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Ketogenic diet suppresses colorectal cancer through reshaping gut microbiota and modulating the intestinal FXR/NF-κB signaling pathway

Qinhan Gao[1], Yuwen Liu[1], Fayu Su[1], Siyu Zhou[1], Tianxiao Mao[2], Majie Wang[3,4], Jialin Duan[1#], Maode Lai[5#], Jiankang Li [1#]

[1]*Xi'an Key Laboratory of Stem Cell and Regenerative Medicine, Institute of Medical Research, Northwestern Polytechnical University, 127 West Youyi Road, Xi'an, Shaanxi 710072, China*

[2] *Department of Pharmacy, Zhongshan Hospital, Fudan University, Shanghai 200000, China.*

[3]*Laboratory of Behavioral Neuroscience, Ningbo Kangning Hospital, Ningbo Institute of Microcirculation and Henbane, School of Medicine, Ningbo University, Ningbo 315201, Zhejiang, PR China*

[4]*Key Laboratory of Addiction Research of Zhejiang Province, Ningbo 315201，Zhejiang, PR China*

[5]*Research Unit of Intelligence Classification of Tumor Pathology and Precision Therapy, Chinese Academy of Medical Sciences (2019RU042); Key Laboratory of Disease Proteomics of Zhejiang Province, Department of Pathology, Zhejiang University School of Medicine, Hangzhou, 310058, China.*

ABSTRACT: Specific dietary patterns are crucial in the incidence and progression of colorectal cancer. Although there is evidence indicating that the ketogenic diet may inhibit colorectal cancer and potentially act as a therapeutic approach, the mechanisms by which it influences gut microbiota and modifies metabolic pathways remain poorly understood. This study aimed to explore the therapeutic effects of a ketogenic diet on colorectal cancer in AOM/DSS model through targeted bile acid metabolomics, transcriptomics, and 16S rDNA gene sequencing analysis. Our results demonstrate that the ketogenic diet provides both preventive and therapeutic benefits against colorectal cancer by reducing inflammatory responses and restoring intestinal barrier integrity. Additionally, serum metabolomic analysis indicated a reprogramming of bile acid metabolism. Furthermore, 16S rDNA sequencing and targeted bile acid metabolomics revealed that the ketogenic diet significantly altered gut microbiota composition and bile acid metabolism, leading to a unique serum bile acid profile characterized by increased primary bile acids and decreased secondary bile acids. Moreover, the combination of a ketogenic diet with antibiotics and fecal microbiota transplantation consistently resulted in higher abundances of *Muribaculaceae, Akkermansia,* and *Lachnospiraceae NK4A136,* while levels of *Parasutterella* decreased following ketogenic diet intervention. The concentrations of CA, CDCA, DCA, and 12-keto LCA in colorectal tissues or feces were consistent with those observed in ketogenic diet-treated subjects. An upregulation of FXR expression in colorectal tissues was noted, which modulated NF-κB metabolic pathways and contributed to a reduction in inflammatory responses. Our findings underscore that the ketogenic diet suppresses colorectal cancer by reshaping gut microbiota and modulating the intestinal FXR/NF-κB signaling pathway

Keywords: Ketogenic diet; Colorectal cancer; Gut microbiota; Bile acid; FXR/NF-κB

1. Introduction

[#]**Corresponding author**
Jialin Duan, duanjl@nwpu.edu.cn; Maode Lai, lmp@zju.edu.cn.
Jiankang Li, Email: lijiankang-1025@nwpu.edu.cn.

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According to the recently published 2022 statistics from the National Cancer Center, there were 517,100 new cases of colorectal cancer, accounting for approximately 10.72% of all newly diagnosed cancer cases, thus establishing it as the second most prevalent cancer following lung cancer[1]. The incidence of early-onset colorectal cancer has seen a significant rise, closely linked to early risk factors such as a Westernized diet, lifestyle modifications, and environmental pollution[2]. Abnormal metabolism is a prominent feature among colorectal cancer patients, with disruptions in various metabolic pathways, including glycolysis, lipid synthesis, and glutamine uptake, facilitating the development and progression of the disease[3]. Consequently, the exploration and development of metabolic therapies aimed at inhibiting the progression of colorectal cancer are at the forefront of contemporary research.

While the exact etiology of colorectal cancer remains unclear, alterations in dietary patterns are recognized as a critical factor in its onset[4]. There is an increasing awareness that a high-calorie, high-fat Western diet can significantly elevate the risk of developing colorectal cancer. However, research focusing on modifying metabolic processes through dietary interventions for the prevention and treatment of colorectal cancer is relatively sparse. Tumor metabolic targets include the targeting of cancer cells, the tumor microenvironment, and the regulation of overall body metabolism[5]. Dietary interventions such as ketogenic diets, calorie restriction, and intermittent fasting have demonstrated potential effects in cancer therapy by influencing whole-body metabolism[6]. The ketogenic diet, which is primarily composed of beta-hydroxybutyrate, is characterized by high fat, low carbohydrate intake, and moderate protein consumption. Recent studies indicate that beta-hydroxybutyrate, a key component of the ketogenic diet, can inhibit the progression of colorectal cancer by suppressing the hcar2-Hopx axis[7]. However, the precise mechanisms through which the ketogenic diet influences whole-body metabolism to suppress colorectal cancer remain to be elucidated.

Bile acids are vital components of bile and play a crucial role in fat metabolism. Primary bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), are primarily synthesized by hepatocytes and possess a simple structure. Secondary bile acids, including deoxycholic acid (DCA) and lithocholic acid (LCA), are generated through the metabolism of primary bile acids by intestinal microorganisms. The increasing incidence of colorectal cancer is closely linked to bile acid levels, making the maintenance of a balanced bile acid profile essential for reducing the risk of colorectal cancer[8]. Secondary bile acids originate from primary bile acids and can intensify intestinal inflammation, significantly contributing to the onset of colorectal adenomas and cancers. Consequently, the composition of bile acids is intricately linked to the incidence and advancement of colorectal cancer. Metabolomic analyses have shown a significant increase in secondary bile acids, such as deoxycholic acid, in fecal samples from patients with colorectal adenomatous polyps and mucosal carcinomas[9]. Animal studies have consistently demonstrated that deoxycholic acid can enhance inflammatory responses and compromise intestinal barrier function, thereby promoting the progression of colorectal cancer. Reports examining the relationship between the ketogenic diet and bile acid

metabolism are relatively limited. Our preliminary results suggest that the ketogenic diet may alter bile acid metabolism in mice, yet the specific mechanisms involved warrant further investigation.

A healthy diet is crucial for maintaining the stability and diversity of the gut microbiota, while dietary changes can disrupt the balance of gut microbes, leading to disturbances in bile acid metabolism[10]. High-fat intake is currently recognized as a significant risk factor for promoting adenoma formation and accelerating the progression of colorectal cancer, primarily due to its impact on gut microbiota composition and bile acid homeostasis[11]. Ketogenic diets demonstrate a significant relationship with gut microbiota. Recent research suggests that the ketogenic diet may have therapeutic potential in managing refractory epilepsy by increasing the abundance of *Akkermansia muciniphila* and *Parabacteroides*[12]. Furthermore, the ketogenic diet may reduce susceptibility to hypoxia-induced cognitive impairment and the risk of Alzheimer's disease by fostering a more balanced microbial environment[13]. The farnesoid X receptor (FXR) is recognized as a key regulator of bile acid synthesis and secretion in both the liver and intestine. Studies have indicated a common reduction in FXR expression among patients with colorectal cancer, leading to metabolic disruptions in bile acids and promoting the development of colorectal cancer[14]. However, whether ketogenic diets can modulate gut microbiota and bile acid metabolism to regulate FXR function and subsequently inhibit colorectal cancer progression remains to be further explored.

A previous study has indicated that the effects of the ketogenic diet on the microbiome are not solely due to the diet itself, but also to its influence on metabolic processes in overall body metabolism, which in turn affects the microbiome[15]. We hypothesize that the ketogenic diet can suppress colorectal cancer by reshaping gut microbiota and mitigating inflammatory responses via intestinal FXR/NF-κB signaling pathway. Therefore, this study aims to investigate potential solutions using animal models for colorectal cancer and fecal microbiota transplantation (FMT) from these models following the therapeutic strategy of the ketogenic diet. Clarifying this issue will contribute to understanding the mechanisms of the ketogenic diet from the perspective of tumor metabolism and provide novel metabolic therapies and treatment strategies for early intervention in colorectal cancer.

2. Materials and methods

2.1 Reagents and chemicals

The FXR inhibitors GW4064 (cat.No.HY-50108) and obeticholic acid (OCA, cat.No.HY-12222) were sourced from MedChemExpress (MCE). The ketogenic diet (KD) (cat.No.D03022101) was obtained from Research Diets, Inc. The sources of Dextran sulfate sodium (DSS), Azoxymethane (AOM), and all antibiotics were described in our previous study[16].

2.2 Ketogenic diet treatment in AOM/DSS mouse model

C57BL/6J male mice (22-25 g, 6-week-old) were purchased from Vital River (Beijing, China). The mice were housed in a specific pathogen-free environment with appropriate temperature and humidity. All protocols were approved by the Institutional Ethics Review Board of Northwest Polytechnical University

(NO.202001016). The AOM/DSS mouse model was previously established by administering 10 mg/kg AOM via intraperitoneal injection, followed by continuous exchange of water and DSS. The mice received 2.5% (w/v) DSS for 7 days and normal water for 10 days as one cycle. All mice were randomly divided into six groups, including B-control, B-model, B-Ketogenic diet (B-KD) and L-control, L-model, L-Ketogenic diet (L-KD). “B” indicates that the KD group received ketogenic diet treatment from the first to the third cycle, “L” indicates that after the third cycle, the KD group received treatment for one cycle. The degree of colon damage was evaluated using the Disease Activity Index (DAI). Portions of the colorectal tissue were utilized for immunohistochemical analysis or H&E staining, while other portions were used for Western blot analysis. Serum samples were used for quantifying cytokines and chemokines, metabolomics, and bile acid analysis.

2.3 Quantification of cytokines and chemokines

The Chemokine Panel (31-Plex) Kit for mice (Product No. 12009-159) from Bio-Rad Laboratories was employed to detect the concentrations of cytokines and chemokines in serum using the Luminex 200™ liquid-phase chip.

2.4 Metabolomics

A total of 100 μL serum samples were combined with 400 μL of an 80% methanol solution in an EP tube. The mixture was vortexed thoroughly, sonicated in ice water for 5 minutes, and then centrifuged at 4℃ at 13,000 r/min for 20 minutes. The supernatant was evaporated to dryness using a centrifugal concentrator to eliminate the organic solvent, and then resuspended in mass spectrometry-grade water. The mixture was ultrasonicated in ice water for 3 minutes, followed by centrifugation at 4℃ at 13,000 r/min for another 20 minutes. The supernatant was collected and injected into the LC-MS for analysis. Detailed information is provided in the supplementary materials.

