Inhibition of the Occurrence and Development of Inflammation-Related Colorectal Cancer by Fucoidan Extracted from Sargassum fusiforme

Xiang Li,‡ Shijun Xin,‡ Xiaojun Zheng,‡ Liqin Lou, Shiqing Ye, Shengkai Li, Qilong Wu, Qingyong Ding, Ling Ji, Chunrong Nan, and Yongliang Lou*

ABSTRACT: Fucoidan has many biological activities, including the inhibitory effect on the development of various cancer types. This study showed that lipopolysaccharide-induced inflammation in FHC cells (normal human colonic epithelial cells) could be reversed using fucoidan at different concentrations. The fucoidan-induced anti-inflammatory effect was also confirmed through in vivo experiments in mice. Compared to the mice of the model group, the ratio of Firmicutes/Bacteroidetes in feces increased and the diversity of gut microbial composition was restored in mice after fucoidan intervention. In colorectal cancer (CRC) cells DLD-1 and SW480, fucoidan inhibited cell proliferation and promoted cell apoptosis. It also blocked the cell cycle of DLD-1 and SW480 at the G0/G1 phase. The animal model of inflammation-related CRC showed that the incidence of tumors in mice was significantly reduced by fucoidan intervention. Furthermore, the administration of fucoidan decreased the expression levels of inflammatory markers such as TNF-α IL-6 and IL-1β in the colonic tissues. Therefore, fucoidan can effectively prevent the development of colitis-associated CRC.

KEYWORDS: fucoidan, colorectal cancer, colitis, intestinal microecology

INTRODUCTION

The International Agency for Research on Cancer (IARC) has shown that colorectal cancer (CRC) has the highest incidence and the second-highest mortality among all cancer types worldwide in 2020.1 In fact, it accounted for 10% of newly diagnosed cancer cases and 9.4% cancer-related deaths worldwide in 2020, making it the second most common cancer of the year. The CRC has complex causes, including factors such as genetics, diet, and inflammation.2,3 Despite the increasing diversity of clinical approaches for the treatment of tumors,4,5 the prognosis of patients with CRC remains unsatisfactory. Therefore, novel therapeutic approaches need to be developed urgently for improving the prognosis of patients with CRC.6

Currently, inflammatory bowel disease-associated colorectal cancer (IBD-CRC) is recognized as the most severe condition that can give rise to IBD-related complications.7 Many reports have suggested that prolonged intestinal inflammation greatly increases the risk of developing CRC.8,9 Patients with inflammation-associated CRC tend to have worse outcomes.10 Progression from colitis to CRC is a continuous dynamic process and is often accompanied by dysbiosis and a compromised gut barrier.11,12

The intestinal microflora is a highly complicated community comprising mainly of bacteria, viruses, fungi, and archaea.13 The rich and diverse intestinal microbes maintain our health in a dynamic balance. As the intestine is the most dominant organ inhabited by human flora, the development of several enteropathies is inextricably linked to microorganisms.14

There is growing evidence that a dysbiosis of the intestinal microflora can trigger and promote diseases mediated by chronic inflammation, including CRC.15—17 Bacteroides fragilis and Fusobacterium nucleatum are the key bacteria responsible for colorectal carcinogenesis.18 In patients with CRC, the ratio of Firmicutes/Bacteroidetes in the stool is significantly higher than that of controls.19 In addition, the bacterial metabolites, including short-chain fatty acids, are essential for protecting the gut mucosal barrier and inhibiting intestinal inflammation.20

