ABSTRACT

The main purpose of the current study was to investigate the ameliorative effects of bovine milk osteopontin (bmOPN) on the gut dysfunction of pregnant rats fed a high-fat diet (HFD). Bovine milk osteopontin was supplemented at a dose of 6 mg/kg body weight. Bovine milk osteopontin supplementation during pregnancy reduced colonic inflammation of HFD dams, and it also increased the colonic expression of ZO-1 and claudin-4 of HFD dams. Bovine milk osteopontin significantly enriched the relative abundance of Bacteroidetes, whereas it decreased Proteobacteria, Helicobacteraceae, and Desulfovibrionaceae in feces of HFD dams. The levels of isobutyric acid and pentanoic acid in the HFD + bmOPN group were higher than that of the HFD group. Functional predication analysis of microbial genomes revealed that bmOPN supplementation to HFD pregnancies changed 4 Kyoto Encyclopedia of Genes and Genomes pathways including bile acid biosynthesis. Further, bmOPN enriched hepatic taurochenodeoxycholic acid and tauroursodeoxycholic acid plus taurohyodeoxycholic acid in the gut of HFD maternal rats. Our findings suggested that bmOPN improved the gut health of HFD pregnant rats partially through modulating bile acid biosynthesis.

Key words: bovine milk osteopontin, intestinal health, gut microbiota, bile acid, pregnancy

INTRODUCTION

During pregnancy, the gut plays a doubly critical role as it not only facilitates nutrient absorption and metabolism that benefit maternal health, but also fetal development. Gut health depends on gut microbiota and intestinal epithelial barrier function. Bacteria reside in the host gut and help to digest dietary ingredients and prevent pathogenic microorganisms’ colonization. In addition, they also participate in the modulation of multiple metabolic processes. Some bacterial taxa, such as Firmicutes and Bacteroidetes, are associated with energy expenditure of the host (Lemons and Liu, 2022). Lipopolysaccharide, a component of gram-negative bacteria, can be transferred to circulation, triggering local and systemic inflammation (Khiaosa-ard and Zebeli, 2018). The gut mucosal barrier, mainly composed of intestinal epithelial cells and tight junction (TJ) proteins, is vital for preventing detrimental microbes and excessive LPS passage from intestinal lumen into the bloodstream (Mohammad and Thiemermann, 2021). In addition, short-chain fatty acids (SCFA), the beneficial metabolites of gut microbiota through fermentation for dietary ingredient, not only act as the energy substrates for enterocytes, but participate in regulating the gut barrier and immunity (Liu et al., 2020). Gut homeostasis is sensitive to the metabolic status and dietary pattern of the host. Mothers consuming a high-fat diet (HFD) suffer from gut dysbiosis and intestinal barrier integrity disruption, resulting in inflammatory response in the gut and peripheral circulation (Wankhade et al., 2017). In addition, the SCFA-producing bacteria and cecal SCFA levels tended to be lower in HFD pregnant mothers (Gohir et al., 2019). Given the important status of the gut, ameliorative interventions for gut health of HFD mothers during pregnancy are urgently needed.

Bile acids, cholesterol-derived endogenous metabolites, have a key role in maintaining gut homeostasis. Bile acids and gut microbiota have a bidirectional relationship. Primary bile acids synthesized in the liver are secreted into intestinal lumen and then partly transformed into secondary bile acids by intestinal flora-derived enzymes (Thomas et al., 2022). Gut microbiota alterations in response to dietary patterns and nutritional status influence bile acid distribution (Swann et al., 2011). In turn, bile acids in the lumen affect intestinal bacteria composition. On the one hand, bile acids are related to RNA secondary structure formation and DNA damage in bacteria (Begley et al., 2005); however, hydrophobic bile acids affect microbial membrane per-

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meability, affecting the growth of intestinal microbes (Stenman et al., 2013). In addition, bile acid signaling is involved in maintaining gut barrier integrity. The protein expression of TJ was decreased in the ileum of bile-duct ligated rats (Verbeke et al., 2015), whereas obeticholic acid treatment enhanced the TJ expression and alleviated microbial dysbiosis and inflammation in the ileum (Übeda et al., 2016). Pregnant dams with HFD consumption were accompanied with alterations in bile acid biosynthesis (Gohir et al., 2015), and cholic acid ingestion ameliorated HFD-related metabolic complication (Watanabe et al., 2006). Thus, improving bile acid metabolism is a promising target for recovering the gut health of HFD dams.

