ORIGINAL RESEARCH

Lactobacillus fermentum ZS09 Mediates Epithelial–Mesenchymal Transition (EMT) by Regulating the Transcriptional Activity of the Wnt/β-Catenin Signalling Pathway to Inhibit Colon Cancer Activity

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Objective: The epithelial–mesenchymal transition (EMT) pathway can mediate tumour migration, and the occurrence of EMT is closely related to the Wnt/β-catenin signalling pathway. The purpose of this paper was to study the effect of *Lactobacillus fermentum* ZS09 (*L. fermentum* ZS09) on the EMT pathway in mouse with azoxymethane/dextran sulfate sodium salt (AOM/DSS) induced colon cancer and the potential underlying mechanism.

Materials and Methods: In this study, a mouse colon cancer model was established through intraperitoneal injection of 10 mg/kg azoxymethane (AOM) and three cycles of 2.5% dextran sulfate sodium salt (DSS) in the drinking water. H&E staining, enzyme-linked immunosorbent assay (ELISA), real-time fluorescent quantitative PCR (RT-qPCR) and Western blotting (WB) were used to study the antitumour mechanisms of *L. fermentum* ZS09 through the EMT pathway.

Results: The results of this study showed that compared with the model group, the high-dose *L. fermentum* ZS09 intervention group exhibited decreased serum levels of MMP-9, TNF-α, IL-6R, Ang-2 and VEGFR-2 and increased contents of DKK1 (P<0.05). The expression of Wnt/β-catenin signalling pathway-related genes (*Dv1, GSK-3β, β-catenin, c-myc, cyclinD1, Vim*, and *MMP-9*) was significantly reduced, and the gene expression levels of *APC, CDH1*, and *Axin* were increased. The levels of related proteins (β-catenin, N-cadherin, and VEGF) were downregulated, and the levels of p-β-catenin and E-cadherin were upregulated.

Conclusion: The results indicate that *L. fermentum* ZS09 could inhibit EMT and angiogenesis pathways by inhibiting the Wnt/β-catenin signalling pathway, which could inhibit tumour metastasis.

Keywords: *Lactobacillus fermentum* ZS09, Wnt/β-catenin signalling pathway, colon cancer, EMT pathway

Introduction

The incidence of colorectal cancer is increasing due to changes in people's eating habits.[1] Colorectal cancer is highly metastatic and aggressive, making it one of the most lethal cancers.[2] Clinically, it has been shown that patients with gastrointestinal diseases often suffer from imbalances in their intestinal flora.[3] The intestinal microbial population and interactions between the host and microbes may be important mechanisms affecting colon cancer. At present, the main treatment plan

for colon cancer is surgery supplemented with chemotherapy. However, patients undergoing chemotherapy experience a series of complications due to the effects of the drugs, such as an imbalance in the intestinal flora as well as vomiting and diarrhoea.[4] All of the results described above show that the intestinal flora has an important effect on intestinal diseases.

Probiotics are microorganisms that are beneficial to the intestinal tract. Probiotics improve the intestinal microecological balance and the health status of patients. In addition, probiotics have good effects in correcting imbalances in the intestinal flora.[5,6] *Lactobacillus* is a common probiotic flora in the human intestine. A number of research results have confirmed that *Lactobacillus* and its products have the potential to promote the apoptosis of colon cancer cells and treat intestinal diseases in animals. A. Adnan verified the effect of *Lactobacillus* on the metastasis and proliferation of cervical cancer cells through 3D cell technology.[7] Escamilla et al stated that cell-free supernatants from probiotic *Lactobacillus casei* and *Lactobacillus rhamnosus* GG decrease colon cancer cell invasion in vitro.[8] The potential of *Lactobacillus fermentum* to inhibit colon cancer metastasis deserves further study.

Studies have found that epithelial-mesenchymal transition (EMT) is closely associated with the invasion and metastasis of tumour cells.[9,10] EMT plays a key role in the initial stage of colon cancer metastatic progression. Current research on EMT suggests that the occurrence of EMT is caused by microenvironmental factors acting on cell receptors, inducing changes in cell pathways and ultimately leading to changes in gene expression.[11] During the process of EMT, cancer cells lose their epithelial characteristics and acquire a mesenchymal phenotype, thereby gaining an enhanced metastatic ability and improving the migration and invasiveness of cancer cells.[12] The Wnt/β-catenin signalling pathway is closely related to EMT and tumour progression. The wnt/β-catenin signalling pathway induces the translocation of β-catenin to the nucleus, thereby regulating the expression of many EMT-related genes and proteins and affecting the process by which normal epithelial polarity and integrity are maintained.[13,14] Therefore, studies about the effects of *L. fermentum* on the wnt/β-catenin signalling and EMT pathways are of value for guiding the use of *L. fermentum* in the treatment of colon cancer.

This article studies the basic mechanism by which *L. fermentum* ZS09 regulates colon cancer metastasis.

L. fermentum ZS09 is an active strain isolated from the traditional fermented yogurt from the Baleksu Kaisk grassland in Zhaosu County, Xinjiang, China, by the Chongqing Collaborative Innovation Center for Functional Food. *L. fermentum* ZS09 is now preserved in the China General Microbiological Culture Collection Center, CGMCC No.: 18225. In a previous study of its activity, *L. fermentum* ZS09 showed good adhesion to TH-29 colon cancer cells, and its viability in artificial gastric juice and 0.3% bile salt reached 79% and 15%, respectively. Drug-induced colon cancer in mouse was treated by ingesting a certain dose of *L. fermentum* by gavage each day. The experimental results show that *L. fermentum* ZS09 can affect EMT pathway by regulating the Wnt/β-catenin signalling pathway, thereby inhibiting the occurrence and metastasis of colon cancer. The results also provide new research materials for studying *L. fermentum* ZS09 as a functional strain in the intervention and treatment of intestinal diseases.

Materials and Methods
Experimental Materials
L. fermentum ZS09 was provided by the Chongqing Collaborative Innovation Center for Functional Food and was deposited in the China General Microbiological Culture Collection Center, CGMCC No.: 18226.

The experimental animals were SPF-grade, 4-week-old male C57BL/6 mouse (body weight 16–18 g) (SCXK 2019-0004) that were purchased from the Chongqing Laibit Biotechnology Co., Ltd. The mouse were raised in a standardized laboratory with a room temperature of 25 ± 2 °C, a relative humidity of 50 ± 5%, and a light cycle of 12-h light/12-h darkness, and the experiment was started after one week of adaptive feeding.