Sargassum fusiforme is perennial temperate seaweed and is mainly found in the southeast coast of China, the Yellow Sea, as well as the neighboring waters. It belongs to the class of brown seaweed (Phaeophyceae) and has unique nutritional and medicinal values.21 It is a major source of dietary fibers and polysaccharides, including fucoidan, alginic acid, and laminaran.21 Fucoidan is a type of sulfated polysaccharide that is mainly extracted from marine brown algae. It is the most potent pharmacological component of seaweed such as S. fusiforme.22 Fucoidan is noncytotoxic to normal cell lines such as 293T and FHC (normal human colonic epithelial cells).23,24 However, it is cytotoxic to various cancer cells because it inhibits their...
proliferation and blocks their cell cycles. In addition, fucoidan is involved in stabilizing the composition of the microbial community in the gut of mice. However, not much is known about the mechanism through which fucoidan inhibits the proliferation of cancerous cells. At present, only a few studies have explored the function and role of fucoidan obtained from S. fusiforme in cancer. In addition, no studies so far have investigated the effect of fucoidan extracted from S. fusiforme on CRC. This study is an attempt to fill in this gap.

**MATERIALS AND METHODS**

**Fucoidan Preparation.** After reflux treatment with 90% ethanol, the S. fusiforme powder was dissolved with water at 70 °C for 2 h (material to water ratio of 1:10). The filter residue was extracted by centrifugation at 3000 rpm for 15 min. The above steps were performed twice. All of the supernatant was collected and filtered. The filtrate was filtered and washed with ethanol to a concentration of 75% after ultrafiltration and then freeze-dried. The dried powder was dissolved into a 2% aqueous solution. Next, HCl was added to achieve a solution of pH 2.0. The filtrate was neutralized with alkali and ethanol was added to 75%. The resulting precipitate was dissolved with acetone and ethanol after filtration and then dried at 40 °C to obtain fucoidan. The total fucoidan assay of the sample was 98.1% fucoidan, with 22% of L-fucose. Barium sulfate turbidimetric analysis revealed the presence of 22.8% of organic sulfates in the fucoidan sample.

**Animal Studies.** Male BALB/c mice with an age of 6–8 weeks (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were fed at the experimental animal center of Wenzhou Medical University. All animal studies were conducted in compliance with the animal experiment guidelines of Wenzhou Medical University. The protocols were approved by the Animal Experimental Ethics Committee (wydw2019-0944).

The animals were divided into three groups by randomization, with six mice in each group. The mice used in the colitis experiment were fed adaptively for a week. Mice in the model group and the treatment group were fed with 4% dextran sulfate sodium salt (DSS, w/v; Aladdin, Shanghai, China) for 7 days. The treatment group was additionally treated with fucoidan at 1 g kg⁻¹ per day. The weight, episodes of diarrhea, bloody stool, and other conditions of the mice were recorded every day. Table 1 shows the criteria of the disease activity index (DAI).

| weight loss (%) | stool consistency | visible blood in feces | score |
|-----------------|-------------------|------------------------|-------|
| none            | normal stool      | none                   | 0     |
| 1–5             | loose stool       | slightly bleeding      | 2     |
| 10–20           | diarrhea          | gross bleeding         | 4     |

After adaptation, mice in both the model and treatment groups were administered with azoxymethane (AOM; 12 mg kg⁻¹; Sigma) through intraperitoneal injection. On the 8th day after AOM injection, the model and treatment groups were fed with 2.5% DSS for 7 days and then given normal water for the next 7 days, in a cycle of three. Mice in the control group were provided with normal water throughout the experiment. After 12 weeks, the mice were euthanized and weighed, and their appearance was noted. The tumor-bearing colorectal tissues of the mice were fixed with 4% paraformaldehyde. The remaining colorectal tissues were filtered and washed with ethanol to a concentration of 75% after centrifugation at 3000 rpm for 15 min. The above steps were performed twice. All of the supernatant was collected and filtered. The filtrate was neutralized with alkali and ethanol was added to 75%. The resulting precipitate was dissolved with acetone and then harvested and resuspended with 1°:10. The filter residue was extracted by ethanol after filtration and then dried at 40 °C for 30 min. Then, the cells were stained with crystal violet (Beyotime, Nanjing, China) for 20 min, washed with sterile water, and then dried in air. The number of cell colonies formed was counted manually.