Osteopontin (OPN), a highly phosphorylated acidic glycoprotein, exists in most tissues, such as bone, brain, kidney, and epithelial tissues. Specifically, OPN expressed in intestinal epithelial cells plays an indispensable role in maintaining TJ complexes, aiding the localization of occludin in TJ and subsequent phosphorylation (Nakase, 2019). In addition, OPN in intestine facilitates gut microbiota homeostasis and functions as an opsonin to activate microbial phagocytosis (Pedraza et al., 2008; Ito et al., 2017). SPP1 gene knockout (SPP1−/−) mice exhibited lower intestinal cholesterol absorption and thereby reduced cholesterol gallstones (Lin et al., 2017). There was a positive correlation between mean OPN levels and fasting insulin in obese patients with obstructive sleep apnea (Sarac et al., 2011). Moreover, OPN exacerbated HFD-induced metabolic disorders by a microbiota-dependent manner in OPN knockout mice (Chen et al., 2022). These evidences suggest that endogenous OPN plays an indispensable role in multiple physiological processes, including intestinal homeostasis and glucolipid metabolism.

Apart from tissues, OPN is also present in body fluids. Osteopontin is one of the 5 most abundant proteins in breast milk, facilitating the development of infants. Both breast milk OPN and bovine milk OPN feeding increased the ratio of villus height to crypt depth of mouse pups at postnatal d 10, indicating that milk OPN promoted the growth of the small intestine (Jiang and Lönnerdal, 2020). Infants fed OPN-supplemented formula had lower prevalence of fever and improved inflammation response against LPS challenge (Jiang and Lönnerdal, 2020). Bovine milk osteopontin regulated gut microbiota and enhanced intestinal barrier function in alcohol-treated mice and, thereby, alleviated hepatic inflammatory response (Ge et al., 2013). The intestinal transcriptome of the rhesus monkey who received formula with bmOPN supplementation was similar to that of breastfed monkeys (Donovan et al., 2014). All of this evidence suggests that oral bmOPN intake helps intestinal protection and anti-inflammation activities. Based on this information, we hypothesized that bmOPN might be a promising candidate for HFD pregnancies to improve intestinal dysfunction.

The aim of the present study is to investigate the ameliorative effects of bmOPN on gut homeostasis, including intestinal inflammation, gut microbiota, and intestinal barrier function of HFD pregnant dams. Also, bile acid metabolism was further analyzed as a key mediator contributing to altered gut microbiota and epithelial health.

MATERIALS AND METHODS

Materials

Bovine milk osteopontin was provided by Arla Co. (Viby J). Normal diet (ND, 10% energy from fat) and HFD (60% energy from fat) were purchased from Research Diets Inc. (New Brunswick, NJ). Primary antibodies against ZO-1, claudin-4, and occludin were obtained from ThermoFisher Scientific (Waltham, MA). The primary antibody against β-actin was purchased from Bioss Inc. (Beijing, China). Secondary antibodies were purchased from Beyotime Biotechnology (Shanghai, China). Enhanced chemiluminescent reagents were from Millipore Co. (Billerica, MA).

Animals and Treatment

Female Sprague-Dawley rats (aged 3 wk old) were purchased from Beijing Vital River Laboratory Animal Technologies Co. Ltd. All rats were housed in the specific animal facility with a temperature at 23°C ± 2°C and a 12-h light-and-dark cycle. The outline of the study design is shown in Figure 1. After 1-wk acclimation, female rats were randomized into 3 groups of 10 each,
including ND group, the HFD group, and the HFD + bmOPN group. Animals in HFD and HFD + bmOPN groups were fed with HFD, whereas animals in the ND group were fed with ND for 8 wk. The BW of female rats was significantly increased by 47.04 g after receiving HFD for 8 wk (Supplemental Figure S1; https://doi.org/10.17632/k866d8xvwrm.1; Han et al., 2023). Then female rats were mated with male Sprague-Dawley rats (aged 10 wk old), and the mating was confirmed by the presence of vaginal smear. Pregnant dams in the HFD + bmOPN group were orally administered with 6 mg/kg BW of bmOPN based on BW. During pregnancy, rats in the HFD group and the HFD + bmOPN group were continually receiving HFD. At gestation d 18.5 (GD18.5), pregnant rats were separately housed in sterile cages, and fresh feces from pregnant dams were collected in sterile tubes and stored at −80°C for 16S rRNA analysis. Then dams were killed. Samples including blood and colon tissues were collected for analysis.

All animal experimental procedures were approved by the Animal Ethical Committee of China Agricultural University (protocol number: AW20212202–4-1).

