, Accurate Biology, China) was utilized. And the primers deployed in this study were: rrs (GGGGGTTTCAACACCTCC and GCAAGGGATGTCAAGTGT) and Roseburia intestinalis (GCGGTRCGGCAAGTCTGA and CCTCCGACACTCTAGTMCGAC), with the rrs acting as an endogenous control gene.

2.8. Statistical analysis

All data were expressed as mean ± standard error (SEM) and statistically analyzed using GraphPad Prism 8.0 (https://www.graphpad.com/scientific-software/prism/) software. All immunofluorescence images were processed and merged using ImageJ (http://es.hongjingcom.cn/ImageJ/s1.html) software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Long-term HFD significantly disrupted the intestinal barrier and intensified inflammation

To simulate the weight gain in females of reproductive age, we selected ICR female mice of reproductive age and established an obesity model using a high-fat diet (HFD) with 60 % of energy from fat. After 36 weeks, the average weight of the mice in the HFD group was 36.87g ± 0.57, compared to 29.71g ± 1.13 in the control diet (CD) group. The weight of the mice in the HFD group was significantly higher than that in the CD group ($P < 0.01$). H&E staining of the jejunum, ileum, and colon illustrated pronounced intestinal damage in the HFD group compared to the CD group. In the CD group, the intestinal villi were well arranged, and the glands were in good condition. In contrast, the HFD group exhibited obvious shedding of villi epithelial cells, expansion beneath the epithelium, separation of the epithelium from the lamina propria, and irregular alignment of crypts (Fig. 1A). Furthermore, both the Chiu's score of the small intestinal mucosa and the pathological score of the colon were higher in the HFD group than in the CD group, suggesting exacerbated intestinal damage. These differences were statistically significant (Fig. 1B and C). The intestinal mucosal surface features a hydration gel barrier, primarily composed of mucin secreted by goblet cells, which effectively protects against mechanical, chemical, and biological aggressions and plays a key role in maintaining intestinal health [25]. Notably, our findings indicated a reduced number of goblet cells in the jejunum, ileum, and colon of HFD mice compared to the CD group (Fig. 1D–F).