**Cell Apoptosis Analysis.** The cultured cells were incubated with fucoidan and then harvested and resuspended with 1x binding buffer. Next, they were dyed with 10 μL of 7-AAD and 5 μL of AnnexinV-PE and incubated in the dark for 5 min at room temperature. Flow cytometry was used to perform cell apoptosis analysis. Finally, the apoptosis rate was evaluated by Flow J software.

**Cell Cycle Analysis.** Cells treated with fucoidan at different concentrations were resuspended with 500 μL of 1x RNase A solution. Next, 5 μL of PI was added. 1x RNase A solution and PI were mixed and the cells were stained for 30 min in the dark. Flow cytometry and CytExpert were used for cell cycle analysis.

**Western Blot.** Bicinchoninic acid (BCA) protein assay was conducted to analyze the total protein concentrations. After sodium dodecyl sulfate polyacrylamide gel electrophoresis, the samples were transferred to the poly(vinylidene difluoride) membranes. Skim milk was used for incubation with specific primary antibodies (1:1000 dilution) at 4 °C overnight. The antibodies included cleaved-caspase3 (Diabio, Hangzhou, China), cleaved-PARP (Diabio, Hangzhou, China), Cdk2 (Diabio, Hangzhou, China), Cyt-c (Diabio, Hangzhou, China), p21 (Diabio, Hangzhou, China), cyclinE1 (Huabio, Hangzhou, China), total STAT3 (s-STAT3; Diabio, Hangzhou, China), phosphorylated STAT3 (p-STAT3; Diabio, Hangzhou, China), β-actin (Abways, Beijing, China), and GAPDH (Abways, Beijing, China). The samples were incubated with secondary antibodies (1:2000 dilution) (Beyotime, Nanjing, China). The Bio-Rad gel imaging system was photographed, and ImageJ software was used for system analysis.

**RT-qPCR.** Trizol was used for the extraction of total RNA. cDNA was generated by reverse transcription. The prepared polymerase chain reaction (PCR) solution and cDNA were placed in PCR 8-strip tubes for conducting RT-qPCR. The primer sequences are shown in Tables 2 and 3. The reaction conditions were as follows: Stage 1: 95 °C for 30 s, Stage 2: 95 °C for 5 s, 60 °C for 60 s, with a total of 40 cycles; and Stage 3: Dissociation.

| prime name (cell) | prime sequence |
|-------------------|----------------|
| TNF-α             | F:5′-ATGGTGTGACACACCTCAGACG-3′ |
|                   | R:5′-GTTGGTTGAGGACACTAC-3′ |
| IL-6              | F:5′-CAATGGAGGAGGACTGCTCTGGT-3′ |
|                   | R:5′-GGTTGTTGACGGGAGGTGTTA-3′ |
| IL-1β             | F:5′-CAGACGCTCTGCTTGGATG-3′ |
|                   | R:5′-GGCTTGAGGCAGCTCTGCT-3′ |
| IL-8              | F:5′-TGGCCAGAGGGTCTAAGAGA-3′ |
|                   | R:5′-GCCCTTCTTCACATTTCTC-3′ |
| GAPDH             | F:5′-TGAGCTCCAGGACTCTCACG-3′ |
|                   | R:5′-ACAGTCTTCCATATGGATC-3′ |
ELISA. First, 10 mg of the colonic tissues of mice was ground with 100 μL of PBS. The supernatant was retained. The IL-6, TNF-α, IL-1β, and iNOS levels were detected by ELISA kits (qzkndbio, Quanzhou, China). The instructions were strictly followed.

**Histologic Analysis.** The colorectal tissues were first fixed in 4% paraformaldehyde for 24 h and then embedded in the paraffin. The wax blocks were cut into 5-μm-thick slices, which were then stained with eosin and hematoxylin (Beyotime, Nanjing, China). The general morphological changes were observed under the microscope. The content of mucin was evaluated under the microscope by staining with nuclear fast red and Alcian blue (pH 2.5) (Beyotime, Nanjing, China).