**Serum Parameter Measurements**

Osteopontin (catalog no. SEA899Ra), LPS (catalog no. IEB526Ge), tumor necrosis factor α (catalog no. HEA133Ra), IL-1β (catalog no. SEA563Ra), and IL-6 (catalog no. SEA079Ra) ELISA kits were obtained from Cloud-Clone Co. Ltd. (Wuhan, China). Serum parameter measurements were carried out using ELISA kits according to manufacturers’ instructions.

**H&E Staining**

Colon tissues were harvested and fixed with 4% paraformaldehyde for 24 h. Subsequently, dehydrated specimens were then embedded in paraffin and sectioned at 4-μm thickness. Hematoxylin and eosin (H&E) staining was carried out with a staining kit (Solarbio, Beijing, China, catalog no. G1120) according to manufacturers’ instructions. The histological score based on inflammatory lesion was performed in a blinded manner by 3 independent investigators. Each H&E staining image was assessed for the extent of inflammatory cell infiltration and allocated a single score based on the following criteria where 0, none; 1, slight; 2, moderate; 3, severe; 4, massive (Erdman et al., 2003; Chen et al., 2023). The 3 grades for each section were combined, and the data from each group of mice were averaged.

**Western Blot Analysis**

Colon samples from 2 rats in the same group were pooled into 1 sample at random. Total protein was extracted using radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) in an ice bath. Fifteen micrograms of protein samples were separated by 10% SDS-PAGE followed by transferring to polyvinylidene difluoride (PVDF) membranes. After being blocked by 5% skim milk, PVDF membranes were incubated with specific primary antibodies overnight at 4°C. Membranes were then probed by appropriate secondary antibodies at room temperature for 1 h. The target bands were visualized using the enhanced chemiluminescent reagents. Tanon imaging system (Tanon, Shanghai, China) was used to take the images, and the intensity of target protein bands was analyzed by bundled software.

**Gut Microbiota Analysis**

Gut microbiota analysis was performed using 16S rRNA high throughput sequencing according to the previous report with some modifications (Jia et al., 2022). Briefly, microbiome DNA was extracted using a DNA Extraction Kit (Qiagen Inc., Valencia, catalog no. 16390-02)}
Liver Bile Acid Composition Analysis

Short-Chain Fatty Acid Measurement

Liver Bile Acid Composition Analysis

MD5115–02B) according to a recommended protocol. After purity and quality inspection, genomic DNA was used to amplify the V3 to V4 regions of 16S rRNA genes, and the Gene-JET Gel Extraction Kit (Thermo Scientific, catalog no. K0691) was applied for PCR product purification. Sequencing was then carried out using an Illumina HiSeq platform according to its instruction. The raw data of 16S rRNA gene sequencing were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive database (Accession Number: PRJNA996078).

In the current study, each sample generated 50,000 to 70,000 raw-sequencing reads on average. The sequences were clustered into operational taxonomic units (OTU) with a cutoff value of 97% using UPARSE software (v7.0.1090). Alpha diversity, including Shannon and Chao1 indices, was carried out by MOTHUR (v1.31.2) at the OTU level. To evaluate the β-diversity, principal component analysis (PCA) in OTU and sample clusters were estimated by QIIME (v1.8.0). The taxonomic assignment of OTU was performed using a Ribosomal Database Project Classifier (v1.8.0). The operating parameters were set according to previous reports (Li et al., 2022c).

Statistical Analysis

All data were displayed as mean ± standard error of the mean. One-way parametric analysis (ANOVA), followed by Duncan corrections, was performed for significant difference analysis (P < 0.05) using SPSS software (version 20.0, IBM Inc., Chicago, IL).

RESULTS

Effects of bmOPN on Litter Size and Fetal Mass of HFD Dams at GD18.5

As shown in Supplemental Figure S2 (https://doi.org/10.17632/k866d8xvwn.1; Han et al., 2023), no significant difference was observed in the number of male, female, and total fetuses of HFD among 3 groups (P > 0.05). Also, maternal HFD and bmOPN intervention had no significant effect on male and female fetal weights (P > 0.05).