Cultivation of Strains
L. fermentum ZS09 and *L. bulgaricus* were inoculated into MRS liquid medium (Becton Dickinson Company, Sparks MD 21152, USA) at a 2% inoculum and cultured in a constant temperature shaker at 37 °C and 100 rpm for 24 h. The bacterial solutions were centrifuged at 12,000 rpm for 10 min, the supernatants were discarded, and the collected bacteria were suspended in 0.9% physiological saline. The purity of the bacterial solutions were microscopically evaluated by the Gram staining method (Solarbio Life Sciences, Beijing, China). Using the gradient dilution method, the *L. fermentum* ZS09 bacterial

solutions were diluted to 10^{11} and 10^9 CFU, and the *L. bulgaricus* solutions were diluted to 10^{11} CFU for using.

Experimental Animals

Sixty 4-week-old C57BL/6 mouse were adaptively housed for 1 week and randomly divided into 6 groups: the normal group (NC), model group (CRC), high-dose *L. fermentum* ZS09 group (ZS09-H), low-dose *L. fermentum* ZS09 group (ZS09-L), *L. bulgaricus bacterial* group (BLA), and sulfasalazine control group (SD). The mouse in the NC and CRC groups were intragastrically administered 0.2 mL of normal saline, and the mouse in the intervention treatment groups (ZS09-H, ZS09-L, and BLA groups) were treated daily with 0.2 mL of 10^{11} CFU, 10^9 CFU and 10^{11} CFU bacterial solution by gavage to administer the indicated number of viable bacteria. The mouse in the SD group were gavaged with a 2.5% sulfasalazine solution at 0.2 mL/day. After 1 week, the mouse were intraperitoneally injected with azoxymethane (AOM) (MP Biomedicals, France) at a dose of 10 mg/kg (except the mouse in the NC group) and were fed purified water for 1 week. One week later, the purified water was replaced with a 2.5% dextran sulfate sodium salt (DSS) (MP Biomedicals, France) solution for 1 week; then, this solution was replaced with purified water again for 2 weeks. Three cycles of these drinking water conditions were conducted. At the end of the experimental period (13th week), all the animals were sacrificed. Whole blood was centrifuged (4 °C, 3000 rpm, 30 min) to obtain serum, and the colon was quickly removed, photographed, and sampled. The samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

Pathological Tissue Section Preparation

Histological observation was performed on colon tissue samples from each animal. The colon tissue samples were fixed in tissue fixative (Sevier Biotechnology Co., Ltd., Chongqing, China) and placed at 4 °C. Within 24 h, a paraffin block was generated after washing, dehydrating, waxing, and embedding. After being cut, the sections were stained with haematoxylin and eosin, and immunohistochemical staining of CD34 (Sevier Biotechnology Co., Ltd., Chongqing, China) was performed. Finally, the pathological tissues were observed under an optical microscope (OLYMPUS, Japan).

Intestinal Mucosal Inflammation Score

Mucosal inflammation was evaluated in a double-blinded manner, and scores were given according to the following 5 grades: grade 0: a small number of inflammatory cells; grade 1: mild inflammation of the lamina propria and submucosa; grade 2: severe inflammation of the lamina propria and submucosa; grade 3: mild inflammation of the entire colon wall; and grade 4: severe inflammation of the entire colon wall.

Determination of MMP-9, TNF-α, IL-6R, Ang-2, VEGFR-2, DKK1 Levels in Serum

To obtain serum, blood was collected from the eyeballs of the mouse, incubated at 4 °C for 1 h, and then centrifuged at 3000 rpm for 30 min at 4 °C. A commercial ELISA kit (mlibio, Shanghai, China) was used to measure the matrix metallopeptidase 9 (MMP-9), tumour necrosis factor (TNF-α), interleukin 6 (IL-6R), angiopoietin 2 (Ang-2), vascular endothelial growth factor receptor 2 (VEGFR-2), and Dickkopf 1 (DKK1) levels.

Total RNA Extraction and Determination of mRNA Levels in Colon Tissue

Real-time fluorescent quantitative PCR (RT-qPCR) was used to detect the mRNA expression of *Dvl*, *GSK-3β*, *β-catenin*, *c-myc*, *cyclinD1*, *Vim*, *MMP-9*, *APC*, *CDH1*, and *Axin*. Total RNA was extracted and cDNA reverse transcription was performed according to the instructions of the RNA extraction kit (Solarbio Life Sciences, Beijing, China) and cDNA reverse transcription kit (Yeasen Technologies, Shanghai, China). A microspectrophotometer (Nano-300; ALLSHENG, Zhejiang, China) was used to determine the purity of the RNA at 260 nm. Actin was used as the standardized internal reference for RT-qPCR analysis (StepOne Plus, ABI, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the final product expression levels as determined by RT-qPCR.

Colon Tissue Protein Extraction and Western Blotting Analysis

Mouse colon tissues were homogenized using a protein extraction kit (Solarbio Life Sciences, Beijing, China), which included phenylmethanesulfonyl fluoride (PMSF), protease inhibitors and phosphatase inhibitors, and then, the homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C. The protein concentration was measured with a BCA protein assay kit (Solarbio Life Sciences, Beijing,

China). For Western blotting analysis, 50 µg of the protein extracts were separated by 10% NuPAGE (NP0302BOX, Invitrogen) and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 1 h at 28 °C and 50 rpm and then incubated with primary antibodies (anti-β-catenin, anti-N-cadherin, anti-VEGF, anti-P-β-catenin, anti-E-cadherin) overnight at 4 °C. The membranes were washed 5 times with 1× TBST (Solarbio Life Sciences, Beijing, China) for 5 min/wash, incubated with HRP-labelled secondary antibodies for 1 h, and then washed with 1x TBST 5 times for 5 min/wash. ECL chemiluminescent solution (Solarbio Life Sciences, Beijing, China) was used to observe antibody binding. ImageJ software (NIH) was used to quantify protein expression.

Data Analysis

Data from different groups are expressed as the mean ± standard deviation (SD). The *t*-test was used to determine the significance of the differences between two groups. To compare two or more groups, ANOVA (analysis of variance) and Dunnett's post-temporary analysis tests were performed, and GraphPad 7.0 (GraphPad Software Company, La Jolla, California, USA) was used for analysis.

Results

Gram Stain Microscopic Examination

The purity of the *L. fermentum* ZS09 and *L. bulgaricus* cultures was identified by Gram staining. As shown in Figure 1, both *L. fermentum* ZS09 and *L. bulgaricus* were gram-positive bacteria with rod shapes. The background of

the field of view was clean, the type of strain was single, and it met the purity standards of the experimental bacteria.