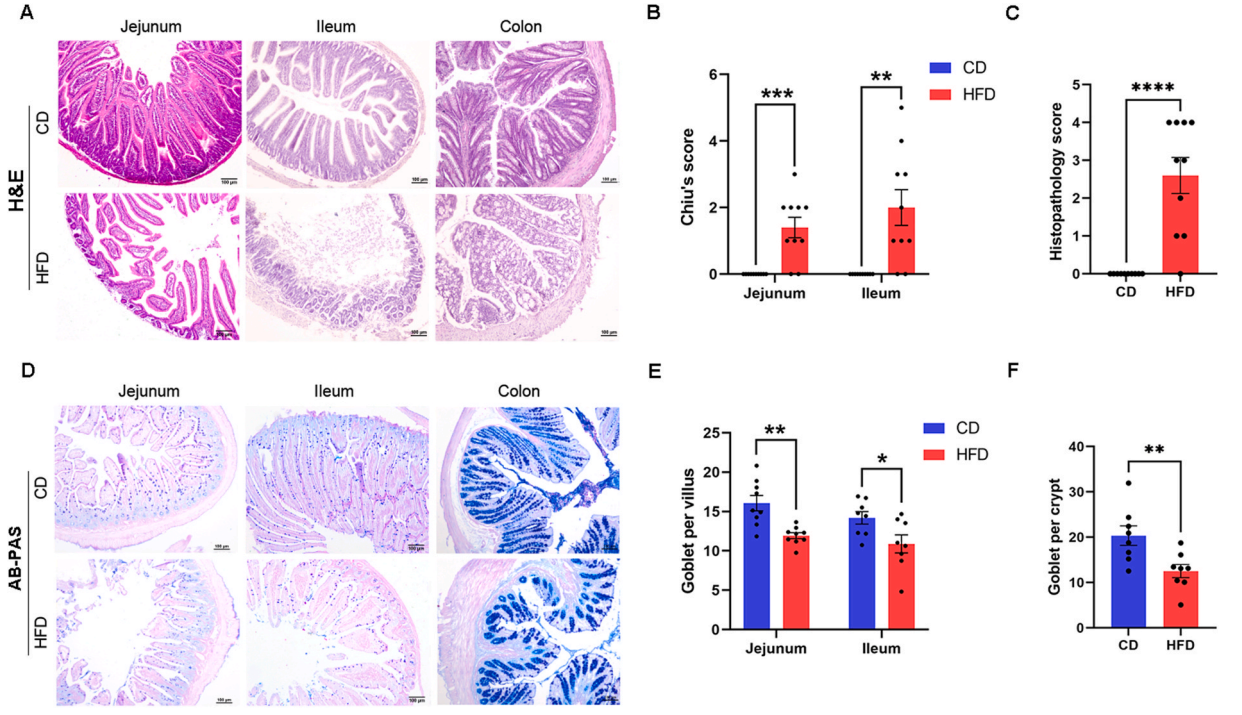

Fig. 1. HFD results in an impaired intestinal barrier. (A) H&E staining. Scale bars: 100 μm. (B) Chiu's score of jejunum and ileum. (C) Histopathology score of the colon. (D) AB-PAS staining. Scale bars: 100 μm. (E) Count of goblet cells per villus in the jejunum and ileum. (F) Count of goblet cells per crypt in the colon. The data are expressed as the mean ± SEM. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

The intestinal epithelium is not only the primary defense line of the gastrointestinal tract but also closely related to the stability and immune balance of the mucosal barrier [26]. Through our study, we found that the expression of two key tight junction proteins, ZO-1 and Occludin, in the ileum and colon of HFD group mice was decreased compared with that of CD group mice (Fig. 2A–C). Significantly, F4/80, a marker for mouse macrophages (cells primarily involved in immune and inflammatory responses), was increased in the intestines of the HFD group (Fig. 2D and E). To summarize, prolonged HFD intake caused significant negative effects on the structure and function of the mouse gut, with particular detriment to the intestinal barrier and heightened inflammatory responses.

3.2. Long-term HFD reduced intestinal butyric acid and Roseburia intestinalis

Butyric acid has anti-inflammatory properties that bolster the intestinal barrier and augment mucosal immunity [27]. In order to explore the effect of HFD on intestinal butyric acid, we collected the ileal contents from two groups of mice for quantitative analysis. It was discerned that the HFD led to diminished levels of butyric acid in the ileum (Fig. 3A). Roseburia intestinalis, a Gram-positive, obligate anaerobic bacterium that produces butyrate, is renowned for mitigating intestinal inflammation and significantly enhancing intestinal barrier resilience through its metabolites [28]. To further confirm the butyric acid alterations, a qRT-PCR test was conducted on the collected feces. The result highlighted a reduced expression level of R. intestinalis in the HFD group when compared to the CD group (Fig. 3B). Collectively, these findings indicate that sustained HFD consumption suppresses the butyric acid-producing bacterium, R. intestinalis, subsequently leading to decreased intestinal butyric acid concentrations.

3.3. Long-term HFD led to ovarian inflammation without significant changes in follicle counts

Next, we sought to investigate the effects of long-term HFD on ovarian physiology in female mice. Fig. 4A depicts the ovarian pathology of both the CD and HFD groups. After an independent validation by blinded observers, a comparative analysis of follicle counts and types revealed a slightly higher proportion of growing follicles in the CD group in contrast to the HFD group. Conversely, the HFD group exhibited a slight uptick in the proportion of primary follicles. However, these variations did not achieve statistical significance (Fig. 4B and C) (with p-values of 0.1736 and 0.1787, respectively). It is worth noting that prior research has posited that HFD might precipitate polycystic ovary syndrome (PCOS), a syndrome associated with increased expression of ovarian inflammatory factors and inhibition of oocyte maturation and ovulatory processes [29]. In order to further explore the possible ovarian inflammatory response induced by HFD, we conducted F4/80 immunohistochemical staining on ovarian tissue samples from both groups (Fig. 4D