bmOPN Supplementation Suppressed Inflammatory Response of HFD Dams at GD18.5

To investigate the effects of bmOPN on intestinal and systemic inflammation of HFD dams at GD18.5, colon histomorphology, serum contents of LPS, and pro-inflammatory cytokines were determined. As shown in Figure 2A, colon tissue in the ND group exhibited normal morphology without inflammation. We observed a severe inflammatory cell infiltration (red arrows) in the colon tissue of HFD dams. However, the inflammatory cell number in the HFD + bmOPN group was significantly reduced. Figure 2B exhibited that bmOPN reversed the histological score of the HFD colon. As shown in Figure 2C-G, contents of serum OPN, LPS, TNF-α, IL-1β, and IL-6 in the HFD group were significantly higher than that of the ND group (P < 0.05). Compared with the HFD group, serum OPN, endotoxin, TNF-α, IL-1β, and IL-6 contents in the HFD + bmOPN group were remarkably reduced (P < 0.05). These results suggested that bmOPN supplementation during pregnancy ameliorated colonic and systemic inflammation of HFD dams.
Figure 2. Bovine milk osteopontin (bmOPN) suppressed colonic inflammatory response and enhanced the intestinal barrier function at gestation d 18.5 in dams fed a high-fat diet (HFD). (A) Representative hematoxylin and eosin (H&E) staining images of colon tissue (n = 3). (B) Histological score of colonic H&E staining ($F[2, 6] = 97.8, P < 0.01$). Serum contents of (C) OPN ($F[2, 15] = 4.209, P < 0.05$), (D) LPS ($F[2, 15] = 36.49, P < 0.01$), (E) TNF-α ($F[2, 15] = 24.081, P < 0.01$), (F) IL-1β ($F[2, 6] = 20.081, P < 0.01$), (G) IL-6 ($F[2, 6] = 112.563, P < 0.01$), and (H–I) Protein expression of claudin4 ($F[2, 6] = 20.081, P < 0.01$), ZO-1 ($F[2, 15] = 19.108, P < 0.01$), and occludin ($F[2, 6] = 3.633, P < 0.093$) in colon. Red arrows point to inflammatory cells. Data are presented as mean ± SEM, n = 6. Values with different lowercase letters (a–c) are significantly different ($P < 0.05$). TNF-α = tumor necrosis factor α; EU = endotoxin units; ND = normal diet; HFD = high-fat diet; HFD+bmOPN = high-fat diet with bovine milk osteopontin supplementation.
**bmOPN Enhanced Intestinal Barrier Function of HFD Dams at GD18.5**

To evaluate the effects of bmOPN supplementation on the intestinal barrier, the expression of TJ was determined. Compared with the ND group, the expression of ZO-1, occludin, and claudin-4 was downregulated in colon tissues of HFD dams ($P < 0.05$). After bmOPN supplementation, the expression of ZO-1 and claudin-4 in colons of HFD dams was significantly elevated ($P < 0.05$), and the expression of occludin exhibited a trend of increase (Figure 2F-G). All these results suggested that bmOPN supplementation restored the intestinal barrier integrity of HFD dams.

**Effects of bmOPN on Intestinal Bacterial Structure of HFD Dams at GD18.5**

To determine the effects of bmOPN on the gut microbiota structure of HFD dams, α-diversity (Shannon index and Chao I index) and β-diversity (PCA analysis) were analyzed. As shown in Figure 3A, no significant change was found in the Shannon index among the 3 groups ($P > 0.05$). We observed a decrease in the Chao I index in the HFD group as compared with the ND group ($P < 0.05$). However, bmOPN supplementation had no effect on the Chao I index of HFD dams (Figure 3B; $P > 0.05$). The microbial community was distinctly separated between the ND group and the HFD group. No separate clustering pattern was generated from the HFD group after bmOPN supplementation (Figure 3C). These results indicated that the overall intestinal bacterial structure of HFD dams was not altered due to bmOPN supplementation.

**bmOPN Modulated Gut Microbiota Composition of HFD Dams at GD18.5**

Although no significant effect was observed in α-diversity and β-diversity after bmOPN supplementation, alterations of specific microbial taxa were observed. Linear discriminant analysis effect size showed that there were marked variations in the constitutions of microbial species among different groups (Figure 4A). Compared with the ND group, **Proteobacteria** (phylum), **Blautia** (genus), **Desulfovibrio** (genus), **Helicobacteraceae** (family), and **Helicobacter** (genus) were highlighted as the top 5 different biomarkers in the HFD group (Figure 4B). However, **Bacteroidaceae** (family), **Bacteroides** (genus), **Clostridium_XIVb** (genus), and **Alloprevotella** (genus) were dominant in the bacteria of the bmOPN supplemented group (Figure 4B).

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Figure 3. Effects of bovine milk osteopontin (bmOPN) on microbiota diversity at gestation d 18.5 in rats fed a high-fat diet (HFD). The α-diversity including (A) Chao I index ($F[2, 19] = 25.783, P < 0.01$) and (B) Shannon index ($F[2, 19] = 0.271, P = 0.766$). (C) β-Diversity presented as principal component analysis (pca). Data are presented as mean ± SEM, n = 6 to 10. Values with different lowercase letters (a, b) are significantly different ($P < 0.05$). OTU = operational taxonomic unit; ND = normal diet; HFD = high-fat diet; HFD+bmOPN = high-fat diet with bovine milk osteopontin supplementation.