Observation of Colon Tissue Morphology

During the experiment, the number of mouse deaths was recorded. After AOM/DSS treatment, 3 mouse in the CRC group died during the experiment, and 1, 1, 1, and 0 mouse in the ZS09-L, ZS09-H, BLA and SD groups died, respectively (Figure 2B). After the experiment, the mouse were dissected, the colons were removed, and tumours of different sizes were observed in the colons of the mouse (Figure 2A). The colons of the CRC group had the largest number and volume of tumours. After ZS09-H intervention, the numbers of tumours in the colons of the mouse were reduced, and the sizes of the tumours were significantly reduced. In addition, compared with the NC group, the CRC group exhibited colonic oedema and congestion, significantly shortened colon length, thicker colon tissue, increased fragility, and lack of elasticity, and the length of colon increased after intervention. The results show that the AOM/DSS method induces a higher rate of pathogenesis in the mouse model of colon cancer, and the pathogenic effect was obvious. After ZS09 intervention, the incidence of colon cancer in the ZS09 control groups was lower than that in the CRC group, and the effect of ZS09-H was more obvious.

Serum Indicators

As shown in Figure 3, compared with the NC group, the CRC group exhibited significantly increased serum levels of MMP-9, TNF-α, IL-6R, Ang-2, VEGFR-2 and decreased levels of DKK1. Compared with CRC, ZS09-H intervention
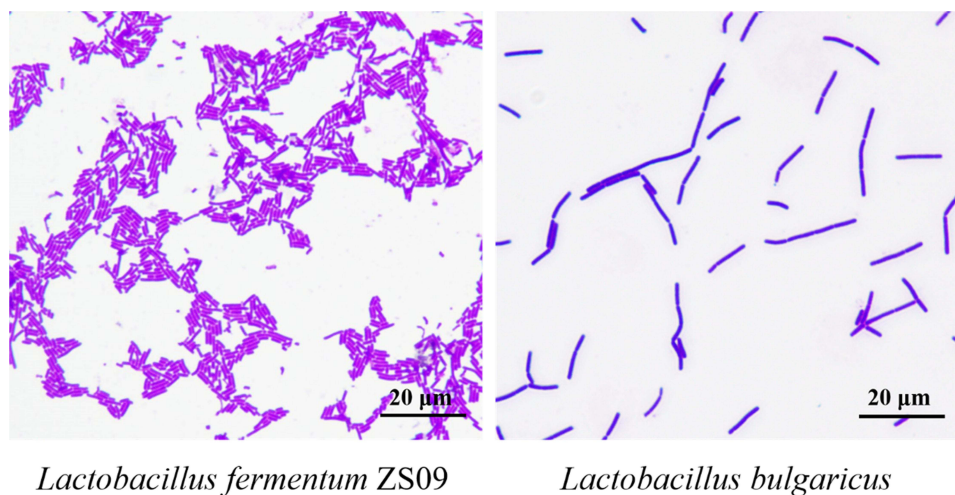

Figure 1 Gram staining microscopic examination. (**A**) *Lactobacillus fermentum* ZS09; (**B**) *Lactobacillus bulgaricus*.
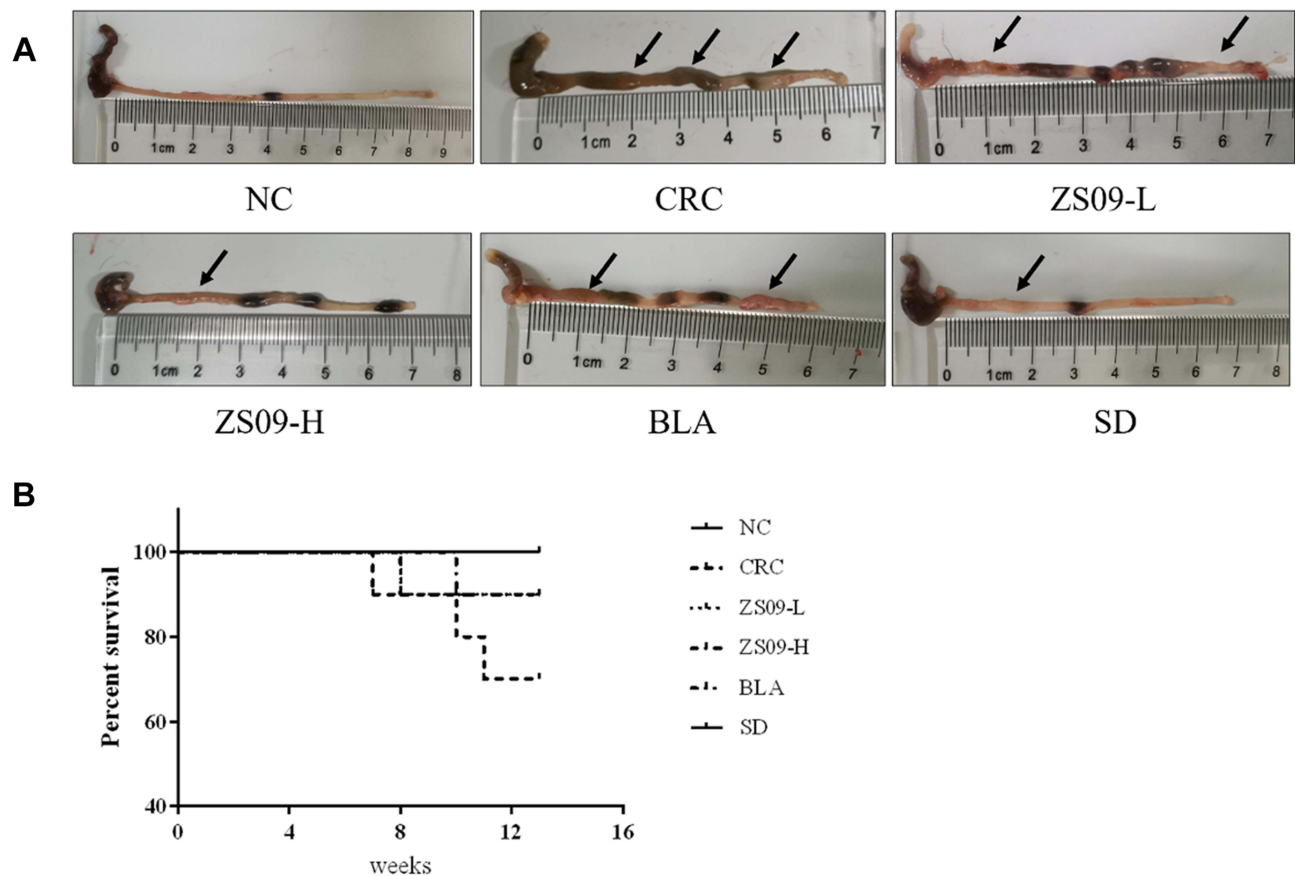
Figure 2 (**A**) Colon tissue morphology. The arrow shows the location of the colon mass. (**B**) Mouse survival curve.
Abbreviations: NC, normal untreated mice; CRC, colon cancer model mouse induced with AOM-DSS; ZS09-L, mouse treated with low-dose *L. fermentum* ZS09 (10^9 CFU); ZS40-H, mouse treated with high-dose *L. fermentum* ZS09 (10^{11} CFU); BLA, mouse treated with *L. bulgaricus* strain (10^{11} CFU); SD, mouse treated with sulfasalazine (2.5%).