**16S rDNA Sequencing.** The total genomic DNA of mice was obtained from their cecal contents. The genes of 16S/18S rRNA were amplified using the specific primer with the barcode. All PCRs steps were conducted in the reaction media (30 μL) containing 15 μL of High-Fidelity PCR Master Mix (New England Biolabs). The following thermocycling conditions were used: prededegeneration at 98 °C for 1

### Table 3. Prime Sequence (Animal)

| prime name (animal) | prime sequence               |
|---------------------|------------------------------|
| TNF-α               | F:5′-CTCCAGGCCGGTGCCTATG-3′  |
|                     | R:5′-GGGCCATAGAAGTGAAGG-3′   |
| IL-6                | F:5′-GCTACAAACTGGATATATACGA-3′|
|                     | R:5′-CAGATGCTATGGTACCTGGA-3′|
| IL-1β               | F:5′-TCACAGGAGCACTCAAAC-3′   |
|                     | R:5′-TGCTGCTACCTGGAAGGT-3′   |
| iNOS                | F:5′-CACCAAGCTGACTTGAAGCC-3′ |
|                     | R:5′-CGTTGCTTGGTCCTGCTC-3′   |
| β-actin             | F:5′-GGCTTGGATTCCTCCTG-3′    |
|                     | R:5′-CCAGTTCTGGAACATGCGAT-3′|

**Figure 1.** Fucoidan is not cytotoxic to FHC cells and relieves LPS-induced inflammation of FHC cells. (A) MTT results after fucoidan treatment of FHC. (B) RT-qPCR results of inflammatory factors after treatment with LPS-induced FHC by fucoidan. (C, D) WB results and statistical analysis of inflammatory factors after treatment with LPS-induced FHC by fucoidan. *P < 0.05, **P < 0.01, ***P < 0.001, ns P > 0.05 vs Control.
min, denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 7 °C for 30 s, with a total of 30 cycles; the final elongation step was carried out at 72 °C for 5 min. The PCR products were mixed and purified for quantification and identification. The sequencing libraries were generated by a TIANSeq Fast DNA Library Prep Kit (Illumina, TIANGEN Biotech, China). The library quality was evaluated on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the sequencing of the constructed library was performed on the Illumina platform using the 2 × 250 bp paired-end protocol and data analysis was conducted.

Statistical Analysis. The above experiment was performed at least thrice. The GraphPad Prism7.0 software was used for graphing and statistical analysis. Apoptosis detection was completed using the BD FACS Aria II flow cytometer, and data were analyzed by Treestar Flowjo 10.0 software. Cell cycle detection and data analysis were conducted by a CytExpert 2.3 flow cytometer. All variables were presented as mean ± SD. The Kruskal–Wallis test was used for analyzing microbiological and physiological indicators. Other statistics were performed by Student’s t-test. The significant statistical difference was accepted at $P < 0.05$.

**RESULTS**

**Cytotoxicity of Fucoidan to FHC.** To investigate what kind of cytotoxic effect fucoidan exerts on normal colonic cells, we first treated normal colonic epithelial cells, FHC, with 0, 50, 100, 250, 500, 750, and 1000 μg mL$^{-1}$ of fucoidan and examined their survival rate after 24, 48, and 72 h of fucoidan treatment by
MTT proliferation assay. There was no significant difference between the survival rate of the FHC cells treated with different concentrations of fucoidan and those treated without fucoidan at different treatment times (Figure 1A). Hence, it can be suggested that fucoidan did not exert any cytotoxic effect on FHC and it could be used for subsequent experiments.