Figure 4. Relative abundance of bacterial phylotypes in the 3 groups was further compared. As shown in Figures 4C and 4D, as compared with the ND group, the relative...
Figure 4. Bovine milk osteopontin (bmOPN) modulated gut microbiota composition at gestation d 18.5 in rats fed a high-fat diet (HFD). Overall taxonomic diversity of gut bacteria analyzed by LEfSe. (A) Taxonomic cladogram plot from LEFSe analysis. (B) LDA score plot. LDA score >3 was considered significant ($P < 0.05$). (C) Relative abundance of Bacteroidetes ($F[2, 17] = 10.345, P < 0.01$), Firmicutes ($F[2, 17] = 3.094, P = 0.069$), and Proteobacteria ($F[2, 17] = 5.770, P < 0.01$). (D) The ratio of Bacteroidetes to Firmicutes ($F[2, 17] = 4.548, P < 0.05$). (E) Relative abundance of Helicobacteraceae ($F[2, 19] = 24.634, P < 0.01$) and Desulfovibrionaceae ($F[2, 19] = 5.945, P < 0.01$). Data are presented as mean ± SEM, $n = 5$ to 9. Values with different lowercase letters (a, b) are significantly different ($P < 0.05$). ND = normal diet; HFD = high-fat diet; HFD+bmOPN = high-fat diet with bovine milk osteopontin supplementation; LEfSE = linear discriminant analysis effect size; LDA = linear discriminant analysis.
abundance of Bacteroidetes was significantly decreased while Firmicutes was elevated in the feces of HFD dams, as a consequence of a reduction in the ratio of Bacteroidetes/Firmicutes (P < 0.05). Moreover, we observed an increase in the relative abundance of Proteobacteria in the HFD group (Figure 4C). However, bmOPN supplementation significantly boosted the relative abundance of Bacteroidetes and decreased the relative abundance of Proteobacteria (P < 0.05), though no significant difference was found in the abundance of Firmicutes (Figure 4C; P > 0.05). We observed no significant difference in the ratio of Bacteroidetes to Firmicutes in the HFD + bmOPN group compared with the ND group (Figure 4D; P > 0.05). At the family level, the relative abundance of Helicobacteraceae and Desulfovibrionaceae were upregulated in HFD dams as compared with the ND group, which was significantly reversed by bmOPN supplementation (P < 0.05; Figure 4E). All these results suggested that bmOPN supplementation during gestation altered the gut microbiota composition at different taxonomic levels.

Effects of bmOPN on Fecal SCFA Levels of HFD Dams at GD18.5

In an attempt to further reveal the effects of bmOPN on gut microbiota, the contents of SCFA, the metabolites of gut microbiota, were measured. As shown in Figure 5A, the total SCFA level was lower in the feces of HFD dams than that of the ND group (P < 0.05). Specifically, the contents of acetic acid, propanoic acid, butyric acid, and pentanoic acid were significantly decreased in the fecal samples of HFD dams compared with the ND group (Figure 5B-F; P < 0.05). No difference was found in isobutyric acid content between the HFD and the ND group (Figure 5E; P > 0.05). Bovine milk osteopontin supplementation had little effect on the contents of total SCFA, acetic acid, propanoic acid, and butyric acid (Figure 5A-D; P > 0.05), but it remarkably elevated the contents of isobutyric acid (Figure 5E) and pentanoic acid (Figure 5F) in the feces of HFD dams (P < 0.05). These data suggested that bmOPN supplementation during pregnancy facilitated bacterial SCFA production of HFD dams.
PICRUSt Analysis and Function Prediction of Microbial Genes

To further investigate the effects of bmOPN supplementation on metabolism of HFD dams, the genomic functional prediction responding to bacterial community alterations was performed based on KEGG pathway database (level 3). As shown in Figure 6, we observed significant decreases in microbial gene abundance involved in primary and secondary bile acid biosynthesis in the HFD group as compared with the ND group ($P < 0.05$). No significant difference was observed in the LPS biosynthesis pathway among the 3 groups ($P > 0.05$). Microbial gene abundance relevant to the glycerolipid metabolism pathway was enhanced by the consumption of HFD. However, bmOPN supplementation significantly increased the bacterial gene abundance of primary and secondary bile acid biosynthesis pathways of HFD dams ($P < 0.05$). In addition, lower bacterial gene abundance of the pathway in glycerolipid metabolism was also observed in the HFD + bmOPN group as compared with the HFD group ($P < 0.05$).