treatment reduced the serum levels of MMP-9, TNF-α, IL-6R, Ang-2, VEGFR-2 and increased the expression level of DKK1 (p <0.05). In addition, compared with the CRC group, the ZS09-L, BLA intervention treatment and SD control groups exhibited decreased serum levels of MMP-9, TNF-α, IL-6R, Ang-2, VEGFR-2 and increased levels of DKK1. The results indicate that *L. fermentum* ZS09 could regulate the serum levels of tumour markers and vascular cytokines in C57BL/6 mouse treated with AOM-DSS.

H&E Staining Observations

The colon is composed of a mucosal layer, submucosal layer, muscular layer and serosal layer. The mucosal layer protrudes into the cavity to form wrinkles. The wrinkled surface is covered with a single layer of columnar epithelium, with a large number of goblet cells in between, and the lamina propria is dense. The colon tissue morphology was observed by analysing H&E-stained sections of colon tissues (Figure 4A). Pathological observation showed that

there were no obvious pathological changes in the colons of the NC group. The mucosal epithelial layers of the CRC group exhibited local defects, obvious inflammatory cell infiltration, loose submucosal structure, oedema, disordered muscle fibre arrangement, and somewhat necrotic muscle fibres. ZS09-H intervention treatment reduced the structural damage and the inflammatory factor accumulation in the colon tissues of the mouse in the CRC group. In addition, compared with the CRC group, the ZS09-L, LBA intervention treatment and BS control groups had significantly reduced inflammatory infiltration in colon tissues.

Colonic Mucosal Inflammation Score

Compared with the colonic mucosa and submucosa in the NC group, the colonic mucosa and submucosa in the CRC group had large amounts of inflammatory cell infiltration, the most severe degree of infiltration, and the muscle layer appeared to be involved. After ZS09-H and SD intervention, the degree of inflammatory cell infiltration in the
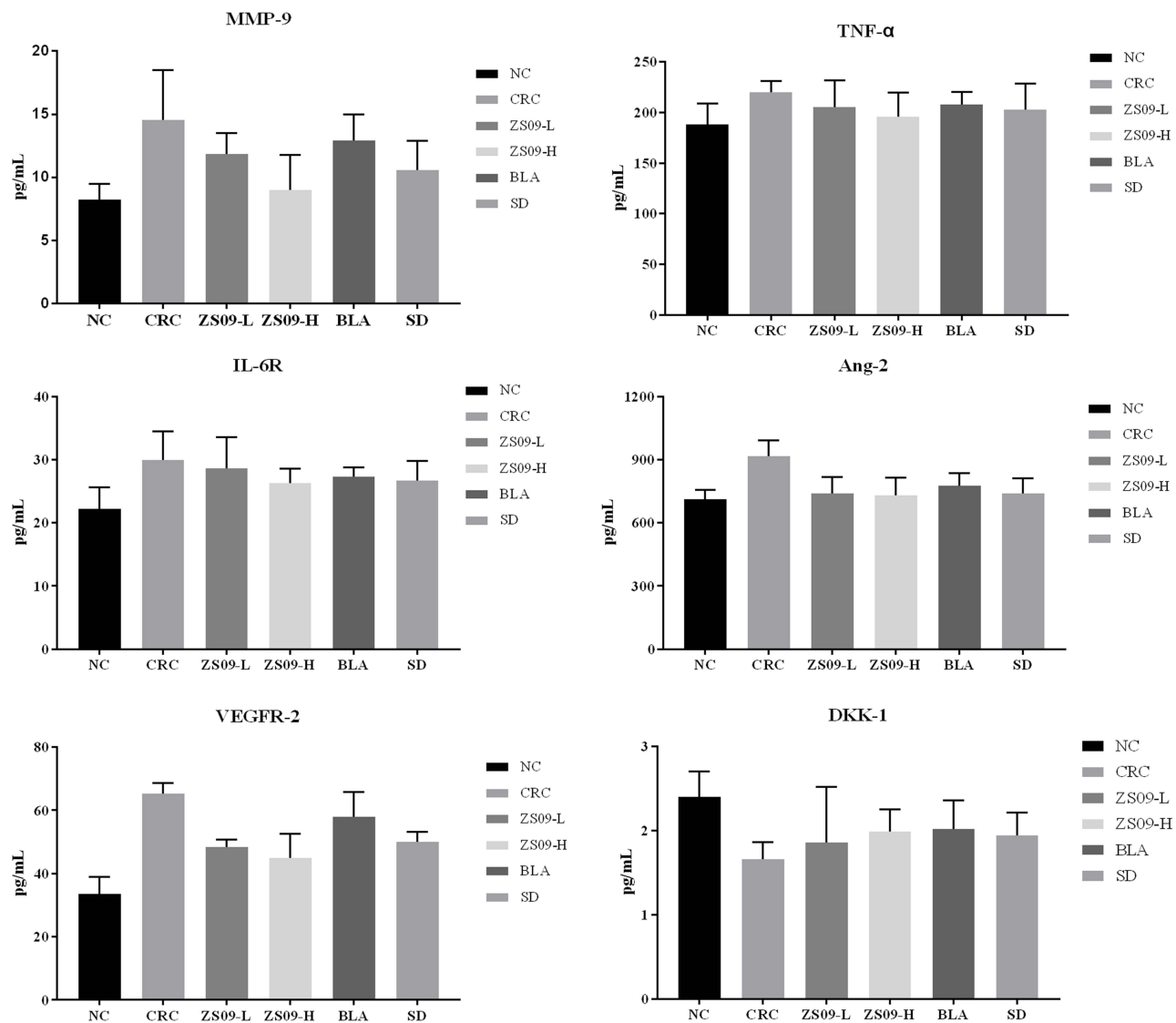
Figure 3 Mouse serum indicators.
Abbreviations: MMP-9, matrix metallopeptidase 9; TNF-α, tumour necrosis factor; IL-6R, interleukin 6; Ang-2, angiopoietin 2; VEGFR-2, vascular endothelial growth factor receptor 2; DKK1, Dickkopf 1; NC, normal untreated mice; CRC, colon cancer model mouse induced with AOM-DSS; ZS09-L, mouse treated with low-dose *L. fermentum* ZS09 (10^9 CFU); ZS40-H, mouse treated with high-dose *L. fermentum* ZS09 (10^{11} CFU); BLA, mouse treated with *L. bulgaricus* strain (10^{11} CFU); SD, mouse treated with sulfasalazine (2.5%).