**Fucoidan Relieves LPS-Induced Inflammation of FHC Cells.** We used 20 μg mL⁻¹ of LPS to induce inflammation in the FHC. Next, the cells were treated with fucoidan at different concentrations. The RT-qPCR results showed that the reexpression levels of IL-8, IL-6, TNF-α, and IL-1β in LPS-stimulated FHC significantly increased. The inflammatory factor expression of FHC administered with 50 μg mL⁻¹ of fucoidan showed a downward trend; however, the difference was not statistically significant (Figure 1B). When 100 μg mL⁻¹ of fucoidan was used, the expression of inflammatory factors in the FHC cells showed a significant decrease (Figure 1B). Meanwhile, WB results revealed that the p-STAT3 levels and the levels of cellular inflammatory factors, including IL-6, significantly increased in FHC cells under LPS stimulation. However, their expression levels reduced a little after fucoidan intervention (Figure 1C,D). Hence, fucoidan can alleviate the LPS-induced inflammation of FHC cells.

**Fucoidan Relieves DSS-Induced Colitis in Mice.** Cellular-level studies showed that fucoidan has an anti-inflammatory effect on FHC. This result prompted us to determine whether fucoidan exerts an inhibitory effect on the generation and progression of mice colitis. The treatment process for mice is shown in Figure 2A, in which we also referred to previous modeling approaches and the concentration of DSS used.⁵⁰ ⁵¹ A significant decrease in body weight and the presence of diarrhea and bloody stools in mice were detected when 4% DSS drinking water was used (Figure 2B,C,E). We scored the mice with DAI for weight loss, diarrhea, and feces containing blood. The results demonstrated a significant increase in the scores of mice with colitis (Figure 2D). However, the mice showed some relief from weight loss, diarrhea, and blood in stool with fucoidan intervention (Figure 2B,C,E). At the same time,
DAI scores significantly reduced (Figure 2D). In Figure 2F, we can see that the cecum of the mice with colitis atrophied. We measured the colorectal length from the lower end of the cecum to the anus and found that the colorectal length was significantly reduced in colitis mice (Figure 2G). However, fucoidan intervention normalized the cecum size and restored the colorectal length.

**Fucoidan Relieves DSS-Induced Colonic Inflammation and Intestinal Barrier Damage in Mice.** To verify the role of fucoidan in affecting the expression of inflammatory factors in the intestinal tissues of mice with colitis, we detected the mRNA levels of inflammatory factors in the colonic tissues of mice in each group. The results are shown in Figure 3A. The daily drinking water containing 4% DSS caused a significant elevation in the relative expression of IL-1β, IL-6, and iNOS mRNA in the colonic tissues of mice. In addition, a rising trend of TNF-α was noted, although no statistical difference was detected. To further explore changes in the protein levels of the abovementioned inflammatory factors, we conducted ELISA experiments. The results were consistent with the gene expression levels. The protein levels of IL-1β, IL-6, TNF-α, and iNOS were significantly higher in the colonic tissues in the colitis model group than that obtained in the control group. The fucoidan intervention can effectively inhibit the expression of protein levels of the abovementioned inflammatory factors (Figure 3B). To further assess the intestinal tissue morphology and intestinal inflammation, we performed the HE staining of colonic tissues.

The results are shown in Figure 3C. The structure of the colonic tissue of mice with colitis was significantly damaged, as shown by the abnormal crypt structure, damaged epithelial cells, and inflammatory cell infiltration. However, fucoidan intervention significantly alleviated the damage in mice. Figure 3D shows changes in the intestinal mucus in the mice of different groups. Alcian blue staining showed the formation of a significantly large lesion of the mucus layer in the colon of the mice of the model group. When fucoidan was supplied to these mice, their intestinal mucus layer was maintained in a more normal state.