Effects of bmOPN on Bile Acid Profiles of HFD Dams at GD18.5

To further identify the effects of bmOPN on bile acid biosynthesis of HFD dams at GD18.5, the hepatic bile acid levels were further analyzed. As compared with the ND group, the contents of TCDCA (Figure 7A), TUDCA + THDCA (Figure 7B), TLCA (Figure 7C), and GCA (Figure 7D) in the liver of HFD dams were significantly decreased ($P < 0.05$) while no significant change was observed in the contents of GCDCA (Figure 7E), TCA (Figure 7F), T-α-MCA (Figure 7G), T-β-MCA (Figure 7H), and TDCA (Figure 7I; $P > 0.05$). However, compared with the HFD group, bmOPN supplementation significantly increased TCDCA (Figure 7A; $P < 0.05$) and TUDCA + THDCA (Figure 7B;
P < 0.05) levels in the liver of HFD dams while it had no remarkable effect on other bile acids (P > 0.05).

**DISCUSSION**

Endogenous OPN is known to increase neutrophil and macrophage infiltration via its Arg-Gly-Asp sequence (Atai et al., 2011; Li et al., 2012). Further, OPN plays critical roles in the functioning of fibroblasts, macrophages, and lymphocytes during inflammation (Koh et al., 2007). Consistently, we found that serum OPN level was increased in HFD pregnant rats (Figure 2C), which was coincident with previous study that plasma OPN contents were increased in obese patients (Gómez-Ambrosi et al., 2007). Moreover, the levels of serum LPS, TNF-α, IL-1β and IL-6 were also upregulated and more inflammatory cell infiltration in colon was observed in HFD dams. These evidences indicated an
inflammatory environment in HFD pregnant rats. However, bmOPN supplementation significantly decreased serum OPN level of HFD dams, accompanied by lower level of serum LPS, TNF-α, IL-1β and IL-6 and less colonic inflammatory cell infiltration. Orally bmOPN intake alleviated hepatic neutrophil infiltration but increased plasma OPN protein expression of mice with alcoholic liver disease (Ge et al., 2013). Different OPN antibody and different model might contribute to this conflicting result. In addition, human milk osteopontin (hmOPN) binds to LPS to limit its availability (Ge et al., 2014). The ameliorative effects of bmOPN on intestinal inflammation were also observed in DSS-treated mice (Kanwar et al., 2016). Thus, we speculated that bmOPN alleviated systemic and colonic inflammation of HFD pregnant rats.

A healthy intestinal epithelial barrier is essential to ensure a homeostatic gut environment and systemic metabolism. Intestinal barrier disturbance aggravated the penetration of LPS and harmful bacteria from intestinal lumen into circulation, leading to systemic inflammation and thereby aggravated intestinal inflammation (Liu et al., 2022). During pregnancy, there is a benign increase in intestinal permeability which increase the absorption of nutrients (Astbury et al., 2015). However, the intestinal permeability during pregnancy is aggravated by maternal HFD, which is partially due to the suppressed expression of TJ, such as ZO-1, occludin, and claudins (Gohir et al., 2019). Iron-saturated bmOPN promoted the proliferation of human intestinal epithelial cells, indicating the potential for intestinal barrier protection (Liu et al., 2019). In alcohol-treated mice, bmOPN decreased gut permeability as evidenced by the elevated expression of ZO-1, occludin and claudin-5 in colon tissues (Ge et al., 2013). In the present study, bmOPN significantly increased the expression of ZO-1 and claudin-4 in the colon tissues of HFD dams, indicating an improved intestinal barrier function.