colon tissue was significantly reduced, and the double-blinded score was not significantly different from that of the normal group (Figure 4B). 0 marks: intact villi and epithelium; Scores standard: 1 marks: slight submucosal or lamina propria swelling and separation; 2 marks: moderate submucosa or lamina propria swelling and separation and plasma cell infiltration; 3 marks: severe submucosal or lamina propria swelling and plasma cell infiltration, local villi atrophy and shedding; 4 marks: intestinal villi disappeared with intestinal wall necrosis. Pathology score ≥ 2, regarded as intestinal injury.

Immunohistochemistry

The staining intensities of CD34 protein (Figure 5A) in colon tissues were observed by immunohistochemistry. As shown in the figure, the staining intensities of CD34 in the colon tissue specimens of the CRC group were higher, and the staining intensities in the ZS09-H, ZS09-L, BLA intervention treatment and SD control groups were lower; among these groups, the staining intensities in the ZS09-H and SD groups were more obviously decreased.

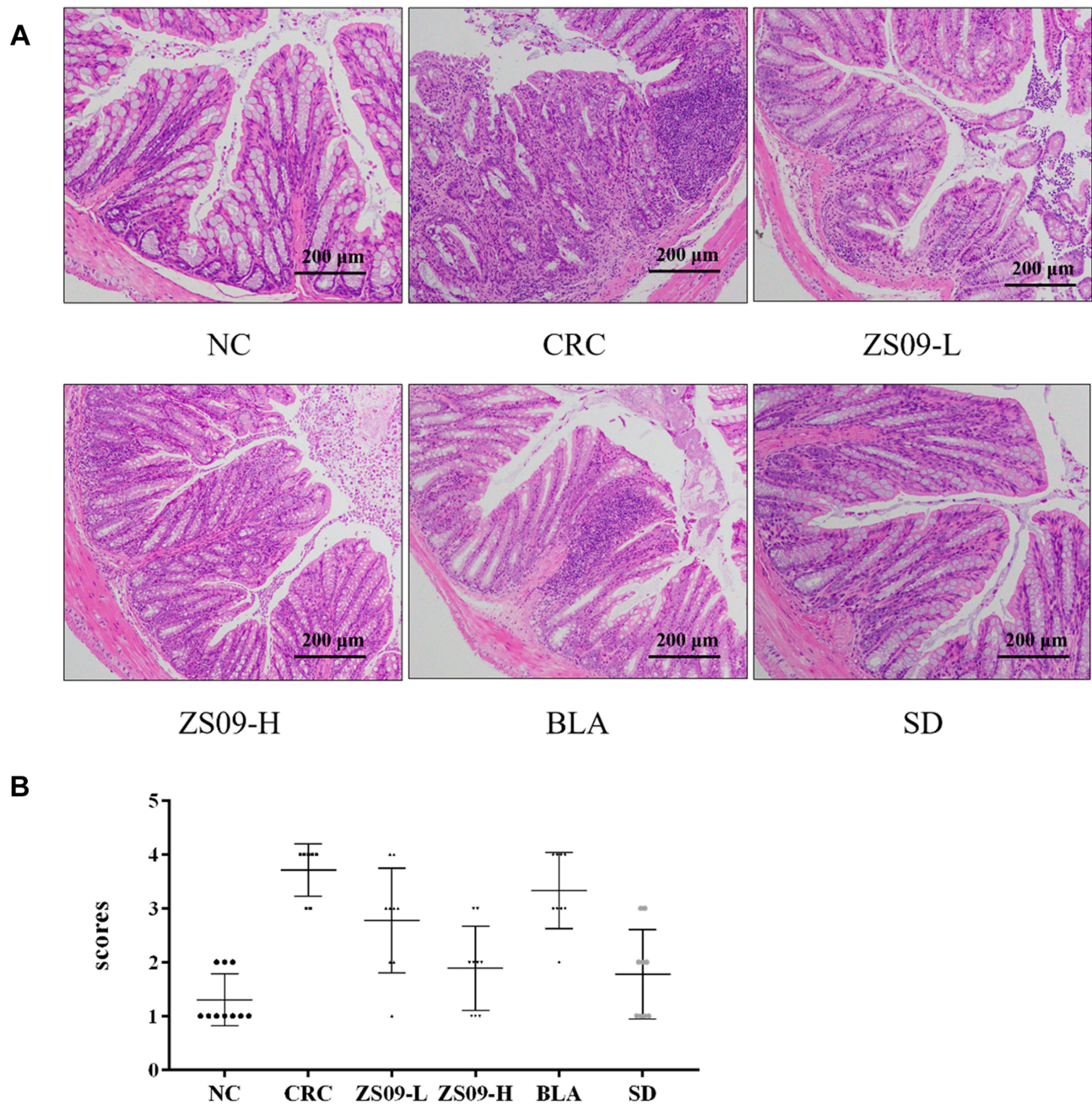The quantitative analysis results of the immunohistochemical pictures (Figure 5B) showed that compared with

Figure 4 (**A**) Pathological section of mouse colon (H&E). Magnification 100×. (**B**) Colon severity pathological scores. 0 marks: intact villi and epithelium; 1 marks: slight submucosal or lamina propria swelling and separation; 2 marks: moderate submucosa or lamina propria swelling and separation and plasma cell infiltration; 3 marks: severe submucosal or lamina propria swelling and plasma cell infiltration, local villi atrophy and shedding; 4 marks: intestinal villi disappeared with intestinal wall necrosis. Pathology score ≥ 2, regarded as intestinal injury.
Abbreviations: NC, normal untreated mice; CRC, colon cancer model mouse induced with AOM-DSS; ZS09-L, mouse treated with low-dose *L. fermentum* ZS09 (10^9 CFU); ZS40-H, mouse treated with high-dose *L. fermentum* ZS09 (10^{11} CFU); BLA, mouse treated with *L. bulgaricus* strain (10^{11} CFU); SD, mouse treated with sulfasalazine (2.5%).