2.5 Targeted measurement of bile acids

The sample preparation protocol was as follows: 50 μL of serum sample was combined with 40 μL of a 1 μg/mL bile acid internal standard solution and 200 μL of methanol. The mixture was vortexed thoroughly, sonicated in ice water for 3 minutes, and then centrifuged at 4℃ at 13,000 r/min for 10 minutes. The supernatant was evaporated to dryness using a centrifugal concentrator to remove the organic solvent, and then resuspended with 100 μL of the initial mobile phase by vortexing. Subsequently, the mixture was ultrasonicated in ice water for 3 minutes, followed by centrifugation at 4℃ at 13,000 r/min for another 10 minutes. The supernatant was collected for analysis..

Approximately 40 mg of colorectal tissue or fecal sample was taken and combined with 500 μL of methanol. After thorough extraction, 200 μL of the supernatant was added to 40 μL of a 1 μg/mL bile acid internal standard solution. The subsequent extraction procedure was consistent with that of the serum sample. Specific details regarding the liquid phase and liquid chromatography conditions are described in the supplementary materials.

2.6 Cell Proliferation Assay and Colony Formation

The detailed experimental methods for the cell proliferation assay and colony formation are described in the supplementary materials. HCT116 and HT29 cells were treated with GW4064 and OCA for 24, 48, 72, and 96 hours in the cell proliferation assay. Three groups of HCT116 or HT29 cells were incubated separately for 10 days using regular medium, medium supplemented with GW4064, and medium supplemented with OCA for colony formation.

2.7 Transcriptome Sequencing

Total RNA was extracted from the cells, after which mRNA was enriched using Oligo (dT) magnetic beads. The purified mRNA was processed with the VAHTS Universal V6 RNA-seq Library Kit obtained from Vazyme, followed by sequencing on the MGI-SEQ2000 platform.

2.8 Fecal microbiota transplantation (FMT)

C57BL/6J mice were orally gavaged with 200 μL of mixed antibiotic solutions (5 mg/mL of metronidazole, streptomycin sulfate, ampicillin, neomycin sulfate, and 2.5 mg/mL of vancomycin hydrochloride) for 7 days after a week of acclimation to establish the PGF (pseudo-germ-free) mouse model. For FMT experiments, C57BL/6J mice were first divided into four groups: control, model, KD, and PGF with ketogenic diet. The ketogenic diet was administered from the first to the fourth cycle during the AOM/DSS modeling process. Fecal samples from each mouse were collected after the fourth cycle and stored at -80 ℃. Subsequently, PGF-C57BL/6J mice were randomly divided into three groups: F-control group, F-model group, and F-KD group. Mice in each group were orally administered a 200 μL suspension of fecal microbiota solution (1 g fecal samples homogenized in 5 mL of PBS).

2.9 16S rDNA gene sequencing

The entire microbiome DNA was isolated from fecal samples using the CTAB method and subsequently amplified by PCR with specific primers. The PCR products were purified using AMPure XT beads and analyzed with the Agilent 2100 Bioanalyzer and Illumina sequencing technology (Kapa Biosciences). Qualified gradient dilutions of each library were mixed in the appropriate ratio for the desired sequencing volume, denatured with NaOH to single-stranded DNA, and sequenced as 2 × 250 bp double-ends.

2.10 Statistics

To assess differences among various groups, statistical analysis was conducted using GraphPad Prism version 9.0 (GraphPad Software, Inc., San Diego, California, USA). Data are expressed as mean values and standard deviations. The Student's t-test was employed for comparing two groups, while two-way analysis of variance (ANOVA) was utilized for multiple group comparisons.

3. Results

3.1 The ketogenic diet suppresses the growth of colorectal cancer

The preventive and therapeutic effects of the ketogenic diet on colorectal cancer were evaluated, with the experimental design illustrated in Fig. 1A. In contrast to a standard diet, the ketogenic diet is characterized by

a significantly higher fat content and lower carbohydrate content (Fig. 1B). During the AOM/DSS modeling process, the ketogenic diet treatment resulted in a notable decrease in DAI scores, which were utilized to assess colonic damage. Additionally, the L-KD group consistently exhibited lower DAI scores compared to the L-model group following tumor development (Fig. 1C). H&E-stained images confirmed the efficacy of the ketogenic diet in both preventing and treating colorectal cancer (Fig. 1D). The B-KD group demonstrated a significant reduction in tumor number and tumor burden during the AOM/DSS modeling process, while the L-KD group showed diminished tumor growth post-tumor development (Fig. 1E-G). The concentrations of 3-hydroxybutyrate across different groups are presented in Fig. 1H. These findings indicate a positive correlation between the levels of β-hydroxybutyric acid and the effectiveness of the ketogenic diet in the treatment of colorectal cancer.

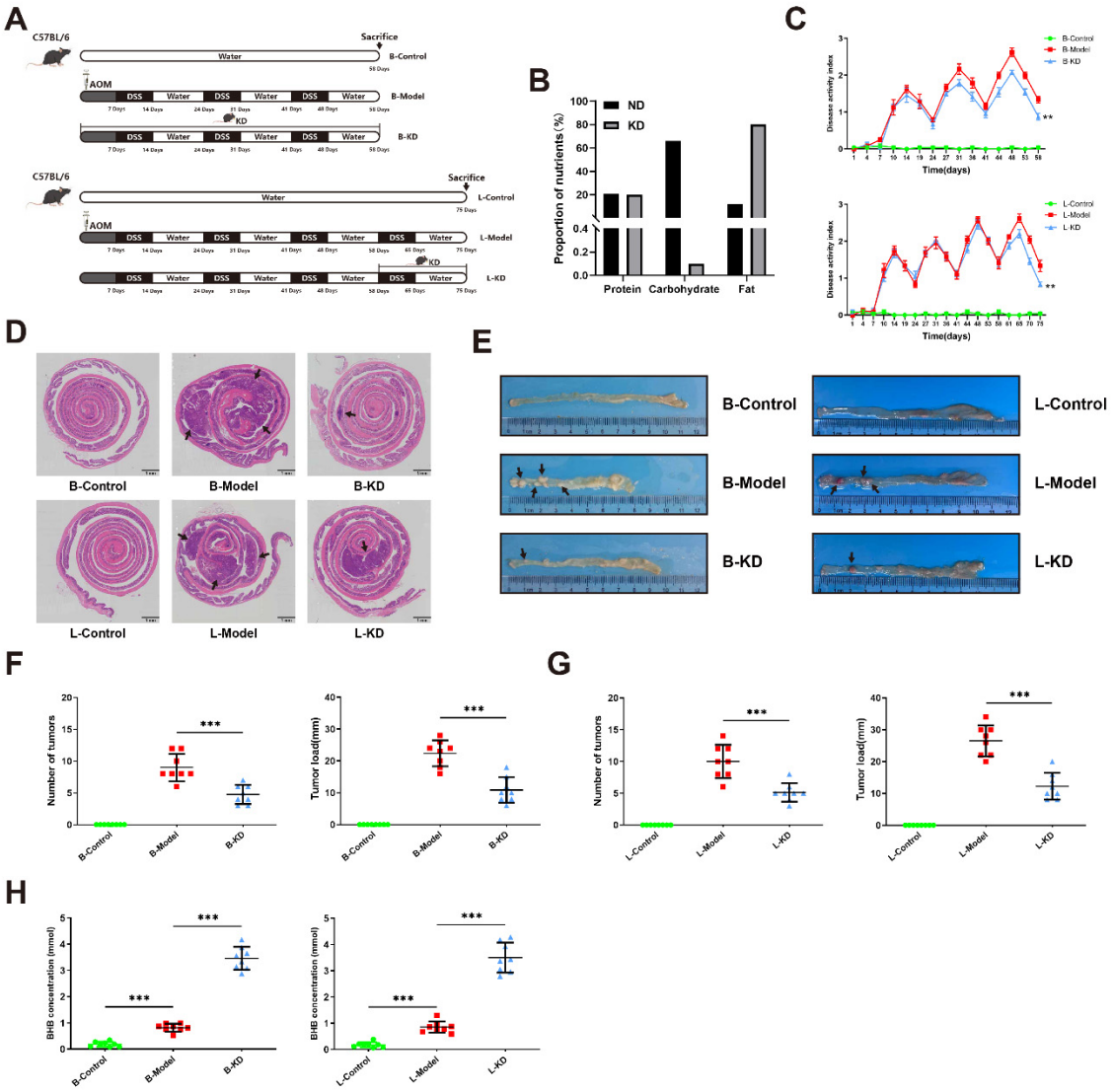
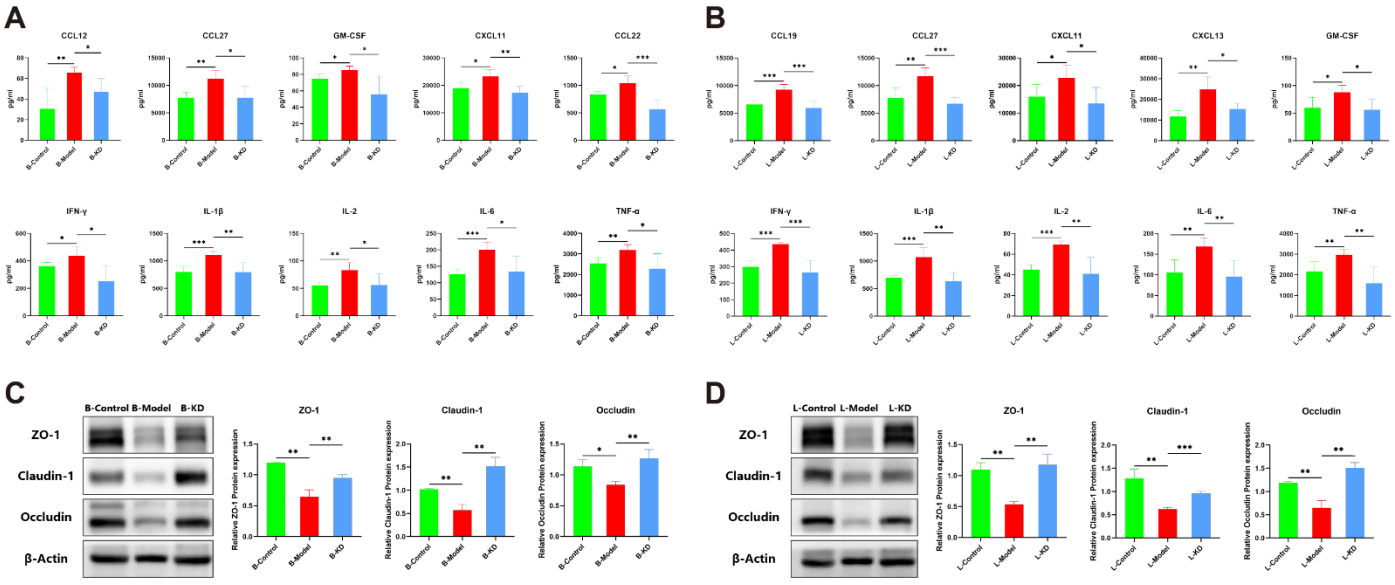


Figure 1. The ketogenic diet inhibits the proliferation of colorectal cancer. (A) Schematic representation of the experimental design to assess the preventive and therapeutic impacts of the ketogenic diet on colorectal cancer. (B) Comparative analysis of the ketogenic diet versus a standard diet composition. (C) Disease activity index (DAI) scores, which include body weight loss, presence of gross blood, and stool consistency across various groups (n = 8). (D) Representative images of colon tissue stained with hematoxylin and eosin (H&E) (n = 3). (E) Macroscopic observations of the colon from different experimental groups (n = 8). (F) Quantification of tumor numbers and (G) tumor volumes recorded across different groups (n = 8). (H) Measurement of 3-hydroxybutyrate concentrations in various groups (n = 8). Data are expressed as mean ± SD. Statistical analysis: unpaired t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. KD: ketogenic diet.