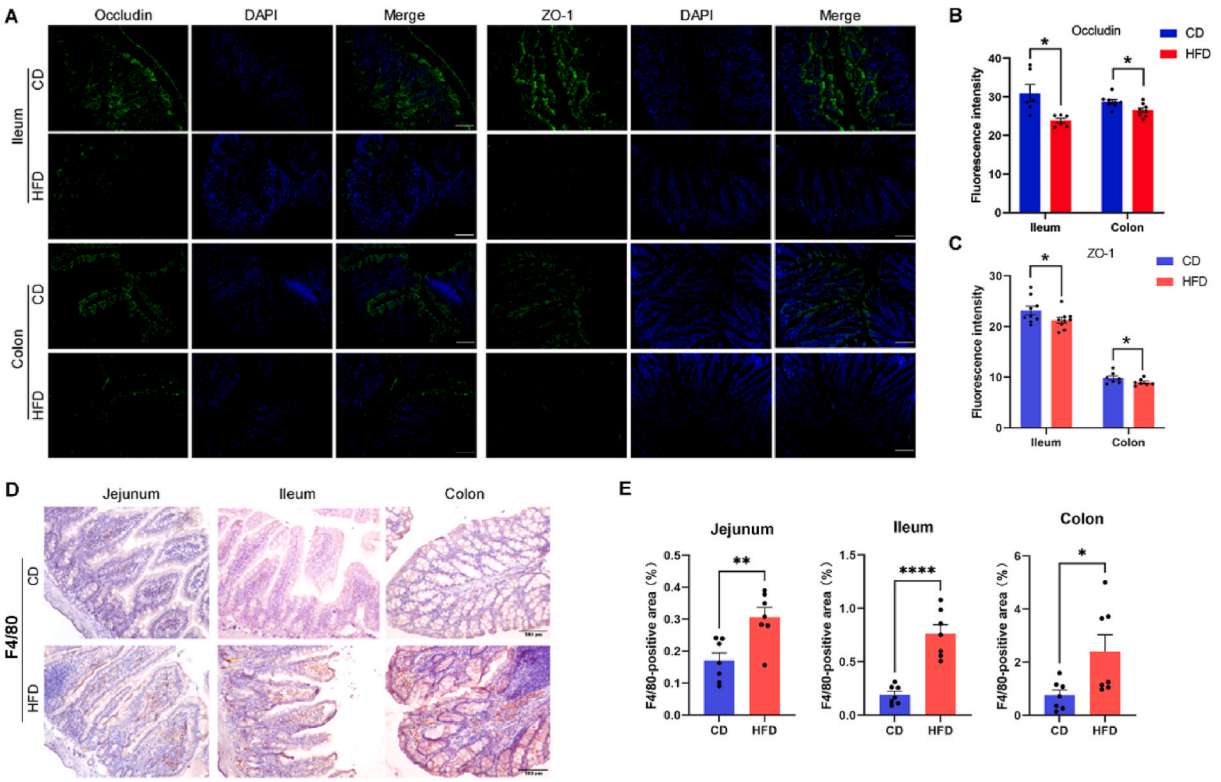

Fig. 2. HFD disrupts the intestinal barrier and intensifies inflammation. (A) Immunofluorescence staining for Occludin and ZO-1. Scale bars: 100 μm. (B) Fluorescence intensity of Occludin. (C) Fluorescence intensity of ZO-1. (D) Immunohistochemical staining of F4/80. Scale bars: 100 μm. (E) Quantification of F4/80. The data are expressed as the mean ± SEM. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