NC, AOD (NC) = 0.325±0.004, the average optical density of CRC was significantly increased, AOD (CRC) = 0.444 ±0.042. The average optical density of ZS09 and SD in the treatment group was reduced to 0.356±0.010 (ZS09-H) and 0.333±0.010 (SD), respectively. These results indicate that the positive expression of CD34 protein occurs in

mouse colon cancer. The expression of protein CD34 was effectively reduced in the ZS09 intervention group.

mRNA Expression in Colon Tissue

The mRNA expression of *Dvl, GSK-3β, β-catenin, c-myc, cyclinD1, Vim, MMP-9, APC, CDH1*, and *Axin* in the Wnt/β-
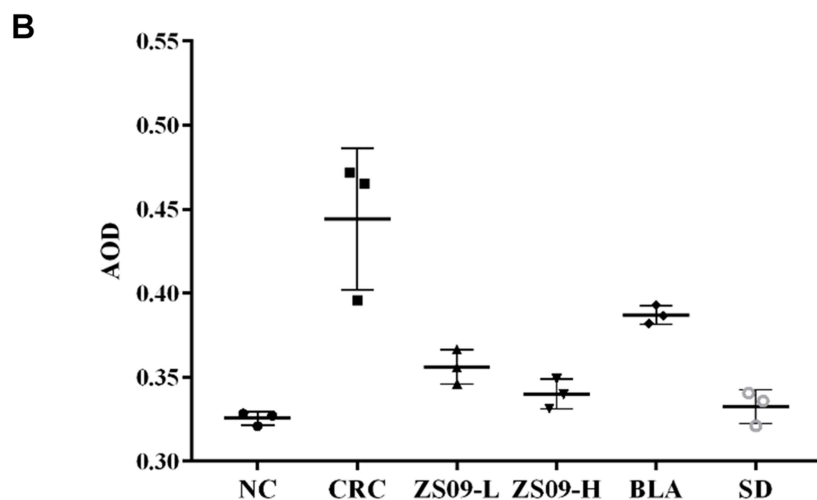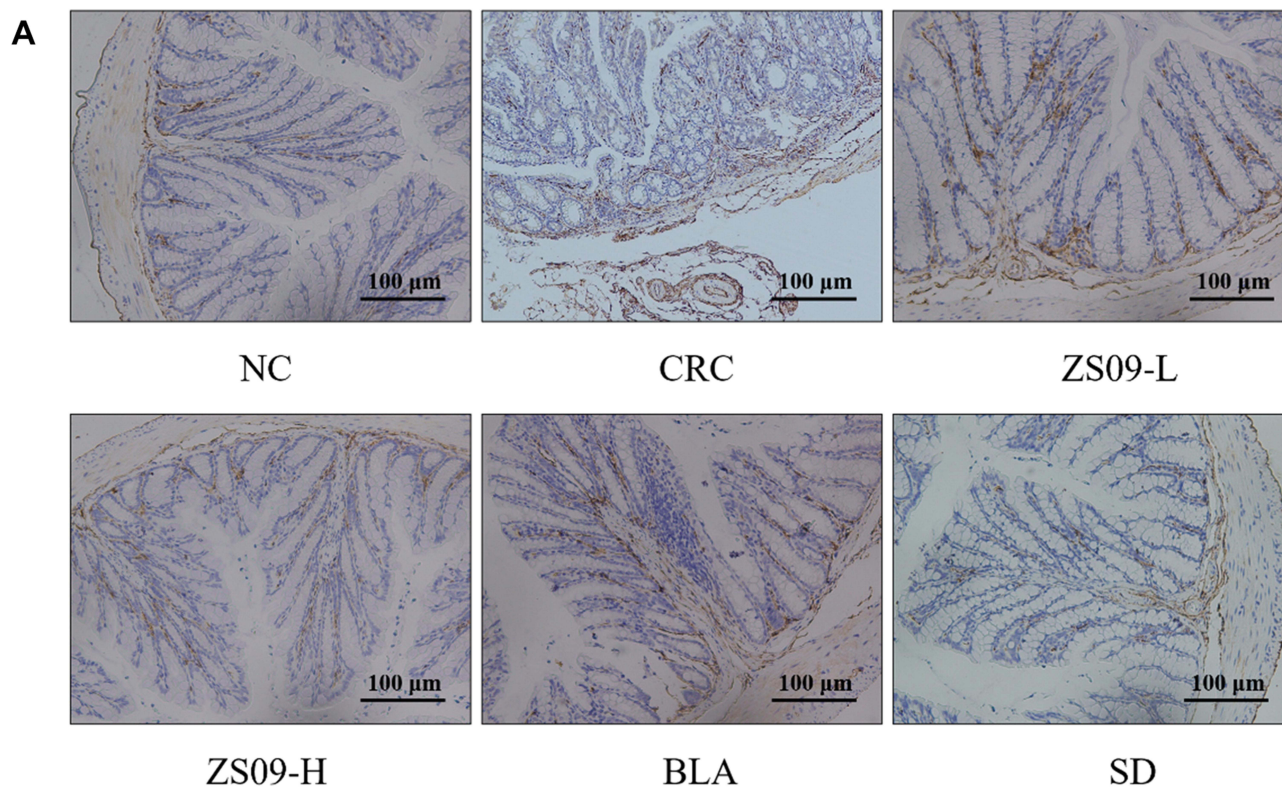
Figure 5 (**A**) Pathological section of mouse colon (CD34 immunohistochemical staining). Magnification 200×. (**B**) Quantitative immunohistochemical density. AOD (Average Optical Density) = IOD/ Area.
Abbreviations: NC, normal untreated mice; CRC, colon cancer model mouse induced with AOM-DSS; ZS09-L, mouse treated with low-dose *L. fermentum* ZS09 (10^9 CFU); ZS40-H, mouse treated with high-dose *L. fermentum* ZS09 (10^{11} CFU); BLA, mouse treated with *L. bulgaricus* strain (10^{11} CFU); SD, mouse treated with sulfasalazine (2.5%).

catenin signalling pathway was analysed by RT-qPCR. The results are shown in Figure 6A. *Dvl, GSK-3β, β-catenin, c-myc, cyclinD1, Vim,* and *MMP-9* genes in the CRC group were highly expressed, and *L.* ZS09 treatment reduced the expression of these genes. *APC, CDH1*, and *Axin* genes shown a lower expressed in the CRC group, and the gene

expression was up-regulated in ZS09-H, ZS09-H and SD had the strongest effects on the expression of these genes.