**Fucoidan Changes the Composition of Gut Microbes in Colitis Mice.** To assess the change in the intestinal internal flora composition in mice with colitis under fucoidan intervention, we analyzed the cecum content of each group of mice by 16S rDNA sequencing. The results showed that ACE and Chao1 indices, which indicate the abundance of microflora communities, decreased significantly in the mice of the model group. Similarly, Shannon and Simpson indices, which represent the diversity of microbial communities, also decreased significantly in the mice of the model group (Figure 4A). The presence of an inflammatory environment reduced both the diversity and abundance of the microflora environment of the mouse gut. However, the fucoidan intervention reversed this decline. In addition, the β-diversity analysis showed a significant difference in the composition of the mouse gut community among the three groups, with more differences between groups than within groups (Figure 4C). PCOA analysis revealed a
difference in the microflora composition of the three groups of mice (Figure 4B). Cluster analysis showed that the control groups were clustered separately from the model and treatment groups, which were clustered together (Figure 4D). Relative abundance composition analysis demonstrated a significant decline in the ratio of Firmicutes/Bacteroidetes in mice of the model group; however, this ratio rebounded in the mice treated with fucoidan (Figure 5). These results suggested that fucoidan may play a role in maintaining the intestinal microecology in mice with colitis.

Figure 5. Fucoidan changes the relative abundance composition of the mouse gut induced by DSS. C: Control; D: 4% DSS Model; F: FUC Treatment. Relative abundance composition at the (A) phylum level, (B) family level, and (C) genus level.

Figure 6. Fucoidan inhibits colony formation and arrests the DLD-1 and SW480 cell cycle. (A) Results of DLD-1 and SW480 colony-formation experiment. (B) Statistical analysis of the colony-formation experiment. (C) Flow cytometry results of DLD-1 and SW480. (D) Statistical analysis of flow cytometry results. (E) Western blot results of DLD-1 and SW480 cycle-related proteins. (F) Statistical analysis of western blot results of DLD-1 and SW480 cycle-related proteins. *P < 0.05, **P < 0.01, ***P < 0.001.
To investigate the effects of fucoidan at different levels on the proliferation capabilities of CRC cells (SW480 and DLD-1), we performed colony-formation experiments. As shown in Figure 6A, the number of colonies formed in the SW480 and DLD-1 groups under different fucoidan concentrations was significantly reduced compared to the control group. The results suggest that fucoidan has a growth inhibitory effect on CRC cells.

**Figure 7.** Fucoidan promotes the apoptosis of DLD-1 and SW480 cells. (A) Flow cytometry apoptosis results of DLD-1 and SW480. (B) WB results of DLD-1 and SW480 apoptosis-related proteins. (C) Statistical analysis of WB results of DLD-1 and SW480 apoptosis-related proteins. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 8. Fucoidan inhibits the occurrence and development of colorectal cancer. (A) Diagram of the animal study design. (B) Appearance of mice in the control group, model group, and treatment group, respectively. (C) Colorectal tissues of mice in the control, model, and treatment groups, respectively. (D) Weight of mice in the control, model, and treatment groups, respectively. (E) Survival curve of the control, model, and treatment groups, respectively. (F) Number of tumors in the mice of the control, model, and treatment groups, respectively. (G) HE staining results of mice rectum tissue. *$P < 0.05$ vs Control, **$P < 0.01$ vs Control, ***$P < 0.001$ vs Control; †$P < 0.05$ vs Model, ‡$P < 0.01$ vs Model.
DLD-1 cells treated with different levels of fucoidan decreased in a dose-dependent manner (Figure 6B). To further explore the influence of fucoidan on the cell cycle of DLD-1 and SW480-human CRC, fucoidan at a concentration of 250 μg mL⁻¹ was used to treat the cells. The results are shown in Figure 6C,D. The DLD-1 and SW480 cells were arrested at the G0/G1 phase by fucoidan. Next, we used western blotting (WB) to measure the expression of related proteins in the signal pathways involved in the cell cycle. Figure 6E,F shows that the expression of Cdk2 and cyclinE1 proteins in DLD-1 and SW480 cells decreased, while that of p21 proteins increased in a dose-dependent manner. Hence, fucoidan can enhance the expression of p21, inhibit that of Cdk2 and cyclinE1, and block the cycle of SW480 and DLD-1.