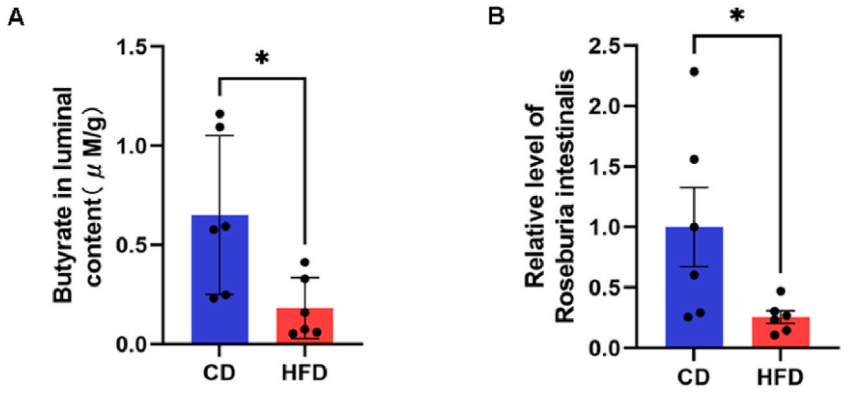

Fig. 3. HFD leads to a decrease in intestinal butyric acid levels. (A) Levels of butyric acid in the ileum. (B) Relative mRNA expression of Roseburia intestinalis. The data are expressed as the mean ± SEM. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

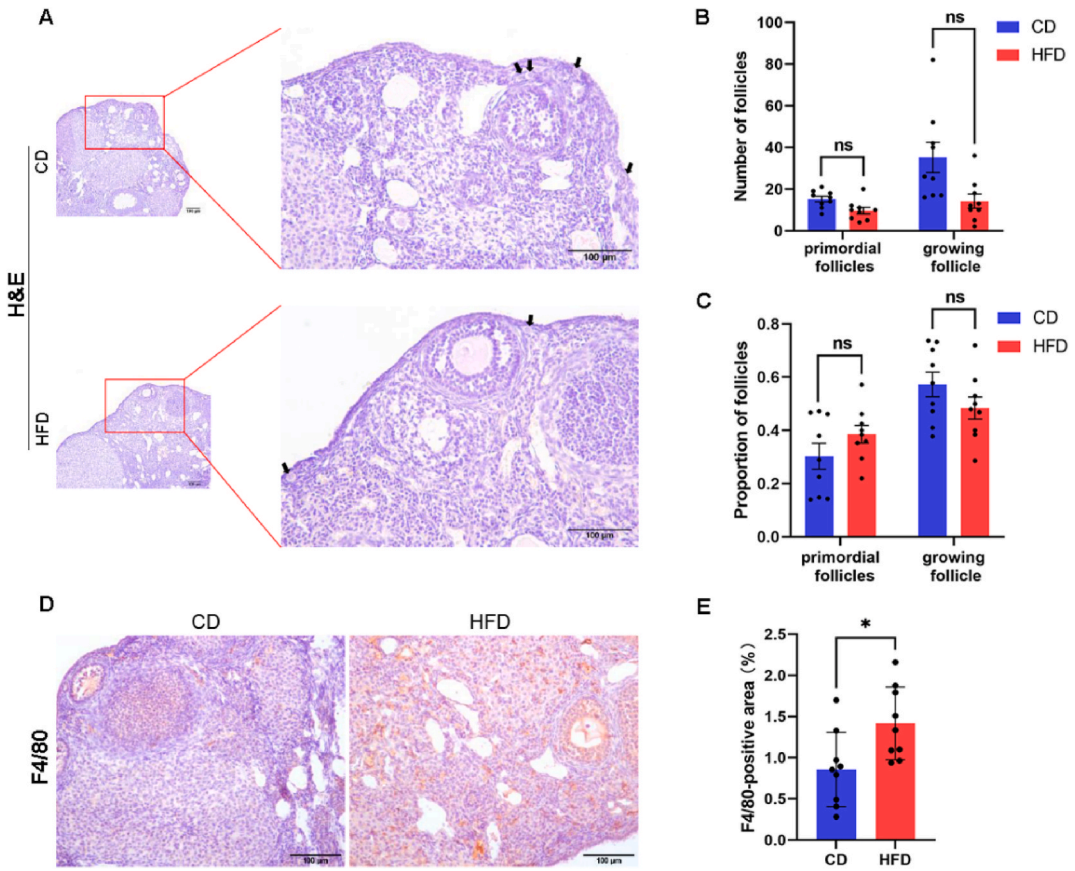

Fig. 4. HFD intensifies ovarian inflammation and impairs follicular development. (A) H&E staining of the ovary; black arrows indicate primordial follicles. Scale bars: 100 μm. (B) Count of follicles. (C) The proportion of follicles. (D) Immunohistochemical staining of F4/80. Scale bars: 100 μm. (E) Quantification of F4/80. The data are expressed as the mean ± SEM. (n.s. $P > 0.05$, *$P < 0.05$).

and E). The results clearly showed a more significant inflammatory response in the ovaries in the HFD group in contrast to the CD group.