Gut microbiota is tightly associated with maternal intestinal function and metabolic adaption during pregnancy. The bacteria colonized in maternal gut is remodelled during gestation to adapt maternal metabolic alteration and benefit pregnant outcomes. However, the remodeling process of microbial community is dependent on maternal diet consumption before and during pregnancy (Gohir et al., 2015). In the present study, bmOPN supplementation did not affect α-diversity and β-diversity of gut microbes in HFD dams, suggesting that the overall structure of gut microbiota in obesity pregnancies remained stable during bmOPN supplementation. Similar results were also reported in previous articles (Kang et al., 2016; Song et al., 2017). *Firmicutes* and *Bacteroidetes* are major phyla that account for 80% to 90% of total gut bacteria. The decreased abundance of *Bacteroidetes* and *Bacteroidetes/Firmicutes* are regarded as indicators of gut microbiota dysbiosis, leading to intestinal inflammation and permeability (Kim et al., 2012). *Bacteroidetes* to *Firmicutes* ratio was decreased with advancing pregnancy. But this ratio in HFD pregnant rodents was even lower than that of pregnancies with a ND (Mahizir et al., 2020). In the present study, bmOPN supplementation to HFD pregnant dams elevated the relative abundance of *Bacteroidetes* and the ratio of *Bacteroidetes/Firmicutes*. During the shift of gut microbes from the second to third trimester, the proportion of *Proteobacteria* was largely increased, contributing to the inflammatory response in pregnancy (Koren et al., 2012). In the current study, the predominance of *Proteobacteria* in pregnancies was further aggravated by HFD consumption. However, bmOPN significantly decreased the relative abundance of *Proteobacteria* in feces of HFD dams, which was partly due to a reduction in the abundance of *Helicobacteraceae* and *Desulfovibrioaceae*. *Helicobacteraceae* and *Desulfovibrioaceae* are LPS-producing bacteria and causative to the development of endotoxemia and pro-inflammation-inflammation cytokines release. Particularly, there exists an interaction between *Desulfovibrio* and intestinal epithelial cells, generating a surface biofilm and hydrogen sulfide (Figliuolo et al., 2018), which affects intestinal epithelial cell apoptosis and physical barrier degradation (Zhao et al., 2020). *Helicobacter* spp. was involved in inflammatory diseases of intestine (Chow et al., 2011). The modulating effects of bmOPN on gut microbiota has been reported in alcohol-treated mice (Das et al., 2022). Thus, bmOPN altered abundance of specific taxa of gut microbiota, ameliorating gut microbes shift and barrier function in HFD pregnant rats.

Short-chain fatty acids are health benefitting metabolites generated by gut microbiota and tightly related to intestinal function and hosts’ health. A reduction in SCFA level decreased the expression of TJ, resulting in the impairment of intestinal epithelial barrier integrity (León Aguílera et al., 2022). Increasing the production of SCFA was associated with strengthening intestinal barrier function and alleviated inflammatory response in HFD mice (Tian et al., 2022). In this study, bmOPN supplementation significantly elevated the contents of isobutyric acid and pentanoic acid in feces of HFD dams, while had no effect on acetic acid, propanoic acid and butyric acid. Acetic acid, propanoic acid, and butyric acid, accounting for over 95% SCFA in mammal, are generated by microbial fermentation of polysaccharides (O’Riordan et al., 2022). Although bmOPN contains sialic acids, we speculated it is insufficient for gut bacteria fermentation to affect the concentration of acetic acid, propanoic acid and butyric acid in HFD dams.
Isobutyric acid, a kind of branched SCFA, is mainly produced from the fermentation of branched chain amino acids-containing proteins and peptides by genera *Bacteroides* and *Clostridium* (Rios-Covian et al., 2020). It not only acts as an energy substrate of intestinal epithelial cells, but alleviates intestinal inflammation (Ye et al., 2020). *Bacteroides* and *Clostridium*._XIVb_ were dominant genera in bacteria of bmOPN supplemented group, which utilized the branched chain amino acids of bmOPN to generate isobutyric acid and benefited gut barrier functions of HFD pregnancies. In addition, pentanoic acid supplementation regulated B10 cells (a kind of regulatory B cells) function to produce IL-10 and inhibit inflammation (Zou et al., 2021). Oral pentanoic acid intake protected against intestinal injury induced by radiation (Li et al., 2020). An increase in pentanoic acid level in bmOPN supplemented rats might be correlated to the alterations in the abundance of *Bacteroides*, *Clostridium* and *Desulfovibrio* (Sun et al., 2021). Therefore, bmOPN facilitated SCFA generation, partially contributing to the enhancement of gut barrier functions and suppression of inflammatory response.

Bile acid metabolism has major effects on gut epithelial homeostasis and gut microbiota composition (Malesza et al., 2021). It has been reported that microbial genes of primary and secondary bile acid biosynthesis are decreased in HFD pregnancies (Gohir et al., 2015). Consistently, primary and secondary bile acid biosynthesis of microbiome in HFD + bmOPN treated group were increased. TCDCA is a primary taurine-conjugated bile acid. Increased TCDCA levels in circulating contributed to ameliorated intestinal inflammation in mice with inflammatory bowel disease (IBD; Wong et al., 2022). TUDCA, a secondary taurine-conjugated bile acid, is converted from TCDCA by 7α-hydroxysteroid dehydrogenase. Wang et al. demonstrated that TUDCA treatment significantly ameliorated intestinal inflammation and barrier function with increased TJ expression in HFD mice (Wang et al., 2018). Long-term HFD consumption is accompanied with an increase in unconjugated bile acids in intestinal bile acid pool, contributing to increased epithelial permeability (Gupta et al., 2020). Conjugated bile acids were demonstrated to form micelles with unconjugated bile acids, which reduces the interaction between unconjugated bile acids and gut epithelial cells, thereby protecting against cell apoptosis and intestinal barrier dysfunction (Li et al., 2022b). Here, we observed that bmOPN enriched the contents of TCDCA and TUDCA + THDCA in hepatic bile acid pool of HFD rats. Therefore, bmOPN enhanced gut barrier function of HFD pregnant rats partially through increasing conjugated bile acid biosynthesis.