Mouse Colon Tissue Protein Expression

Western blotting was used to analyse the protein expression of components of the Wnt/β-catenin signalling

pathway and EMT pathway in mouse colon tissues (Figure 6B). Compared with the colon tissue of the NC group, the colon tissue of the CRC group exhibited significantly increased protein expression levels of p-β-catenin, VEGF and N-cadherin, and the expression levels of β-catenin, and E-cadherin were significantly decreased. In addition, ZS09-H, ZS09-L, BLA and SD regulated the expression of these proteins in the tissues, and the ZS09-H and SD treatments had the most obvious effects.
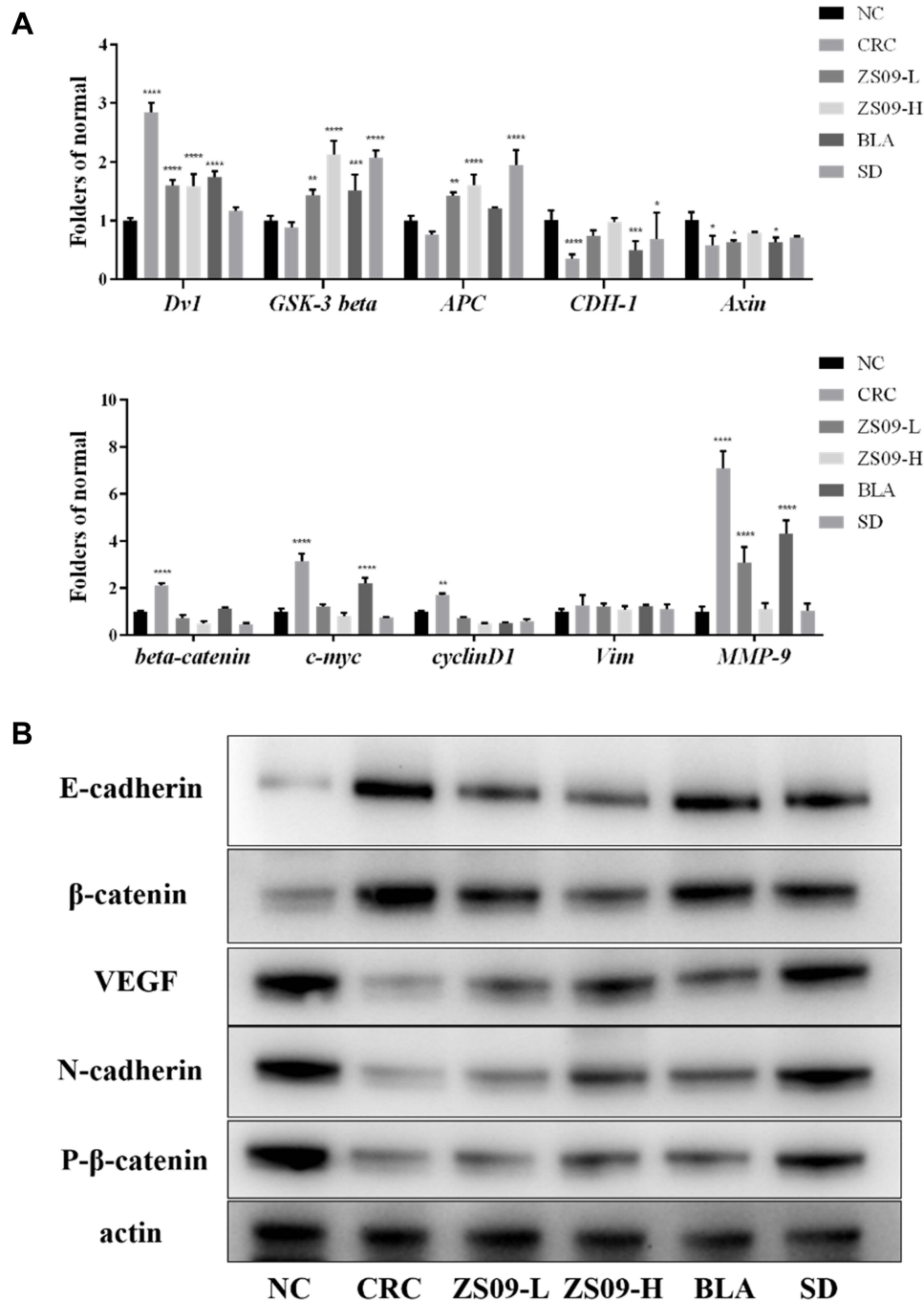

Figure 6 (**A**) mRNA expression levels of inflammatory factors in colon tissue. *Represents a significant difference from the NC group. *p <0.05, **<0.01, ***<0.001, ****<0.0001 (**B**) Expression of key proteins in signaling pathway in colon tissue.

Abbreviations: NC, normal untreated mice; CRC, colon cancer model mouse induced with AOM-DSS; ZS09-L, mouse treated with low-dose *L. fermentum* ZS09 (10^9 CFU); ZS40-H, mouse treated with high-dose *L. fermentum* ZS09 (10^{11} CFU); BLA, mouse treated with *L. bulgaricus* strain (10^{11} CFU); SD, mouse treated with sulfasalazine (2.5%).

Discussion

Patients with colon cancer, a common form of advanced cancer, experience abdominal pain, diarrhoea, and blood in the stool as the main symptoms. Colon cancer is difficult to completely treat because of its highly metastatic and invasive nature. At present, the prevention and treatment of diseases through diet is an important research topic in the new era of the field of food health. Studies have shown that the daily intake of a certain dose of active lactic acid bacteria plays an important role in disease prevention.[15] The lipopolysaccharide and teichoic acid produced during the growth and metabolism of lactic acid bacteria have been proven to have the ability to inhibit tumour activity and regulate the intestinal mucus barrier.[16] Therefore, this study hypothesizes that lactic acid bacteria inhibit colon cancer metastasis. Therefore, it is necessary to study the effect of lactic acid bacteria in preventing the onset of colon cancer and inhibiting metastasis by establishing a chronic animal model similar to human colon cancer. The establishment of an AOM/DSS-induced animal model of colon cancer is simple and economical and has a high success rate.[17–19] This model can accurately replicate the characteristics of human colon cancer disease. This experiment required 13 weeks to establish a mouse model of colon cancer. During the experiment, *L. fermentum* ZS09 was used to treat the mouse during the model establishment process. After the experiment, the experimental mouse were sacrificed to observe the pathological changes induced by AOM/DSS in the mouse colon cancer model and the effects of treatment with *L. fermentum* ZS09. The experiments showed that mouse with AOM/DSS-induced colon cancer showed symptoms such as blood in the stool and weight loss. Pathological sections of the colon revealed that a large amount of inflammatory cell infiltration into the intestinal mucosa and submucosa, and plasma cells and lymphocytes infiltrated the muscular tissues, suggesting that intestinal proinflammatory cytokines were produced in large quantities, and the intestinal mucosal inflammation score reached its highest level. In the *L. fermentum* ZS09 intervention group, it was observed under a microscope that muscle fibre hyperplasia was present in the muscle layer, the structure was normal, the oedema and congestion of the colon were alleviated, and the treatment effect was significant.