3.4. Long-term HFD led to down-regulation of 5 mC and H3K36me3 in follicles

Histone methylation modification plays an indispensable role in gene expression during embryonic development by regulating the

activity of specific genes. To evaluate the impact of HFD on histone methylation in mouse follicles, we analyzed the expression levels of DNA methylation (5 mC) and histone methylation (H3K36me3) using immunofluorescence staining. The results showed that, compared with the CD group, the expression of 5 mC and H3K36me3 in the follicles of the HFD group was decreased (Fig. 5A–C). Subsequently, we conducted a correlation analysis between butyric acid and 5 mC as well as H3K36me3. The results indicated a significant positive correlation between intestinal butyric acid levels and the epigenetic modifications in the ovaries (Fig. 5D and E). From these observations, it can be deduced that HFD can reduce the levels of 5 mC and H3K36me3 in the ovarian follicles of obese mice, potentially through the reduction of butyric acid levels.

4. Discussion

HFD-induced obesity exerts a negative impact on the female gut and reproductive system. Our study found that obesity leads to decreased levels of Roseburia intestinalis and butyric acid, an increased inflammatory response, and weakened mucosal barrier function, all of which are strongly associated with abnormal intestinal function. In addition, our observations in obese female mice revealed increased ovarian inflammation and alterations in epigenetic markers in ovarian follicles, particularly a decrease in 5 mC and H3K36me3 modifications. We believe that long-term HFD intake may adversely affect ovarian function and reduce reproductive efficacy by lowering butyric acid levels and damaging the mucosal barrier. The epigenetic governance of DNA and histone methylation by butyric acid could be involved in this process. These insights open avenues for developing novel therapeutic interventions targeting obesity-associated gut and reproductive disorders, especially through gut microbiota adjustments or supplementation with butyric acid and its derivatives.

Butyric acid and butyrate both play important roles in enhancing the mucosal defense barrier by enhancing the expression of mucin-encoding genes and inducing the activity of trefoil factors, heat shock protein, antimicrobial peptide, and transglutaminase [30]. Experiments have shown that the infusion of sodium butyrate can effectively improve the intestinal microbiome structure, enhance the intestinal mucosal barrier, and reduce the inflammatory response in HFD mice [18]. Interestingly, some studies have indicated that changes in butyric acid can alter host epigenetics. The addition of butyrate to human cancer cell lines diminished miR-17-92a levels through epigenetic mechanisms, which in turn restricted cell growth and promoted apoptosis [31]. Butyrate also enhances the antitumor activity of cytotoxic T lymphocytes (CTL) and chimeric antigen receptor (CAR) T cells through metabolic and epigenetic reprogramming [32]. Acting as an inhibitor of histone deacetylase (HDAC), butyrate prevents HDAC from removing acetyl groups from histones, resulting in chromatin relaxation and the consequent gene activation [33]. Zhang and colleagues observed diminished butyric acid levels in PCOS patients, especially in obese ones [34]. Supplementation of butyric acid in such patients ameliorated ovarian health and reduced local ovarian inflammatory markers [35]. Although the role of butyric acid in several