In addition to intestinal barrier function, bile acids are also involved in gut microbiota regulation. A higher level of bile acids in intestinal lumen is beneficial to the growth of microbial taxa with 7α-dehydroxylase activity, such as genera *Clostridium* and *Bacteroides*, whereas a lower level facilitates the growth of gram-negative microbes (Guo et al., 2022). Bile acids were also demonstrated to inhibit the microbial overgrowth by activating inducible NO synthase and IL-18 (Inagaki et al., 2006). Specifically, TUDCA treatment not only reduced the relative abundance of *Firmicutes* and *Proteobacteria* but increased the relative abundance of *Bacteroidetes* of HFD mice. Similar alterations in gut microbiota composition in response to TUDCA treatment were also observed in IBD mice (Van den Bossche et al., 2017). In addition, some bacterial taxa possess dehydroxylation activity, such as *Clostridium*._XIVb_. Increases in TCDCA and TUDCA contents create a substrate-abundant environment, which contributed to the bloom and dominant status of *Clostridium*._XIVb_ in bmOPN supplemented HFD dams (Van den Bossche et al., 2017). Therefore, we speculated that the conductive effect of bmOPN on bile acid metabolism contributed to healthier gut microbiota composition of HFD pregnant rats.

Endogenous OPN level was considered as a potential biomarker of inflammation in some obesity-related diseases (Catalán et al., 2016; Nacaroglu et al., 2017) and positively related to obesity and insulin resistance (Lin et al., 2017). The OPN gene aggravated metabolic disorders and gut dysbiosis induced by 24-weeks HFD intervention (Chen et al., 2022). However, there were also controversial results. Silencing OPN promoted adipogenic differentiation of adipose-derived mesenchymal stem cells, and intravenous injection of OPN-expression adenovirus delayed the development of HFD-induced obesity and insulin resistance (Tang et al., 2019). These conflicting reports indicate that OPN exerts different roles under different condition. Moreover, OPN exists in different isoforms, which have diverse functions (Sarosiek et al., 2015). As exogenous protein, bmOPN is hydrolyzed by digestive enzymes through gastrointestinal tract (Liu et al., 2019), which might generate bioactive small fragments. We speculated that these small fragments regulated intestinal dysfunction and metabolism of HFD pregnant dams.

In the current study, maternal HFD and bmOPN supplementation had no significant effect on litter size and fetal weights. Our findings were similar to the study that metformin intervention during pregnancy had no effect on litter parameters characteristics (Huang et al., 2022a). Controversially, Huang et al. (2022b) reported that maternal HFD increased fetal weights at embryonic d 21, which was reversed by butyrate administration.
during pregnancy. Berberine supplementation during gestational d 7 to 20 decreased the number of dead and absorptive fetuses and fetal weights of HFD-induced gestational diabetes mellitus rats (Li et al., 2022a). Multiple factors, such as duration of maternal HFD, fetus collection time and macronutrient composition of diet, jointly contributed to these discordant results. We failed to assessed number of implants, resorptions and placental weights. Indeed, it has been reported that maternal HFD/obesity not only adversely affects implantation (Brewer and Balen, 2010), but increases the average number of resorption sites (Hayes et al., 2012). Placental weights of maternal HFD/obese dams are reduced compared with normal dams (Ye et al., 2017; Lin et al., 2019). In current study, we mainly focus on the effects of bmOPN on intestinal health of HFD pregnancies. It warrants further investigation into the effects of bmOPN on the reproductive ability of HFD dams and their offspring development in future research.

CONCLUSIONS

Bovine milk osteopontin supplementation during pregnancy suppressed colonic inflammation and enhanced intestinal barrier function. Bovine milk osteopontin also modulated gut microbiota populations with increased SCFA generation. Furthermore, bmOPN facilitated bile acid biosynthesis which partially contributes to the improvement of bmOPN on gut health of HFD dams. It is worth to investigate the effects of maternal bmOPN supplementation on the development of HFD offspring to get a holistic understanding of bmOPN in future research.

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