**Fucoidan Promotes Apoptosis of SW480 and DLD-1 Cells.** To further explore the influence of fucoidan on CRC cell apoptosis, we used flow cytometry to measure the occurrence of apoptosis at different concentrations of fucoidan. The results showed that fucoidan can promote the apoptosis of both SW480 and DLD-1 cells (Figure 7A). To further study the molecular mechanism through which fucoidan promotes this apoptosis, we detected relevant proteins in the apoptosis pathway by WB. The WB results showed that the proteins related to the apoptosis (cleaved-caspase3, cleaved-PARP, and Cyt-c) of DLD-1 and SW480 cells increased with an increase in the fucoidan concentration (Figure 7B); the difference was statistically significant (Figure 7C). Hence, fucoidan promotes the apoptosis of SW480 and DLD-1 cells.

**Fucoidan Inhibits the Occurrence and Development of CRC In Vivo.** To assess the effect of fucoidan on the inhibition of colorectal tumor formation in vivo, we established a CRC mouse model using the classical CRC modeling approach (Figure 8A). At the same time, gavage intervention with fucoidan was performed on the treated group. Figure 8B shows the difference in the appearance of the mice in each group. Compared to the mice in the control group, the mice in the model group were thin and had poorer hair color; however, this poor condition significantly improved in the fucoidan-intervention group. As shown in Figure 8D, the DSS intervention significantly decreased the body weight in the model group mice, while the fucoidan intervention reversed this trend. During the entire experimental period, the model group showed the phenomenon of individual mice death. In addition, the survival rate decreased in the model group, whereas no mice died in the treatment group (Figure 8E). Assessment of tumor formation in the colorectal tissues of mice showed a significant decline in the number of tumors formed in the fucoidan-intervention group (Figure 8C,F). To further confirm the tumor, we performed a histological evaluation of the rectal tissues of mice. The results of HE staining are shown in Figure 8G. The control mice had normal morphology of tissues, while the rectal tissues of the mice in the model group were structurally disorganized with obvious epithelial hyperplasia. Infiltration by a large number of inflammatory cells and lymph node hyperplasia were also visible. In contrast, the tissues under fucoidan intervention showed no significant hyperplasia, and the infiltration of inflammatory cells and lymph node hyperplasia were significantly alleviated. Next, we performed further validation to confirm the expression of the proteins related to the cell cycle and inflammation-related function in the intestinal tissue of mice in each group. The results of RT-qPCR demonstrated that the relative expression of inflammatory factors significantly increased in the mice of the model group, whereas it decreased in the fucoidan-treated mice (Figure 9A). The results of WB revealed that the levels of inflammation-related proteins (p-STAT3, TNF-α, IL-1β, and IL-6) and cycle-related proteins (Cdk2 and cyclinE1) showed an upward trend in the mice of the model group. Compared with the mice in the model group, the expression of these proteins in the mice of the treatment group significantly decreased (Figure 9B,C). Hence, fucoidan plays an essential role in inhibiting inflammation-related tumors.

**DISCUSSION**

Intestinal inflammation is a key risk factor for the progression of enterocolitis to CRC. In addition, intestinal microbial dysbiosis and impaired intestinal barrier are key to this inflammatory...
expression levels of related inflammatory factors in the intestinal barrier, a mechanical barrier, an immune barrier, and a biological one, both in vitro state of colitis mice (Figure 2) and downregulated the cytokotoxic effect of fucoidan on FHC cells (Figure 1B) but also alleviated the pathological phenomenon. We found that in mouse tissues affected by CRC, the relative expression of the p-STAT3 protein, which mediates the STAT3-mediated pathway is closely associated with the expression of inflammatory factors IL-6, IL-1α, and TNF-α significantly increased in the colorectum of mice (Figure 8). The levels of highly expressed cycle-related proteins in the tissues of mice in the model group also significantly decreased because of the intervention of fucoidan (Figure 9B,C). Thus, it is evident that S. fusiforme, a source of fucoidan, can inhibit tumor cell proliferation and angiogenesis, in addition to playing an essential role in the inhibition of metastasis of tumor cells. We also noted an interesting phenomenon. We found that in mouse tissues affected by CRC, the relative expression of the p-STAT3 protein, which mediates inflammation, and the relative mRNA expression of inflammatory factors IL-6, IL-1α, and TNF-α significantly increased in the model mice. The fucoidan intervention resulted in a significant regression of the expression of the abovementioned inflammation-related proteins and inflammatory factors (Figure 9A). The STAT3-mediated pathway is closely associated with the functions of apoptosis and proliferation of cancer cells.