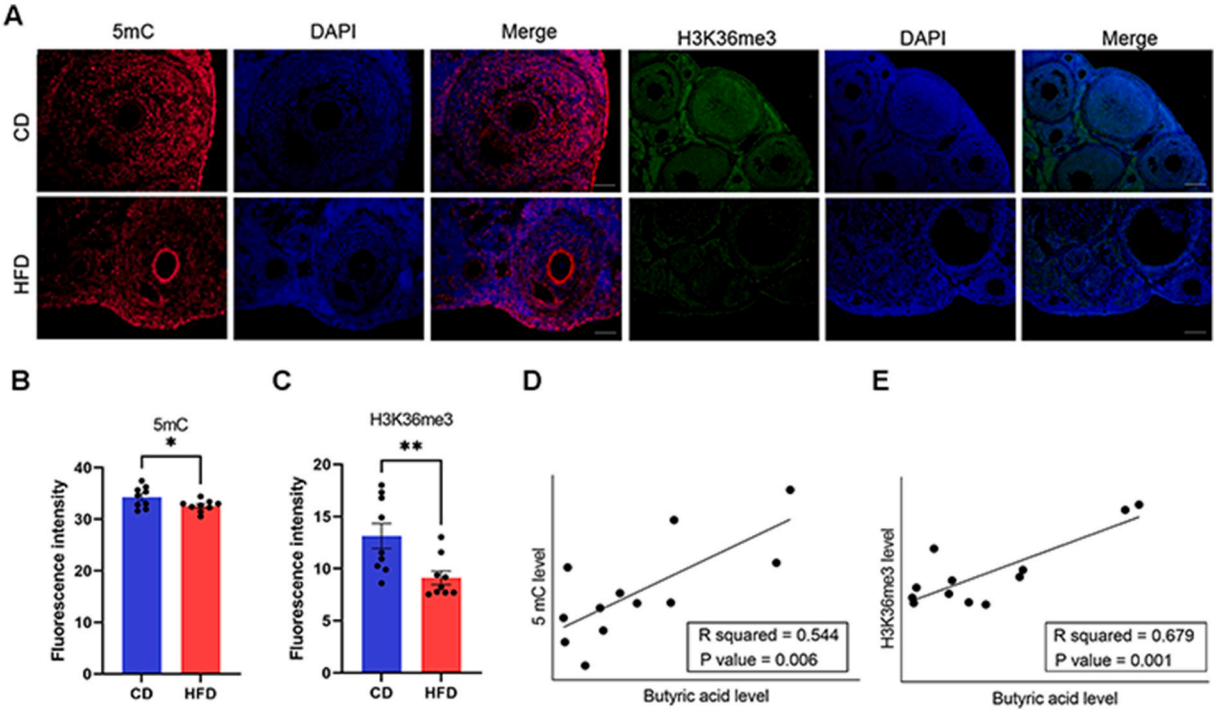

Fig. 5. HFD results in decreased expression of ovarian 5 mC and H3K36me3. (A) Immunofluorescence staining for 5 mC and H3K36me3. Scale bars: 100 μm. (B) Fluorescence intensity of 5 mC. (C) Fluorescence intensity of H3K36me3. (D) Correlation analysis of butyric acid with 5 mC. (E) Correlation analysis of butyric acid with H3K36me3. The data are expressed as the mean ± SEM. (*$P < 0.05$, **$P < 0.01$).

physiological and pathological processes has been investigated, its role in ovarian epigenetic modification is not yet fully understood. Further research is necessary to elucidate these modifications as well as potential therapeutic strategies for treating reproductive disorders by modulating the gut microbiome.

In recent years, numerous scientific studies have pointed to a significant decline in the quality of oocytes in obese women. Mitochondrial dysfunction, meiosis abnormalities, and sustained oxidative stress can all contribute to this adverse phenomenon and have lasting adverse effects on the metabolic function of the offspring [36–38]. It has been proposed that HFD depletes the primordial reserve by increasing follicular atresia [39], and HFD-induced changes in intestinal flora promote primordial follicle activation through ovarian macrophage infiltration and inflammatory cytokine secretion in mice [23]. Although HFD and CD mice in our experiment did not show a statistically significant difference in the number of primordial and growing follicles, the observed results were in the same direction as those previously reported in the literatures. This phenomenon may be a compensatory response to chronic HFD in mice of childbearing age. Alternatively, the lack of statistical significance could be attributed to the inadequate sample size in our study. However, the exact mechanisms involved are unclear. Macrophages play a central regulatory role in ovarian follicular development, ovulation, and luteolysis [40]. A previous study has indicated that in obese rats, cumulus cells within the atria and preovulatory follicles exhibited an upregulation of inflammatory genes and the proinflammatory mediator Egr-1, hinting at a potential intraovarian inflammatory cascade due to obesity [41]. Consistent with this, our study identified a significant increase in the expression of the inflammatory marker F4/80 in the ovarian tissue of obese mice fed HFD. This underscores the possibility that escalating ovarian inflammation might be instrumental in the subsequent, and potentially irreversible, damage to the follicles.