3.2 The ketogenic diet mitigates inflammatory response and restores intestinal barrier integrity

A total of 31 chemokines and cytokines were quantified in serum using the Luminex 200™ liquid-phase chip. Compared to the B-control group, a significant increase in 10 cytokines and chemokines, including CCL19, IL-1β, CCL27, CXCL11, IL-2, CXCL13, IL-6, GM-CSF, IFN-γ, and TNF-α, was observed in the B-model group. The concentrations of these cytokines and chemokines were significantly reduced in the B-KD group compared to the B-model group (Fig. 2A). The concentrations of the remaining chemokines and cytokines are detailed in Fig. S1. Similarly, the levels of 10 chemokines and cytokines, including CCL12, IL-2, CCL22, IL-1β, CCL27, IL-6, CXCL11, GM-CSF, IFN-γ, and TNF-α, were significantly elevated in the L-model group compared to the L-control group. However, these levels decreased in the L-KD group following KD diet treatment (Fig. 2B). The concentrations of the remaining chemokines and cytokines are shown in Fig. S2. The integrity of the intestinal barrier was assessed by examining the expression of claudin-1, occludin, and ZO-1 in intestinal tissues. Western blot analysis revealed a significant reduction in the expression of tight junction (TJ) proteins in both the B-model and L-model groups. During the AOM/DSS modeling process or after tumor development, there was a significant increase in TJ protein expression in the intestinal epithelial cell membrane with KD diet treatment (Fig. 2C-D). Consistent results were obtained through immunofluorescence staining, which showed a significant reduction in claudin-1, occludin, and ZO-1 expression in the model group compared to the control group. Following KD diet treatment, there was a significant increase in TJ protein expression, indicating a more pronounced effect on restoring intestinal barrier integrity (Fig. 2E-F). These findings suggest that the ketogenic diet may effectively reduce inflammatory responses and restore intestinal barrier function.



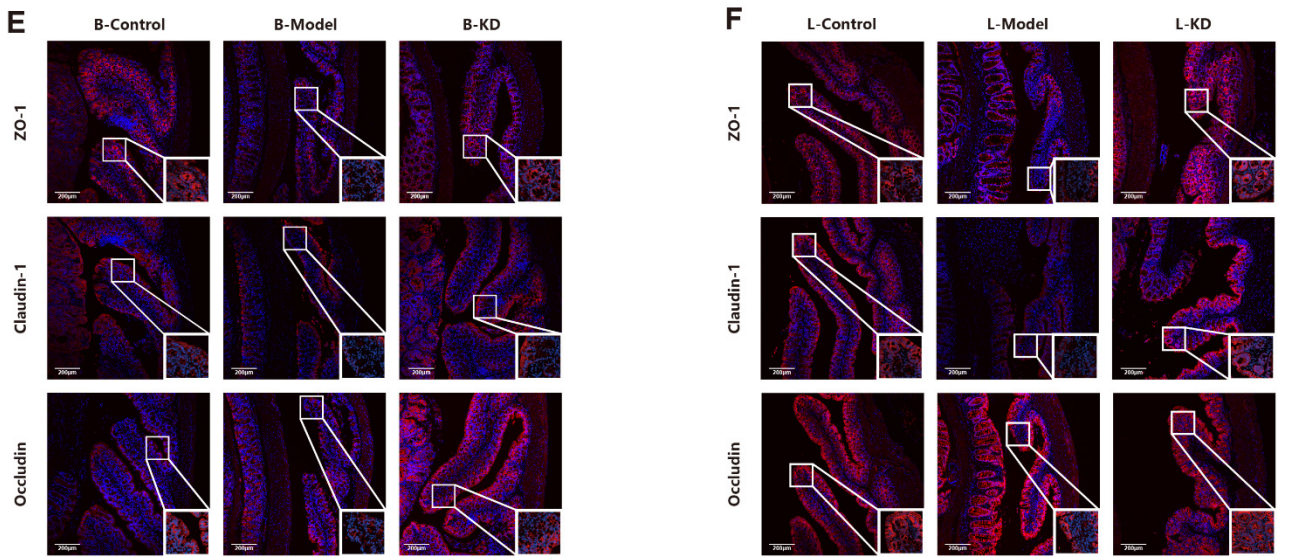


Figure 2. The ketogenic diet mitigates inflammatory responses and restores intestinal barrier integrity. (A) Concentrations of 10 cytokines and chemokines, including CCL19, IL-1β, CCL27, CXCL11, IL-2, CXCL13, IL-6, GM-CSF, IFN-γ, and TNF-α, significantly altered during the AOM/DSS modeling process with KD treatment, as determined by Luminex 200™ liquid phase chip (n = 6). (B) Concentrations of 10 cytokines and chemokines, including CCL12, IL-2, CCL22, IL-1β, CCL27, IL-6, CXCL11, GM-CSF, IFN-γ, and TNF-α, significantly altered post-tumor development with KD treatment (n = 6). (C) Representative immunoblots and relative expression levels of Claudin-1, Occludin, and ZO-1 proteins during AOM/DSS modeling (n = 3). (D) Representative immunoblots and relative expression levels of Claudin-1, Occludin, and ZO-1 proteins post-tumor development (n = 3). (E) Immunofluorescence images depicting in situ expression of Claudin-1, Occludin, and ZO-1 during AOM/DSS modeling (n = 3). Scale bar = 300 μm. (F) Immunofluorescence images showing in situ expression of Claudin-1, Occludin, and ZO-1 after tumor development (n = 3). Scale bar = 300 μm. Data are presented as mean ± SD. Statistical analysis: unpaired t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. KD: ketogenic diet.

3.3 The ketogenic diet regulates bile acid synthesis in colorectal cancer

We performed non-targeted metabolomics analysis to investigate the impact of the ketogenic diet on the preventive and therapeutic benefits against colorectal cancer. Significant differences in metabolites were observed in serum among the three groups during the AOM/DSS modeling process or after tumor development, as indicated by principal component analysis (PCA) in Fig. 3A-B. Subsequent OPLS-DA model analysis revealed distinct differences between each pair of groups (Fig. S3), with a total of 30 differential metabolites selected based on the criteria of VIP > 1.0 and FDR < 0.05 following ketogenic diet treatment during the modeling process and after tumor development. Pathway analysis identified primary bile acid biosynthesis as a key perturbed metabolic pathway based on the differential metabolites shown in Fig. 3C. Therefore, we hypothesized that the effects of a ketogenic diet on colorectal cancer were associated with the levels and composition of bile acids. Subsequently, targeted metabolomics using UPLC-QQQ-MS/MS was employed to measure serum bile acid levels, utilizing a method established in our laboratory. The major bile acids measured in serum are described in Fig. 3D. During the AOM/DSS modeling process, total bile acid concentrations did not significantly change in the B-KD group compared to the B-model group. However, primary bile acid concentrations decreased while secondary bile acids increased significantly in the B-model group compared to the B-control group. Following KD treatment, primary bile acids showed a significant increase while secondary bile acids exhibited a significant reduction (Fig. 3E). Significantly altered bile acids, including CDCA, HDCA, TUDCA, T-β-MCA, β-MCA, CA, DCA, and 7-keto-LCA, are shown in Fig. 3F.

The concentrations of other bile acids are summarized in Fig. S4. Similarly, the results demonstrate consistency in both the levels and composition of bile acids following KD treatment after tumor development (Fig. 3G-I, Fig. S5).

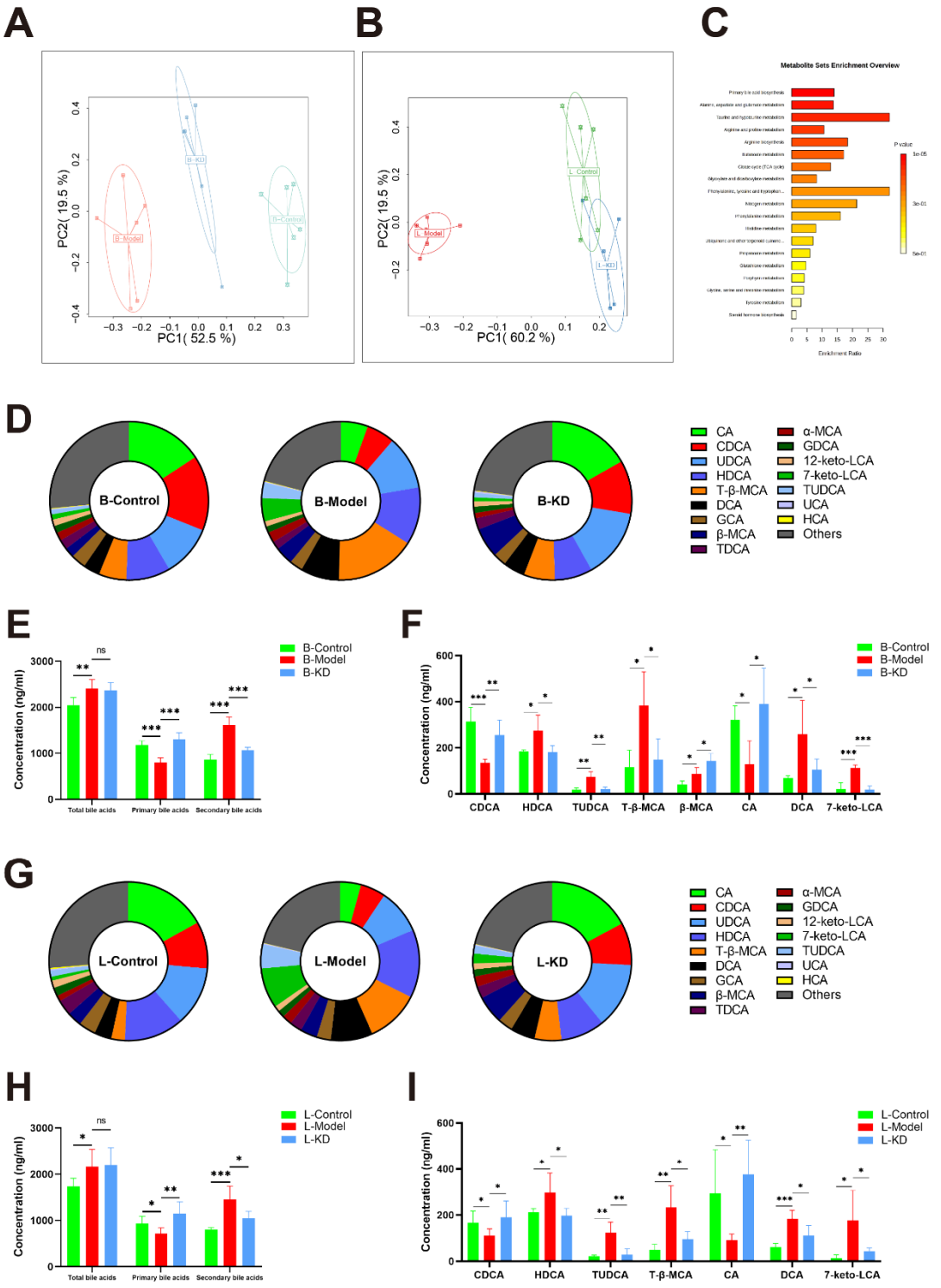
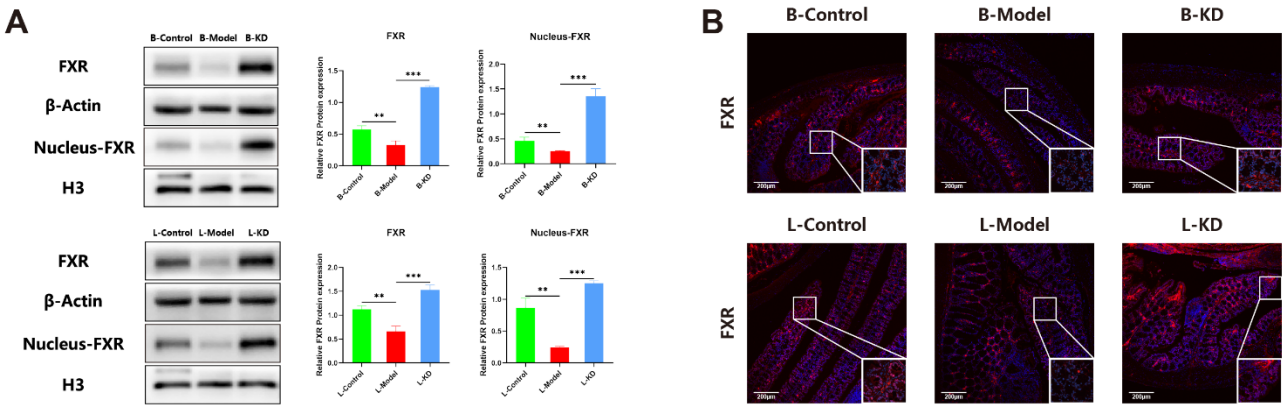