The evidence from our study is also sufficient to make the following reasonable speculation: fucoidan obtained from S. fusiforme may exert a series of tumor-suppressing effects through the STAT3 pathway.

Although the diverse anticancer properties of fucoidan increase its chances of producing increasingly more valuable clinical effects in future trials, it remains somewhat uncertain in light of the current research results. For instance, fucoidan extracted from Fucus vesiculosus can arrest the cell cycle of HT29 at the G0/G1 phase by downregulating the protein expression of Cdk4. However, this is different from the arresting of the G0/
G1 phase in SW480 and DLD-1 cells, which resulted from the downregulation of the protein expression of Cdk2 by fucoidan. This phenomenon may occur because of the following facts.

(1) The source of our fucoidan is different from that used in other research studies. In addition, S. fusiforme of different origins may also have differences in its nutritional components.

(2) There are huge differences in the effective components owing to the use of different extraction processes.

(3) The cells that fucoidan acts on are different.

Therefore, we believe that the mechanism of fucoidan obtained from different sources using different processes on cancer cells may also be different.

In conclusion, we not only clarified the functional mechanism through which fucoidan inhibits CRC in vitro but also successfully corroborated it in vivo experiments. This study confirmed that fucoidan extracted from S. fusiforme could inhibit the development of CRC through inhibiting the process of colitis, promoting the apoptosis of tumor cells, and blocking the cycle of cancer cells.

■ AUTHOR INFORMATION

Corresponding Author
Yongliang Lou — Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; Colorectal Cancer Research Center, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; Phone: +86 577 86699652; Email: louyongliang2013@163.com

Authors
Xiang Li — Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; Colorectal Cancer Research Center, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Shiqing Ye — Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; Colorectal Cancer Research Center, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Shengkai Li — Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; Colorectal Cancer Research Center, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Qilong Wu — Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; Colorectal Cancer Research Center, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Qingyong Ding — Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; Colorectal Cancer Research Center, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Ling Ji — Colorectal Cancer Research Center, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China; The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Chunrong Nan — Wenzhou Key Laboratory of Sanitary Microbiology, Key Laboratory of Laboratory Medicine, Ministry of Education, China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035 Zhejiang, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.2c02357

Author Contributions
X.L., S.X., and X.Z. contributed equally to this work. Study concept and design: X.L., Y.L., and L.J. Cell studies: L.J., C.N., S.X., and X.Z. Analysis and interpretation of data: S.X., L.L., S.L., and Q.D. Manuscript draft: S.X., X.L., and Y.L.

Notes
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ABBREVIATIONS
AOM, azoxymethane; BCA, bicinchoninic acid; DAI, disease activity index; CRC, colorectal cancer; DMEM, Dulbecco’s Modified Eagle Medium; DMSO, dimethyl sulfoxide; DSS, dextran sulfate sodium; FBS, fetal bovine serum; IBD, inflammatory bowel disease; LEfSe, linear discriminant analysis
effect size; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrizolium bromide; PChOA, principal coordinates analysis; PCR, polymerase chain reactions; p-STAT3, phosphorylated STAT3; RNI, reactive nitrogen intermediates; ROS, reactive oxygen species; WB, western blot

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