In mammalian gametogenesis and early embryonic development, epigenetic regulation in oocytes plays a pivotal role. Among these, 5 mC stands out as the principal DNA methylation form in eukaryotes and garners the most research attention [42]. Stella, an essential maternal effect gene in early mouse development, inhibits the demethylation of 5 mC [43]. Research by Han et al. indicated a significant reduction of Stella protein in oocytes from HFD mice, promoting the oxidation of 5 mC to 5-hydroxymethylcytosine (5hmC) and increasing the likelihood of specific defects in oocytes in those oocytes [44]. Consistently, we observed a significantly lower expression of 5 mC in the ovaries of mice in the HFD group compared to the CD group. Histone methylation, largely on histone H3's lysine residues, functions variably based on its modification site. For instance, aberrant reprogramming of H3K27me3 can disrupt zygotic genome activation (ZGA) in somatic cell nuclear transfer (SCNT) embryos, leading to developmental abnormalities [45]. Moreover, disruptions in H3K4me3 modifications are identified as critical factors in the challenges associated with IVF embryo implantation and in placental development complications [46]. Methylation of H3K36 (H3K36me) is highly conserved from yeast to humans and is closely related to transcriptional accuracy, RNA splicing, and DNA repair [22]. Intriguingly, H3K36me3 is closely coupled with DNA methylation on the gene ontology of mature oocytes and can positively regulate the expression of oocyte development-related genes [22]. Depletion of the H3K36 methyltransferase SETD2 in oocytes results in a genome-wide loss of H3K36me3 and DNA hypomethylation, leading to oocyte maturation abnormalities and post-fertilization single-cell arrest, among other severe consequences [22]. Delving into the relationship between N6-Methyladenosine (m6A) and histone modifications, Huang's team unveiled a significant colocalization between H3K36me3 and m6A alterations [47]. In mouse embryonic stem cells, the absence of H3K36me3 leads to a significant decrease in m6A levels in the transcriptome and pluripotent transcripts [47]. Additionally, m6A is instrumental in female reproductive disorders, including oogenesis, ovarian premature aging, endometriosis, and PCOS [48]. In our study, the level of H3K36me3 in oocytes from HFD mice was significantly reduced, suggesting that the transcriptional activity of the oocyte genome was disrupted, and this aberrant methylation may affect the developmental potential of embryos. Based on these findings, we posit that the decreased levels of 5 mC and H3K36me3 induced by HFD may be key factors contributing to the reduced fertility in obese mice.

On another note, butyrate and its salts play primary roles in histone acetylation and deacetylation and also have some connections with DNA methylation, histone methylation, and m6A modifications [35,49,50]. As an HDAC inhibitor, butyrate has been shown to disrupt and downregulate the expression of the Enhancer of Zeste Homolog 2 (EZH2), leading to a reduction in the H3K27me3 mark around lineage-specific genes [51]. By supplementing with tributyrin, an ester that can rapidly transform into butyrate in vivo, this affects the H3K27me3 mark in satellite cells on a genome-wide level [52]. Previous studies have shown that different cell types and concentrations of butyrate salts can affect the content of 5 mC [36,53]. Further studies revealed that another HDAC inhibitor, valproic acid (VPA), can reduce 5 mC levels and increase 5hmC abundance by upregulating the expression of the TET2 gene and protein [54]. Although there is no direct evidence linking butyrate with 5 mC, given its role as an HDAC inhibitor, it may indirectly modulate chromatin structure and gene expression. Based on this, we hypothesize that butyrate might exert indirect effects on epigenetic events related to 5 mC, but this remains to be experimentally verified. Similarly, although there are no reports directly linking butyrate with H3K36me3, recent research indicates that butyrate can ameliorate granulosa cell inflammation in PCOS by regulating m6A modification of FOS-like 2 (FOSL2) mediated by methyltransferase-like 3 (METTL3) [35]. Given the interaction between m6A and H3K36me3 and butyrate's ability to enhance histone acetylation, we speculate that it might influence the modification and function of H3K36me3. As a molecule with anti-inflammatory and epigenetic modification-regulating properties, although the epigenetic modification role of butyrate in the ovary has not been explicitly revealed, our research has indicated that HFD-induced changes in butyrate levels may have potential effects on the ovarian epigenetic modifications, providing a new direction for future research.