Figure 3. The ketogenic diet influences bile acid synthesis in colorectal cancer. (A) Principal component analysis (PCA) plots of serum bile acid profiles for each group during AOM/DSS modeling with KD treatment (n = 6). (B) PCA plots of serum bile acid profiles for each group post-tumor development with KD treatment (n = 6). (C) Identification of disrupted metabolic pathways based on differential metabolites from the four groups. (D) Measurement of major bile acids (E) concentrations of total bile acids, primary bile acids, and secondary bile acids (F) significantly altered bile acids in serum during AOM/DSS modeling with KD treatment (n = 6). (G) Measurement of major bile acids (H) concentrations of total bile acids, primary bile acids, and secondary bile acids (I) significantly altered bile acids in serum post-tumor development with KD treatment (n = 6). Data are presented as mean ± SD. Statistical analysis: unpaired t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. KD: ketogenic diet.

3.4 The ketogenic diet modulates the intestinal FXR/NF-κB signaling pathway

FXR plays a crucial role in regulating the synthesis and secretion of bile acids in both the liver and intestine. Therefore, we examined the expression of FXR and nuclear FXR in intestinal tissues. Our results demonstrated that the expressions of FXR and nuclear FXR in the model group were significantly decreased compared to controls. However, following KD treatment, there was a considerable increase in the expressions of FXR and nuclear FXR compared to the model group (Fig. 4A). Immunofluorescence staining further supported the ability of the ketogenic diet to reverse the expressions of FXR and nuclear FXR in the intestines (Fig. 4B). Additionally, we utilized FXR agonists (GW4064 and OCA) to confirm the impact of FXR on colorectal cancer cells proliferation. The results from CCK8 and colony formation assays revealed that GW4064 and OCA significantly decreased HCT116 and HT29 cell proliferation (Fig. 4C). To further explore the mechanism of FXR-mediated cancer inhibition, we conducted transcriptome sequencing on HCT116 and HT29 cells treated with the FXR agonist GW4064, as well as colorectal cells. PCA analysis revealed distinct segregation between the colorectal cells group and the GW4064 group (Fig. 4D). Fig. S6 depicts the hierarchical clustering analysis of the differentially expressed genes in HCT116 and HT29 cells, while volcano plots demonstrate the changes in gene expression between the two groups (Fig. 4E). The KEGG and GO classification of differentially expressed genes are summarized in Fig. S7. Pathway analysis indicated that multiple signaling pathways may be involved in the tumor-suppressive mechanism of activating FXR. Among these, the NF-kappa B signaling pathway drew our attention due to the negative regulatory effect of FXR on NF-κB signaling (Fig. 4F). Therefore, we chose to further investigate the NF-κB pathway. Western blotting analysis demonstrated that the abnormal expression of NF-κB-related proteins was effectively reduced in the AOM/DSS model with KD (Fig. 4G). Additionally, the protein expression of downstream pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β was assessed, revealing a significant reduction in related inflammatory factors in the KD group compared to the model group (Fig. 4H). These findings indicate that the ketogenic diet can suppress NF-κB activation by activating FXR, thereby mitigating the associated inflammatory response.



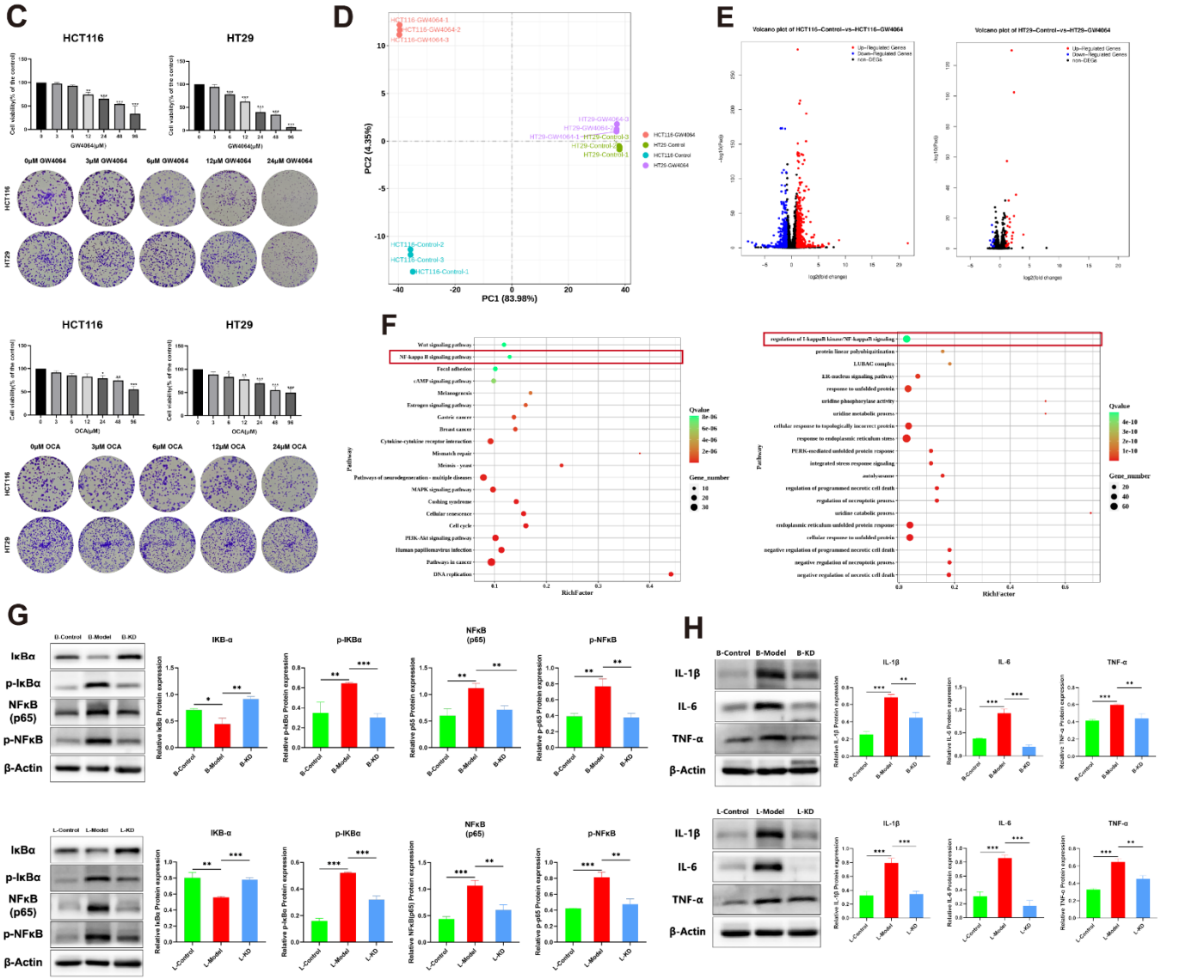
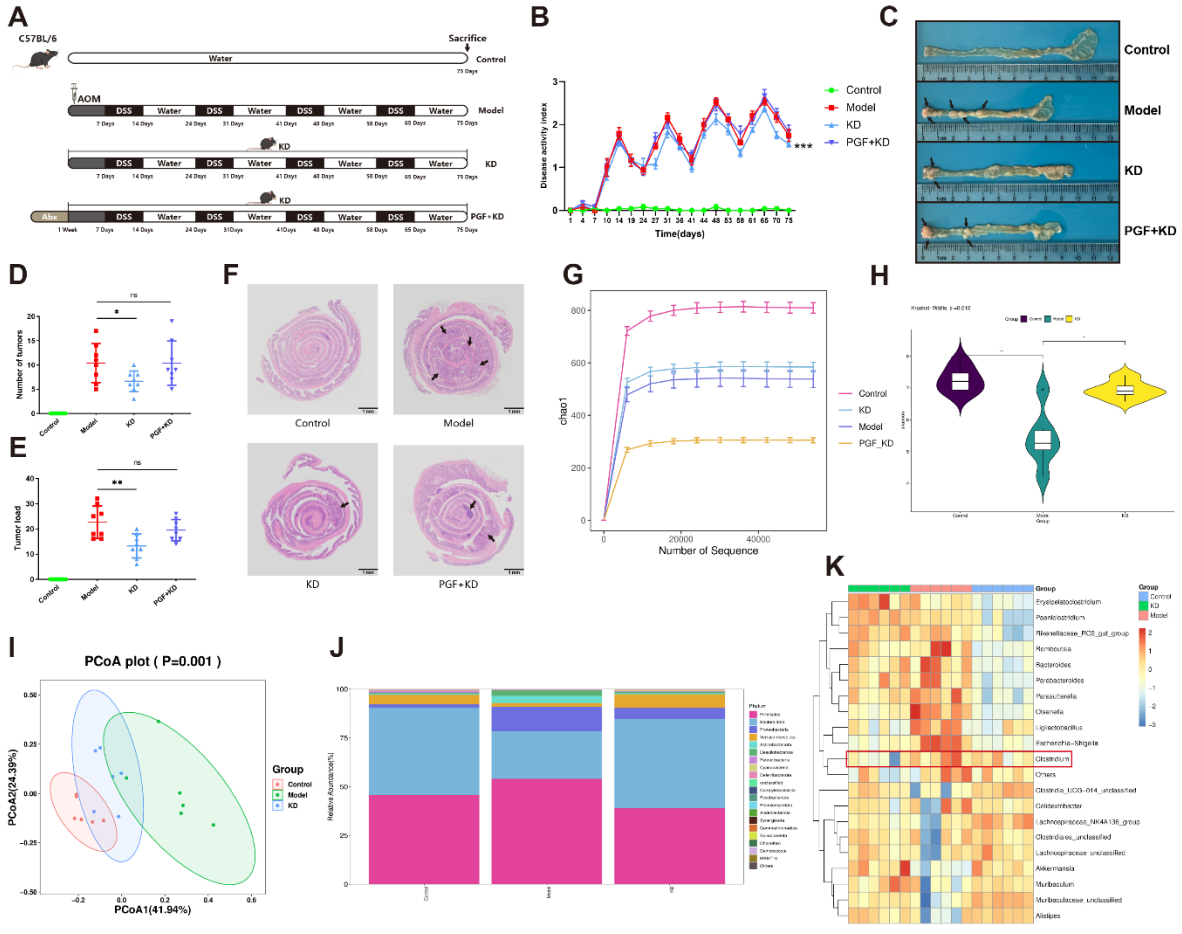


Figure 4. The ketogenic diet modulates the intestinal FXR/NF-κB signaling pathway. (A) Representative immunoblots and relative expression levels of FXR and nuclear FXR in intestinal tissues (n = 3). (B) Representative immunoblots and relative expression levels of FXR and nuclear FXR in intestinal tissues (n = 3). (C) Effects of FXR agonists (GW4064 and OCA) on the proliferation and colony formation of HCT116 and HT29 cells in vitro (n = 3). (D) PCA analysis of HCT116 and HT29 cells treated with FXR agonist GW4064, along with colorectal cells analyzed by transcriptome sequencing (n = 3). (E) Volcano plots of differentially expressed genes in HCT116 and HT29 cells treated with FXR agonist GW4064 (n = 3). (F) Pathway analysis based on differentially expressed genes between the two groups. (G) Representative immunoblots and relative expression levels of NF-κB activation-related proteins, including NF-κB, p-NF-κB, IκBα, and p-IκBα (n = 3). (H) Representative immunoblots and relative expression levels of proteins associated with inflammatory factors, including IL-1β, IL-6, and TNF-α (n = 3). Data are presented as mean ± SD. Statistical analysis: unpaired t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. KD: ketogenic diet.

3.5 The ketogenic diet suppresses colorectal cancer by reshaping the gut microbiota

Growing evidence suggests that the microbiome is associated with tumorigenesis in colorectal cancer. To explore the potential effect of the ketogenic diet in regulating gut dysbiosis in colorectal cancer, a PGF mouse model was established, with the experimental design illustrated in Fig. 5A. The ketogenic diet significantly reduced DAI scores, lowered tumor burden, and alleviated colorectal injury and tumor development as previously noted. In contrast, the PGF group did not achieve the same effect after being fed the ketogenic diet, indicating that the therapeutic effect of the ketogenic diet on colorectal cancer is associated with gut

microbiota (Fig. 5B-F). No significant morphological changes were detected in the major organs through histological analysis using H&E staining, indicating the safety of the ketogenic diet (Fig. S8). The successful establishment of a PGF mouse model was confirmed through comparison of the average rarefaction curves among the four groups with an equal number of 16S sequences (Fig. 5G). The Good's coverage in each group approached 1, indicating that the sequencing results could be considered reliable (Fig. S9A). Fecal 16S sequence analysis revealed that the diversity of gut microbiota showed no significant difference in the ketogenic diet group compared to the model group (Fig. 5H). However, PCoA indicated significant differences among the ketogenic diet, model, and control groups, suggesting distinct microbiota compositions among these groups (Fig. 5I). The Venn diagram illustrates the differential microbiota in the three groups. At the phylum level, *Bacteroidota* and *Verrucomicrobiota* were more abundant in the ketogenic diet group than in the model group, while *Firmicutes* and *Proteobacteria* exhibited opposite trends (Fig. 5J). A heat map illustrating the markedly altered bacteria at the genus level for the three groups was generated (Fig. 5K). Specifically, *Clostridium*, which was enriched in the model group, has been reported to be closely associated with secondary bile acid production in the intestine. Compared to the model group, the relative abundances of *Muribaculaceae*, *Akkermansia*, and *Lachnospiraceae NK4A136* were increased in the ketogenic diet group, while Parasutterella exhibited opposite trends (Fig. 5L-M). The correlation and relationship of differential microbiota in the three groups are shown in Fig. S9C-D. The Sankey diagram of the three groups is shown in Fig. S9E. These results suggest that the ketogenic diet effectively reshapes the gut microbiota in colorectal cancer.



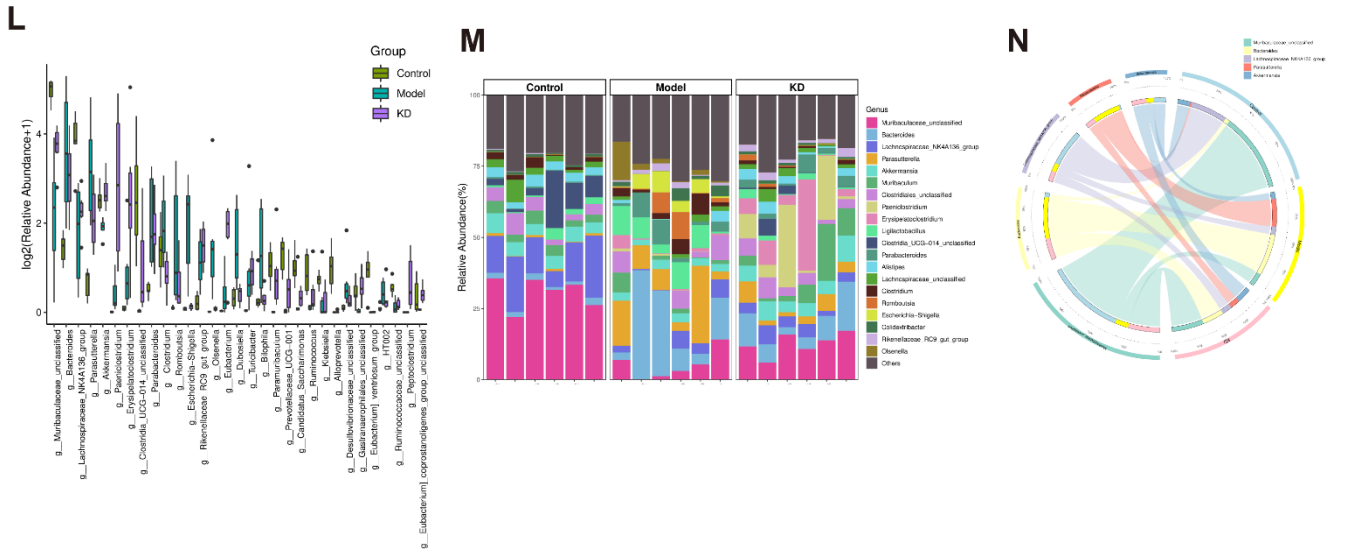


Figure 5. The ketogenic diet suppresses colorectal cancer by reshaping the gut microbiota. (A) Experimental schematic for evaluating the potential effects of the ketogenic diet on gut dysbiosis in colorectal cancer. (B) Disease activity index (DAI) scores, including body weight loss, gross blood, and stool consistency across different groups (n = 8). (C) Representative macroscopic observations of the colon from various groups (n = 8). (D) Tumor numbers and (E) tumor volumes recorded across different groups (n = 8). (F) Representative images of H&E-stained colon tissue (n = 3). (G) Average rarefaction curves in different groups based on identical numbers of 16S sequences (n = 6). (H) Shannon indices in different groups based on 16S sequences (n = 6). (I) Unweighted UniFrac distance heatmap for control, model, and KD groups. (J) Dominant significantly altered bacteria at the phyla level across three groups. (K) Heatmap based on significantly altered bacteria at the genus level across three groups. (L) Relative abundances of the top 30 significantly altered bacteria at the genus level across three groups. (M) Relative abundances of significantly altered bacteria in each group. (N) Circos plot for different groups based on 16S sequences. Data are presented as mean ± SD. Statistical analysis: unpaired t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. KD: ketogenic diet; PGF: pseudo-germ-free.

3.6 The ketogenic diet regulates FXR/NF-κB and bile acid composition via gut microbiota

Western blotting experiments revealed that the ketogenic diet was able to restore intestinal barrier function, activate FXR expression, regulate the NF-κB pathway, and reduce inflammatory factor protein expression. Conversely, the PGF group did not exhibit similar restoration and effects (Fig. 6A-D). Due to the limited entry of bile acids synthesized in the liver into systemic circulation, the majority are reabsorbed from the intestine. Therefore, targeted metabolomics analysis of bile acids in colorectal tissue and feces was conducted in each group using UPLC-QQQ-MS/MS (Fig. 6E). The results consistently indicated that the levels of primary bile acids were significantly decreased, while the concentrations of secondary bile acids were significantly increased in the model group compared to the control group in both colorectal tissue and feces. The ketogenic diet can alter the composition of bile acids by elevating primary bile acids and reducing secondary bile acids (Fig. 6F). Additionally, bile acids that antagonize FXR function, including T-β-MCA and DCA, were significantly decreased, while those that agonize FXR function, including CDCA and CA, were significantly increased following ketogenic diet treatment in colorectal tissue and feces (Fig. 6G). Furthermore, a notable disparity was observed in the concentrations of 12-keto LCA in the intestinal tissues and feces of AOM/DSS mice following treatment with the ketogenic diet. The concentrations of the remaining bile acids in colorectal tissues and feces, which were determined not to be significantly changed, are shown in Fig. S10. The conversion of the differential bile acids in colorectal tissues and feces is described in Fig. 6H. The results of correlation analysis indicate a strong relationship between differential bile acids in colorectal

tissues and feces (Fig. S11). The correlation between gut microbiota and bile acids in feces is shown in Fig. 6I. Although PGF mice with ketogenic diet treatment exhibited significant changes in primary and secondary bile acids, it did not significantly alter the conversion from primary to secondary bile acids. The concentrations of CA and CDCA were low, while T-β-MCA was high compared to the ketogenic diet group, suggesting that the activation of FXR by the ketogenic diet is primarily related to CA, CDCA, and T-β-MCA. These results demonstrate that the ketogenic diet can regulate FXR/NF-κB and bile acid composition by altering gut microbiota.