Abnormal methylation and histone modifications can influence the expression of specific genes and proteins, disrupting body weight management and oocyte maturation and reducing oocyte quality. These disruptions may further lead to defects in embryonic development. These observations suggest that obesity could result in diminished intestinal butyric acid levels, inducing alterations in ovarian histone modifications. These alterations could detrimentally affect follicle functionality and essential maturation mechanisms, leading to reproductive anomalies. However, it's crucial to highlight that, although there is substantial evidence to support this mechanism, additional clinical studies are imperative to cement these findings and chart optimal therapeutic avenues.

We acknowledge several limitations in our research. The 36-week duration of our murine model, though adequate for preliminary observations, may not fully represent the chronic consequences of dietary interventions on reproductive health. Furthermore, the epigenetic analysis presented here is preliminary, and a more exhaustive profiling of epigenetic marks could offer deeper insights into the functional genomics of ovarian function under the influence of HFD and butyric acid. Future studies should aim to overcome these limitations through longitudinal research designs that assess the prolonged effects of HFD and butyric acid on ovarian integrity. The inclusion of butyric acid intervention groups will be pivotal in evaluating its therapeutic potential. Translational research involving human subjects is essential to confirm these findings and explore their clinical implications. Expanding the scope of epigenetic profiling to encompass a broader spectrum of modifications and conducting mechanistic studies to decipher the influence of butyric acid on these epigenetic changes, will be vital. Additionally, employing a variety of animal models could offer a more holistic perspective on the dietary impact on reproductive epigenetics.

5. Conclusions

Our research indicates that HFD negatively affects reproductive capacity in mice through epigenetic changes in the ovary, potentially linked to alterations in gut microbiota, particularly Roseburia intestinalis and butyric acid levels. It is important to note that our research was specifically concentrated on Roseburia intestinalis due to its established role in butyric acid production and its relevance to gut health. Consequently, our results do not purport to offer a comprehensive characterization of the entire gut microbiota, which is a complex and diverse ecosystem. Future studies should aim to explore the broader implications of the gut microbiota, beyond Roseburia intestinalis, to fully understand the multifaceted interactions between diet, gut health, and reproductive outcomes.

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Consent for publication

Not applicable.

Data availability statement

Source data were included in article, and the data associated with this study have not been deposited in a publicly available repository.

Ethics statement

This study was reviewed and approved by the Animal Experimental Ethics Committee of Xi'an Jiaotong University with the approval number: No. XJTUAE2023-2193.

CRediT authorship contribution statement

Jia Qi: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Congcong Xia:** Writing – review & editing, Resources, Methodology, Funding acquisition, Data curation. **Yulin Zhang:** Writing – review & editing, Investigation, Data curation. **Ruike Ding:** Writing – review & editing, Formal analysis, Data curation. **Yanru Zhang:** Writing – review & editing, Resources, Investigation, Data curation. **Wenbin Cao:** Writing – review & editing, Resources, Conceptualization. **Chenjing Duan:** Validation, Project administration, Methodology, Conceptualization. **Zijing Yao:** Validation, Software, Methodology, Conceptualization. **Hongyu Qin:** Writing – review & editing, Visualization. **Yun Ye:** Writing – review & editing, Validation, Methodology, Funding acquisition, Conceptualization. **Pengxiang Qu:** Writing – review & editing, Software, Formal analysis. **Yandong Li:** Writing – review & editing, Validation, Supervision, Software, Conceptualization. **Enqi Liu:** Writing – review & editing, Visualization, Supervision, Data curation, Conceptualization.

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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