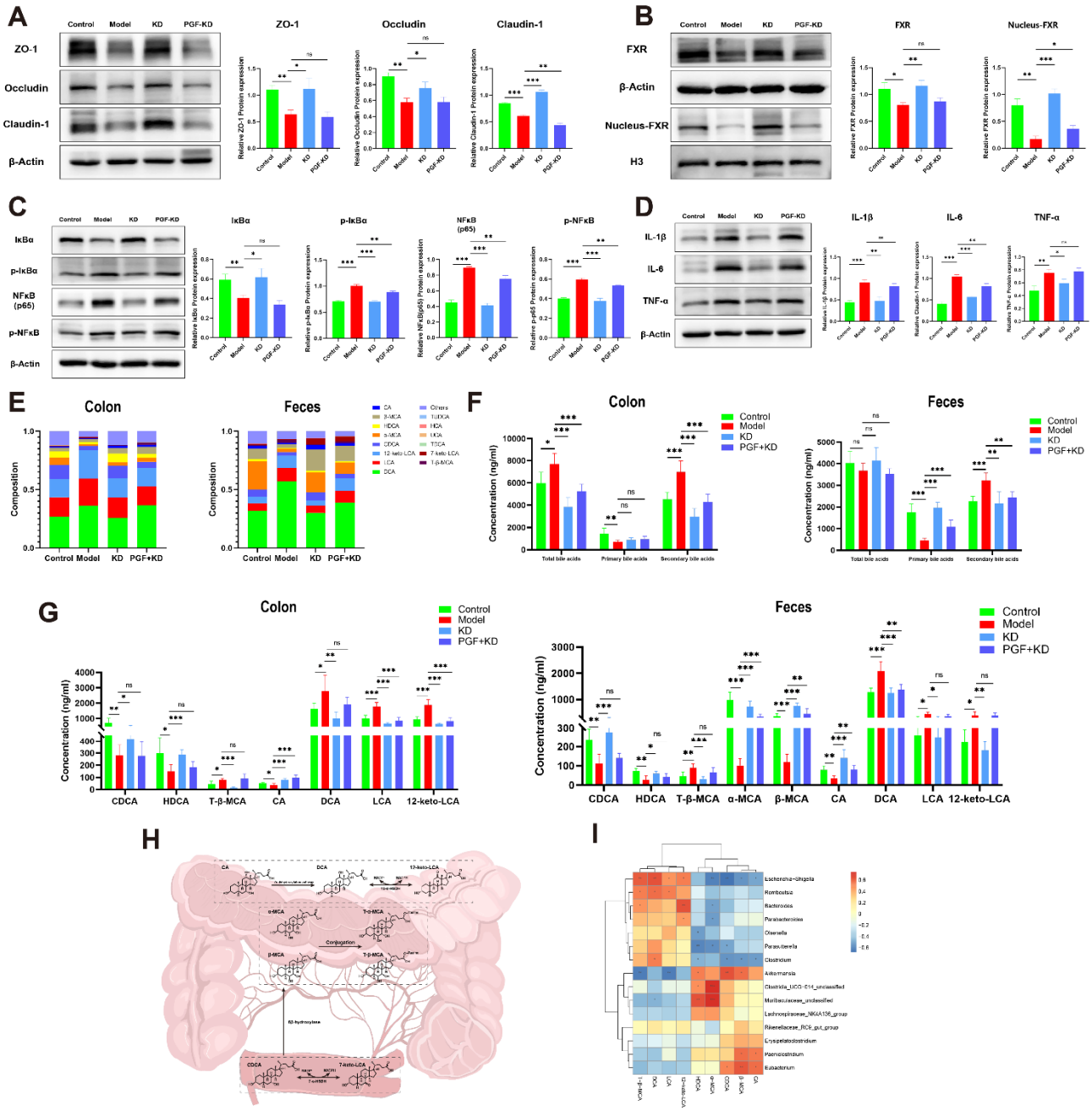
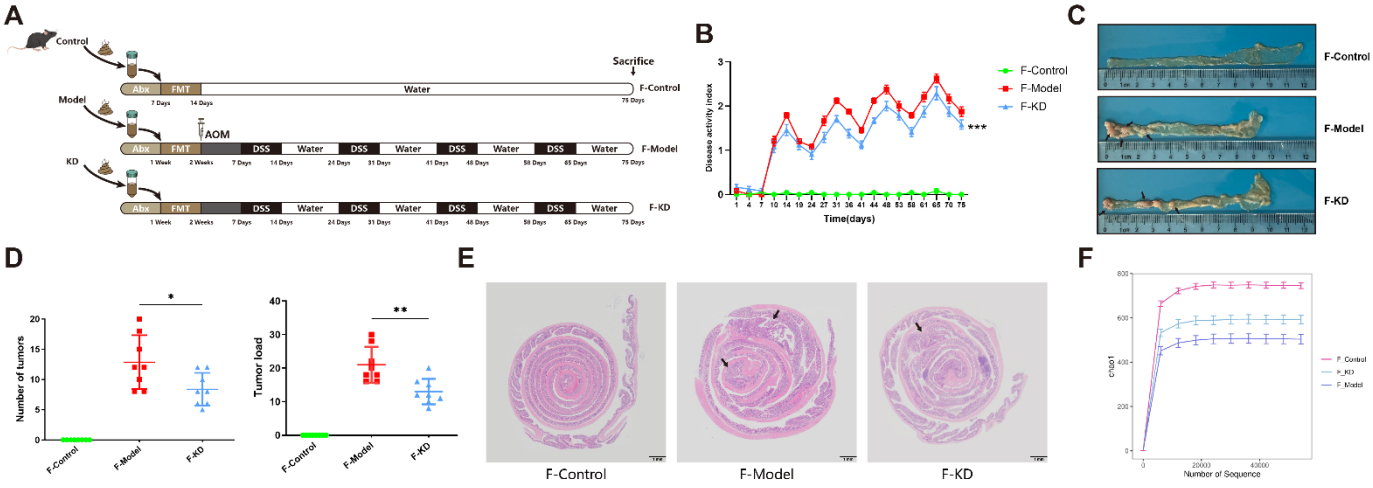


Figure 6. The ketogenic diet regulates FXR/NF-κB signaling and bile acid composition via gut microbiota. Representative immunoblots and relative expression levels of (A) Claudin-1, Occludin, and ZO-1 proteins (B) FXR and nuclear FXR (C) NF-κB activation-related proteins, including NF-κB, p-NF-κB, IκBα, and p-IκBα (D) proteins related to inflammatory factors, including IL-1β, IL-6, and TNF-α (n = 3). (E) Measurement of major bile acids (F) concentrations of total bile acids, primary bile acids, and secondary bile acids (G) significantly altered bile acids in colorectal tissues and feces. (H) Conversion of major significantly altered bile acids in colorectal tissues and feces. (I) Correlation between gut microbiota and bile acids described in feces. Data are presented as mean ± SD. Statistical analysis: unpaired t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. KD: ketogenic diet; PGF: pseudo-germ-free.

3.7 FMT-KD alleviates colorectal progression by altering gut microbiota

To further validate the involvement of gut bacteria and bile acids in mitigating colorectal cancer, we conducted fecal transplantation into PGF mice from the control group, model group, and mice treated with the ketogenic diet. The experimental design is illustrated in Fig. 7A. Compared with the F-model group, fecal transplantation resulted in decreased DAI scores (Fig. 7B) and notable amelioration of gross symptoms associated with colorectal cancer in the F-KD group (Fig. 7C). The F-KD group exhibited significantly fewer tumor numbers and tumor burden compared to the F-model group (Fig. 7D). Additionally, H&E stained images demonstrated that fecal transplantation from the KD group mitigated tumor progression and colorectal injury (Fig. 7E). No significant morphological changes were detected in the major organs through histological analysis using H&E staining after fecal transplantation from the KD group (Fig. S12). The Good's coverage of the three groups is shown in Fig. S13A, and the average rarefaction curves of the 16S rDNA sequences in different groups are described in Fig. 7F. The Shannon indices and PCoA following fecal transplantation are presented in Fig. 7G-H. The Venn diagram illustrates the differential microbiota in the three groups after fecal transplantation (Fig. S13B). At the phylum level, the relative abundance of *Bacteroidetes* and *Verrucomicrobiota* in the F-KD group was significantly increased compared to the F-model group, while *Proteobacteria* showed an opposite trend (Fig. 7I). Heat maps illustrating alterations of each group at the genus level are displayed in Fig. 7J. The correlation and relationship of differential microbiota after fecal transplantation from the KD group in the three groups are shown in Fig. S13C-D. The F-KD group exhibited a notable increase in the relative abundance of *Muribaculaceae*, *Lachnospiraceae NK4A136*, and *Akkermansia* compared to the F-model group. Conversely, Parasutterella exhibited a decreased abundance (Fig. 7K-M). A circos plot depicting the relationship between species and sample abundance can be utilized to visualize associations within microbial community studies (Fig. S13E). These findings are consistent with previous experiments and further support our hypothesis that the ketogenic diet exerts its anticancer effects by regulating gut microbial disorders.



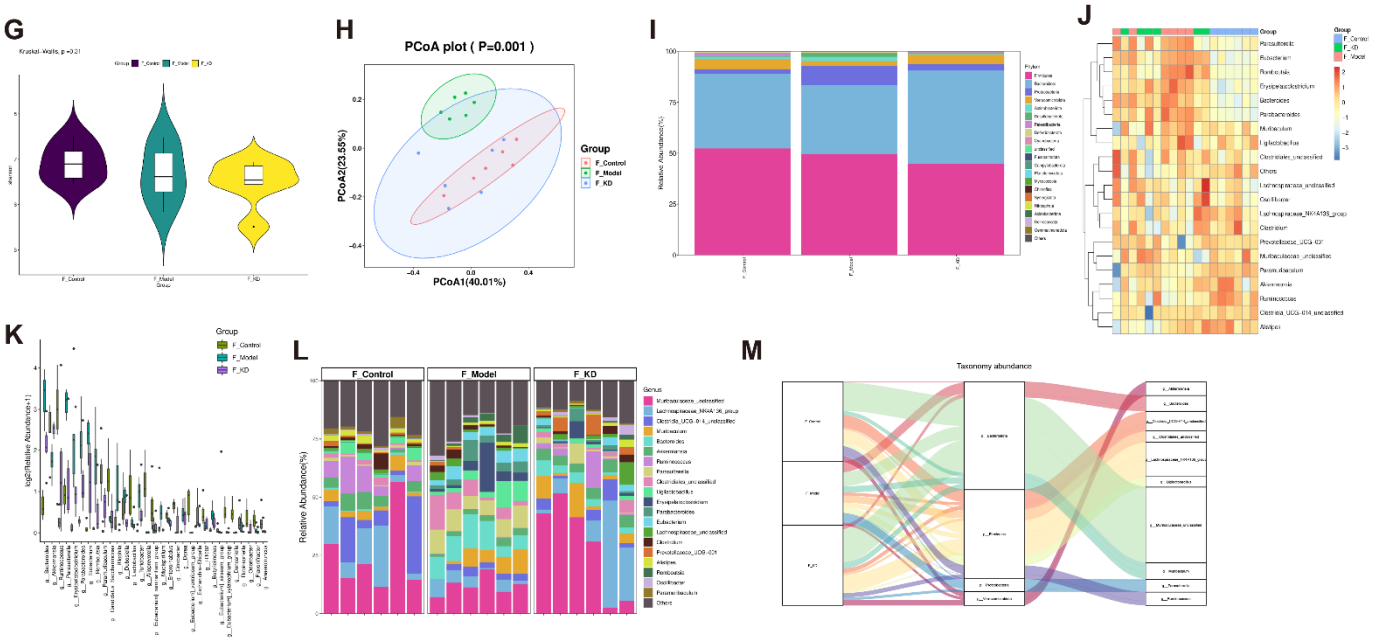


Figure 7. FMT-KD alleviates colorectal progression by altering gut microbiota. (A) Experimental schematic for validating the effect of the ketogenic diet on gut microbiota following fecal transplantation. (B) Disease activity index (DAI) scores, including body weight loss, gross blood, and stool consistency across different groups post-fecal transplantation (n = 8). (C) Representative macroscopic observations of the colon from various groups (n = 8) after fecal transplantation. (D) Tumor numbers and tumor volumes recorded across different groups post-fecal transplantation (n = 8). (E) Representative images of H&E-stained colon tissue after fecal transplantation (n = 3). (F) Average rarefaction curves in different groups based on identical numbers of 16S sequences after fecal transplantation (n = 6). (G) Shannon indices in different groups based on 16S sequences after fecal transplantation (n = 6). (H) Unweighted UniFrac distance heatmap for F-control, F-model, and F-KD groups. (I) Dominant significantly altered bacteria at the phyla level across three groups after fecal transplantation. (J) Heatmap based on significantly altered bacteria at the genus level across three groups after fecal transplantation. (K) Relative abundances of the top 30 significantly altered bacteria at the genus level across three groups after fecal transplantation. (L) Relative abundances of significantly altered bacteria in each group after fecal transplantation. (N) Sankey diagram for different groups based on 16S sequences after fecal transplantation. Data are presented as mean ± SD. Statistical analysis: unpaired t-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. KD: ketogenic diet; FMT: fecal microbiota transplantation.

3.8 FMT-KD modulates bile acid and intestinal FXR/NF-κB

Following fecal transplantation, targeted metabolomics analysis of bile acids in colorectal tissue and feces was conducted using UPLC-QQQ-MS/MS in three groups of mice. The effect of fecal transplantation from mice treated with the ketogenic diet on the composition of bile acids was similar to that of the ketogenic diet in colorectal tissue and feces (Fig. 8A). Compared with the F-model group, the composition of bile acids was altered by elevating primary bile acids and reducing secondary bile acids (Fig. 8B). The levels of two FXR activators, CDCA and CA, were notably increased, while the levels of two FXR antagonists, T-β-MCA and DCA, were significantly decreased following transplantation of feces from the KD group in colorectal tissue and feces compared to the model group (Fig. 8C). The levels of 12-keto LCA in the intestinal tissues and feces were consistently decreased significantly after fecal transplantation from the KD group. The concentrations of the remaining bile acids in colorectal tissues and feces, which were determined not to be significantly changed, are summarized in Fig. S14. After fecal transplantation from mice treated with a ketogenic diet, intestinal barrier function was restored compared to the F-model group through the expression of TJ proteins (Fig. 8D). The activation of FXR was consistently observed in the F-KD group (Fig. 8E), which was caused by regulating gut microbiota-bile acid crosstalk, leading to suppression of NF-κB pathway

activation and decreased expression of inflammatory factors through modulation of the intestinal FXR/NF-κB signaling pathway (Fig. 8F-G). The results of correlation analysis indicate a strong correlation between differential bile acids in colorectal tissues and feces following fecal transplantation (Fig. S15). The correlation between gut microbiota and bile acids in feces is shown in Fig. 8H.

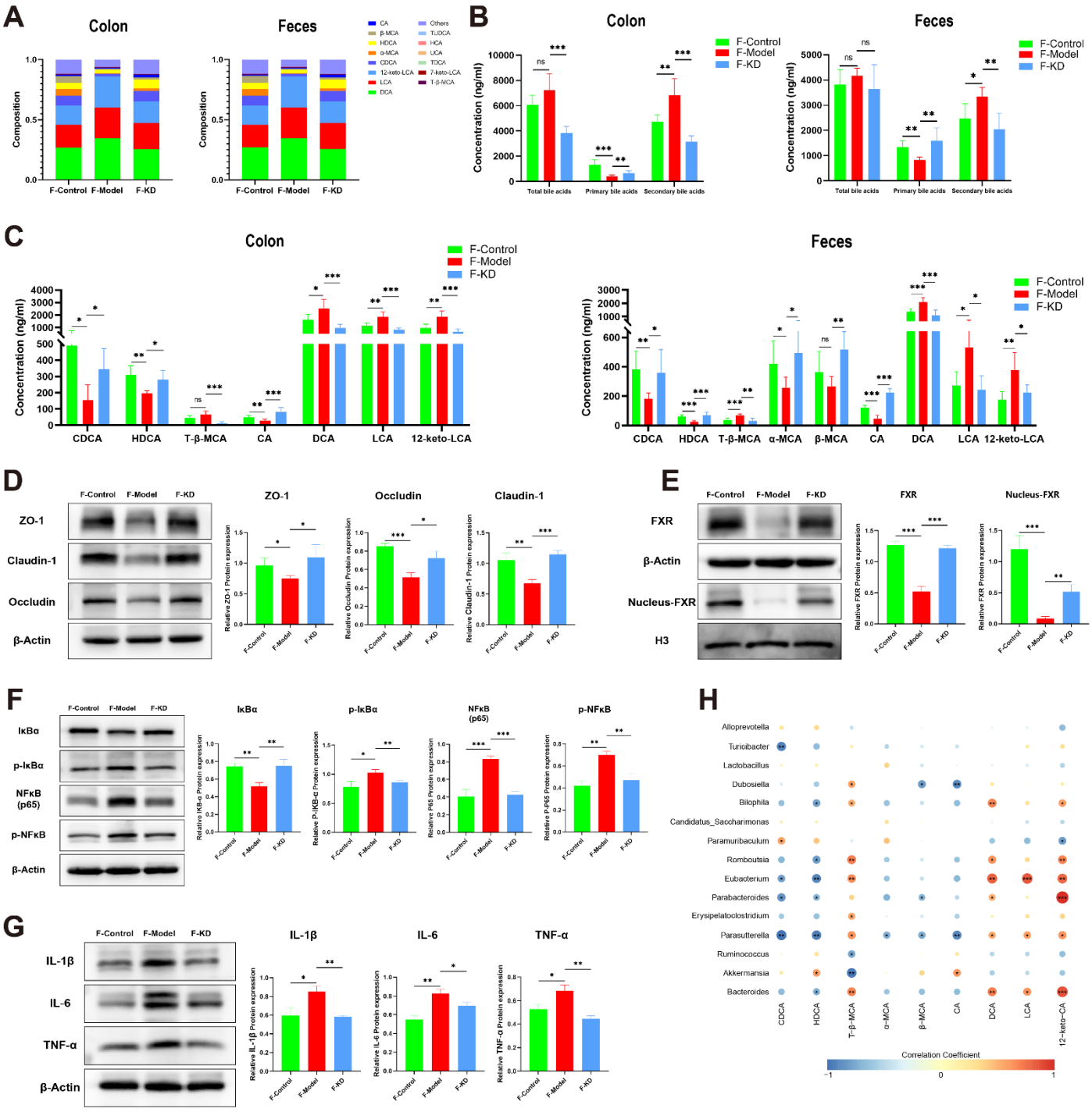


Figure 8. FMT-KD modulates bile acid and intestinal FXR/NF-κB signaling. (A) Measurement of major bile acids (B) concentrations of total bile acids, primary bile acids, and secondary bile acids (C) significantly altered bile acids in colorectal tissues and feces following fecal transplantation (n = 6). Representative immunoblots and relative expression levels of (D) Claudin-1, Occludin, and ZO-1 proteins (E) FXR and nuclear FXR (F) NF-κB activation-related proteins, including NF-κB, p-NF-κB, IκBα, and p-IκBα (G) proteins related to inflammatory factors, including IL-1β, IL-6, and TNF-α in colorectal tissues after fecal transplantation (n = 3). (H) Correlation between gut microbiota and bile acids described in feces after fecal transplantation. Data are presented as mean ± SD. Statistical analysis: unpaired t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. KD: ketogenic diet; FMT: fecal microbiota transplantation.

4. Discussion

Abnormal metabolism is a prominent feature observed in patients with colorectal cancer. Factors such as being overweight or obese, along with an unbalanced diet which characterized by insufficient intake of vegetables and fruits and a high frequency of processed and red meat consumption, contribute significantly to this condition[17-19]. Consequently, dietary modifications are regarded as crucial in the onset and progression of colorectal cancer[20]. Tumor growth is dependent on the nutrients supplied by the host, leading to a growing interest in adjusting dietary regimens to enhance cancer treatment outcomes. Currently, dietary approaches such as the Mediterranean diet, low-carbohydrate diet, and ketogenic diet have demonstrated both preventive and therapeutic benefits against cancer[7, 21-24]. However, the Mediterranean diet has not gained traction in China due to varying dietary customs and logistical challenges in food distribution.

The ketogenic diet, which is characterized by high fat, moderate protein, and very low carbohydrate content, may exert its anti-tumor effects by modulating metabolism, oxidative stress, and fatty acid oxidation. Compared to a high-fat diet, a ketogenic diet also contains a high level of fat. However, the high-fat diet has been shown to significantly promote colorectal cancer. The primary difference in their effects may stem from the low carbohydrate content. Research indicates that a high carbohydrate intake can lead to chronic inflammation in the body, triggering insulin resistance and altering the gut microbiome. In this study, the normal diet consisted of over 60% carbohydrates, and the ketogenic diet, despite containing 80% fat, included only 0.1% carbohydrates. While diet is intricately linked to gut microbiota, the potential of the ketogenic diet to influence gut microbiota in the context of colorectal cancer treatment remains uncertain.

Nonetheless, the preventive and therapeutic roles of the ketogenic diet in colorectal cancer are relatively well established[25-26]. To further explore the differential effects of varying concentrations of β-hydroxybutyric acid on colorectal cancer, we performed a metabolomic analysis of serum. Our results indicated that elevated levels of β-hydroxybutyric acid significantly disrupted lipid metabolic pathways, while the ketogenic diet group and high-dose ketogenic diet group primarily influenced bile acid synthesis pathways. Abnormal bile acid levels are recognized as a carcinogenic factor for colorectal cancer[27-29]. Previous studies have shown that a high-fat diet can elevate specific bile acids, such as T-β-MCA and DCA, by disrupting the balance of bile acids in the intestines, thereby inhibiting FXR activity and promoting the abnormal proliferation of intestinal stem cells, which can lead to colorectal cancer development[30]. It remains uncertain whether a ketogenic diet can modify bile acid metabolism. In our study, we observed alterations in bile acid composition, characterized by a reduction in primary bile acids and an increase in secondary bile acids in the serum of AOM/DSS mice. Following treatment with the ketogenic diet or 3-HB, we noted a shift in bile acid composition ratios, with an increase in the proportion of primary bile acids and a decrease in secondary bile acids. Given that most bile acids are retained in enterohepatic circulation, with only a small fraction entering peripheral circulation, we also evaluated bile acid concentrations in mouse intestines and feces. Consistent with serum findings, the ketogenic diet resulted in a reversal of bile acid proportions from primary to secondary in both intestinal tissues and feces.

Disruptions in bile acid metabolism can lead to a range of diseases, making it essential to maintain metabolic balance[31]. FXR, a ligand-dependent nuclear receptor, is activated by endogenous ligands such as bile acids. Among these, CDCA is the most potent activator, followed by CA and DCA, while T-β-MCA, T-α-MCA, and UDCA have been identified as natural antagonists of FXR. The liver and intestines exhibit the highest levels of FXR expression. Furthermore, FXR inhibits the activity of enzymes involved in bile acid production and serves as a negative regulator of bile acid synthesis. In our study, we observed a significant increase in the concentrations of CDCA and CA in serum, intestinal tissues, and feces following ketogenic diet treatment in AOM/DSS model mice, while levels of DCA and T-β-MCA were notably reduced. Subsequent analysis of FXR expression in colorectal cancer tissues revealed significant upregulation induced by the ketogenic diet. The secondary bile acid deoxycholic acid (DCA) is linked to intestinal carcinogenesis[30,32]. Treatment with DCA has been shown to alter gut microbiota composition in Apcmin/+ mice, and transferring fecal microbiota from DCA-treated mice to another group increased tumor multiplicity[33]. These changes led to inflammation and activated the Wnt/β-catenin signaling pathway associated with tumorigenesis, thereby affecting cell cycle regulation, apoptosis, and proliferation. Recent research has indicated that 7-keto LCA promotes epithelial repair by inhibiting signal transduction through the intestinal bile acid receptor FXR. It has been confirmed as an FXR antagonist, promoting Wnt signaling transduction and enhancing the self-renewal capacity of intestinal stem cells[34]. Our study revealed a significant decrease in 7-keto LCA levels in serum following ketogenic diet treatment, while levels of 12-keto LCA in colorectal tissues and feces were significantly reduced. Additionally, 12-keto LCA suppresses ILC3 secretion of the pro-inflammatory cytokine IL-17A by promoting VDR expression to mitigate colitis exacerbation[35]. However, its role in colorectal cancer remains unreported, necessitating further research to clarify its relationship with FXR.

FXR serves as the primary regulator of bile acid synthesis and excretion in both the liver and intestines. Studies have shown that FXR expression is generally diminished in colorectal cancer patients, leading to disrupted bile acid metabolism. The altered bile acid profile shapes distinct intestinal microbiota, promoting an increase in the abundance of toxicogenic bacteria, which contributes to carcinogenesis and multi-level inflammatory responses. Dysregulated bile acid metabolism also stimulates the production of secretory immunoglobulin A, thereby facilitating the occurrence and progression of colorectal cancer[14]. Comparisons between intestinal epithelial cells and colon polyps or adenocarcinomas reveal lower expression levels of nuclear FXR, contributing to the onset and progression of colorectal cancer[29, 36]. Research has suggested a connection between FXR and the Wnt/β-catenin signaling pathway. Downregulated FXR expression levels are associated with poor prognosis in colorectal cancer tissues[30]. Experimentally, suppressing FXR expression enhances the proliferation and invasive capabilities of colorectal cancer cells, while restoring FXR expression exerts a tumor-suppressive effect[37]. Aberrations in the Wnt/β-catenin signaling pathway significantly contribute to the pathogenesis of colorectal cancer, with 80% of patients exhibiting abnormal activity in this pathway. FXR effectively attenuates the formation of the β-catenin/TCF4 complex by antagonizing the Wnt/β-catenin signaling pathway, thereby exerting its tumor-suppressive effect[38].

Transcriptomic analysis was conducted to explore the mechanism of GW4064 as an FXR activator in colorectal cancer cell lines. Our findings indicate that GW4064 primarily modulates the Wnt pathway and NF-κB pathway. Given the relatively abundant literature on FXR-Wnt pathways in colorectal cancer, we further examined the effects on the FXR-NF-κB pathway axis based on previous evidence suggesting that a ketogenic diet could inhibit inflammatory responses. The results demonstrate that ketogenic diets can activate FXR expression through modulation of NF-κB metabolic pathways, leading to a reduction in inflammatory responses and inhibition of colorectal cancer progression.

The gut microbiota has been found to have a causal relationship with the development of colorectal cancer[39-40]. Studies have implicated harmful bacteria, including *Fusobacterium nucleatum*, *anaerobic streptococci*, and *Firmicutes*, in inducing tumor cell proliferation and promoting inflammation, thereby facilitating the progression of colorectal cancer. Conversely, beneficial bacteria such as *Lachnospira*, *bifidobacterium*, and *Streptococcus thermophilus* are often diminished in colorectal cancer cases[41]. While the ketogenic diet has shown potential in treating refractory epilepsy and Alzheimer's disease by altering gut microbiota, its efficacy in preventing or treating colorectal cancer through this mechanism remains uncertain[42-46]. Additionally, the microbial community significantly impacts the regulation of bile acid synthesis and metabolism in an FXR-dependent manner[8,47]. Gut bacteria can promote the conversion of primary bile acids into secondary bile acids through clostridium with 7α-dehydroxylation enzymes[48]. In our research, we observed that ketogenic diets have the potential to convert primary bile acids into secondary bile acids in colorectal cancer. Consequently, we conducted an analysis of its impact on gut microbiota. The findings indicated a reduction in the abundance of clostridium with 7α-dehydroxylation function following ketogenic diet treatment, leading to decreased production of secondary bile acids from primary bile acids. Additionally, there was an observed increase in *Muribaculaceae*, *Akkermansia*, and *Lachnospiraceae NK4A136*, while *Parasutterella* decreased significantly in the ketogenic diet group.All of these gut microbiota play crucial roles in colorectal cancer development[48-52]. To validate the involvement of gut microbiota, fecal transplantation from ketogenic diet-treated mice was administered to PGF models. The abundances of *Muribaculaceae*, *Akkermansia*, and *Lachnospiraceae NK4A136* were significantly higher, while *Parasutterella* remained lower in the F-KD group compared to the F-model group. Furthermore, the alterations in FXR and NF-κB expression, primary and secondary bile acid composition, as well as the levels of CA, CDCA, DCA, and 12-keto LCA in colorectal tissues or feces were consistent with those treated with the ketogenic diet.

In conclusion, the ketogenic diet exerts both preventive and therapeutic effects on colorectal cancer by reducing inflammatory responses and restoring intestinal barrier function. Following treatment with the ketogenic diet, bile acid metabolism was reprogrammed. The ketogenic diet suppresses colorectal cancer through reshaping gut microbiota and mitigating inflammatory responses via intestinal FXR/NF-κB signaling pathway. Our results may provide further evidence from the perspective of tumor metabolism for the ketogenic diet as a novel therapeutic strategy for early intervention in colorectal cancer (Fig.9).

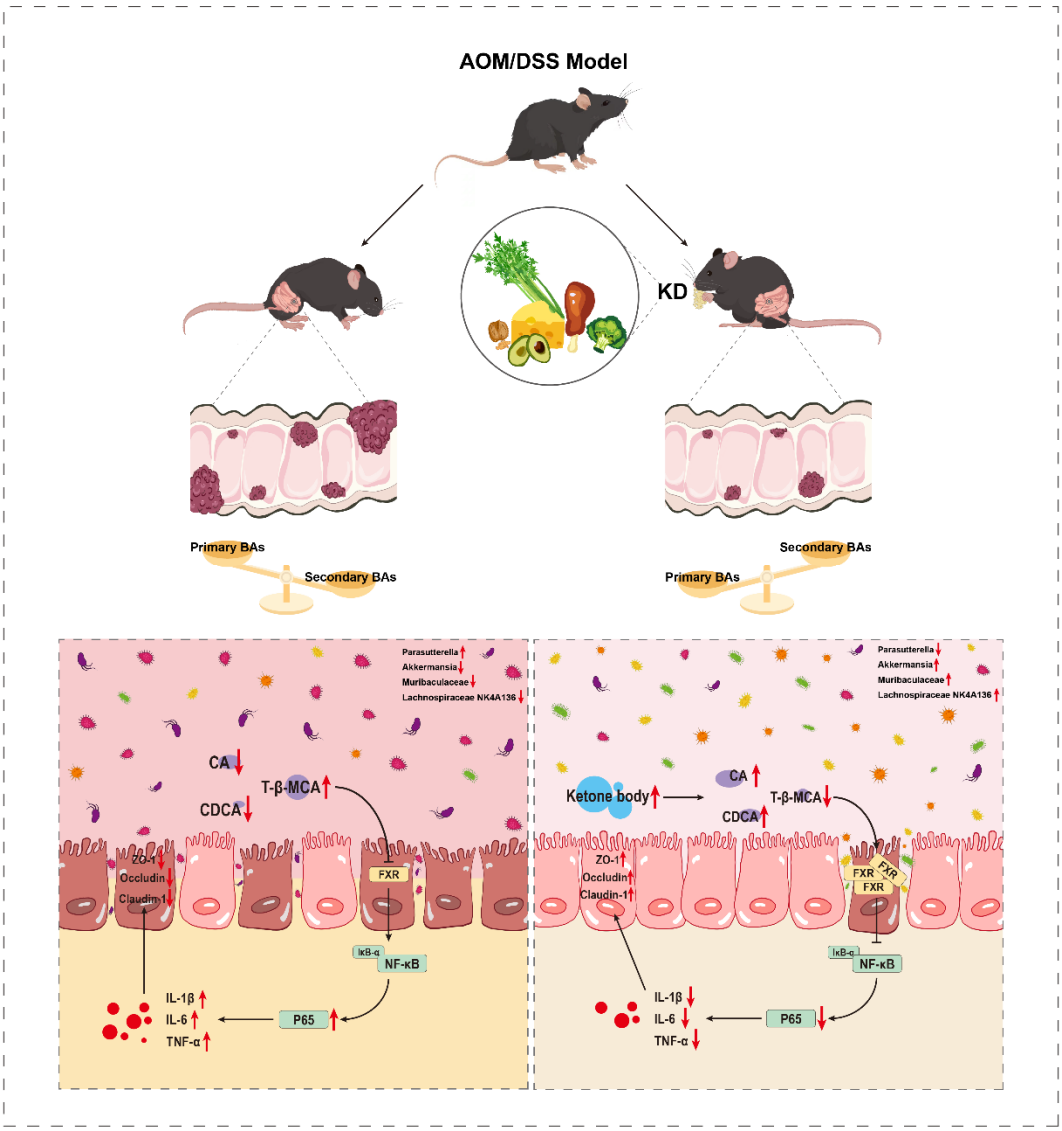


Figure 9. Schematic summary for the proposed Ketogenic diet suppresses colorectal cancer reshaping gut microbiota and modulating the intestinal FXR/NF-κB signaling pathway.

Consent for publication

All authors have approved submission of the manuscript to this journal.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. The accession number for the entire 16S rDNA sequencing dataset reported in this manuscript is CNCB CRA017241.

Competing interests

The authors declare that they have no competing interests.

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