The survival rate of experimental mouse was counted throughout the test cycle. The pathogenesis of colon cancer induced by the combination of AOM-DSS is mainly an inflammatory colorectal cancer model stimulated by drugs under the induction of long-term intestinal inflammation. Sulfasalazine is an antibacterial drug and has been used as a positive control drug in many studies on colitis. It is mainly used for the treatment of inflammatory bowel disease, ulcerative colitis, and non-specific chronic colitis. Adding edible *L. fermentum* ZS09 can enrich the structure of the intestinal flora, enhance the digestive and motility of the intestines, and stimulate the body's immunity to achieve the purpose of intervening in the occurrence and development of cancer. The phenomenon of our experiment is in accordance with the experimental principle. The mouse in the CRC group are thin and weak, and 3 eventually died; only 1 mouse was found dead in the treated groups ZS09. The mortality of mouse was reduced, which shows that the probiotic ZS09 has achieved the effect of intervention and regulation of colorectal cancer.

In addition, this study revealed that compared with the CRC mouse, the mouse treated with *L. fermentum* ZS09 exhibited significantly reduced levels of VEGFR-2 and Ang-2 in the serum. Studies have shown that VEGFR-2 mainly regulates the differentiation, survival, proliferation and migration of endothelial cells.[20] In addition, the expression of VEGFR-2 has also been proven to be related to the formation of blood vessels.[21] Ang-2 can promote angiogenesis by disrupting endothelial cell-pericyte interactions in the primary tumour and enhancing blood vessel permeability.[22] Tumour angiogenesis refers to the process of forming new blood vessels from existing blood vessels.[23] The prerequisite for tumour growth and metastasis is the formation of new blood vessels. Therefore, inhibiting tumour angiogenesis to a certain extent is a very promising strategy to limit the further development of tumours. Matrix metalloproteinases (MMPs) contribute to the degradation of extracellular matrix (ECM) and cause tumours to lose their epithelial polarity characteristics; in particular, matrix metalloproteinase-9 (MMP-9), which is one of the main matrix metalloproteinases, is crucial for these phenomena and participates in promoting tumour metastasis.[24,25] In our experimental results, the content of MMP-9 in the serum of mouse in the CRC group was higher, and the serum indexes of the mouse treated with *L. fermentum* ZS09 were reduced after intervention. This result also verifies the inhibitory effect of *L. fermentum* ZS09 on colon cancer metastasis.

The EMT pathway is a complex process of phenotypic transformation between epithelial cells and mesenchymal cells.[26] EMT is widely involved in the invasion and migration of tumour cells and is closely related to malignant biological manifestations, such as the distant metastasis of tumour cells. When EMT occurs, it causes changes in the expression of E-cadherin and N-cadherin, markers of EMT, and epithelial cancer cells begin to expression mesenchymal phenotype markers (N-cadherin) to induce metastasis. Then, this metastatic ability is enhanced, β-catenin is overexpressed in the nucleus, and the expression of epithelial cell adhesion molecules, such as E-cadherin, is downregulated.[27] E-cadherin is downregulated in many human malignant tumour cells, its expression level is closely related to tumour cell invasion, metastasis and prognosis, and its expression is regulated by the Wnt signalling pathway.[28] Another important marker of EMT, N-cadherin, is mainly involved in regulating the adhesion between cells, weakening the adhesion of epithelial cells and reducing cell polarity, thereby enhancing cell aggregation and migration and enhancing the remote migration and invasion abilities of tumour cells; thus, N-cadherin is closely related to the malignancy of tumour cells. As an important effector in the positive regulation of the Wnt signalling pathway, E-cadherin participates in signal transduction.[29] Downregulation of the protein expression of E-cadherin and upregulation of the expression of N-cadherin are distinguishing features of EMT, and these changes can enable tumour cells to acquire migration and invasion abilities.[30] Therefore, in this study, we explored the potential mechanism by which *L. fermentum* ZS09 inhibits the migration of colon cancer cells by blocking the EMT pathway. The results show that *L. fermentum* ZS09 can effectively downregulate the protein expression levels of mesenchymal markers (N-cadherin) and upregulate the expression levels of epithelial markers (E-cadherin), significantly reducing the migration and invasion of colon cancer cells. In addition, we also found that treatment with a high concentration of *L. fermentum* can significantly block EMT and inhibit colon cancer migration compared to treatment with a low concentration of *L. fermentum*. This result further shows that *L. fermentum* ZS09 can inhibit colon cancer metastasis, and all these results are consistent with our expectations.

An increasing number of studies have proven that the Wnt/β-catenin signalling pathway is closely related to the migration of tumour cells.[31,32] In this complex regulation process, a pivotal molecule in the Wnt signalling pathway,

namely, β-catenin transduces signals from the cell membrane to the nucleus, under normal physiological conditions, the E-cadherin-β-catenin complex interacts with cytoskeletal actin to maintain adhesion between epithelial cells. When the canonical Wnt signalling pathway is activated, β-catenin in the cytoplasm is phosphorylated and degraded by the proteasome complex to promote the transcription of downstream target genes. The transcription and expression of cyclinD1, c-myc, VEGF, Cox-2, etc., reduce the stability of the E-cadherin-β-catenin complex and decrease the adhesion ability mediated by E-cadherin, weakening the connections between tumour cells, mediating the EMT pathway, and regulating tumour metastasis and other cellular processes.[33] Our research results showed that intervention with *L. fermentum* ZS09 can promote the phosphorylation and degradation of β-catenin, regulate the transcription and expression of downstream target genes and proteins, and inhibit the Wnt/β-catenin signalling pathway, thereby blocking the EMT pathway. Finally, intervention with *L. fermentum* ZS09 effectively inhibited the metastasis of colon cancer.

In summary, this experiment proved that *L. fermentum* ZS09 can mediate the EMT pathway by blocking the activation of the classic Wnt/β-catenin signalling pathway, thereby inhibiting the migration and invasion of colon cancer cells and ultimately preventing colon cancer. *L. fermentum* ZS09 may control tumour angiogenesis and further inhibit tumour migration. Therefore, *L. fermentum* is expected to be a potential anticancer drug for the treatment of metastatic colon cancer, but its mechanism of action needs to be further studied.

Conclusion

Inhibition of colon cancer metastasis and expansion can be achieved by regulating the Wnt/β-catenin signaling pathway to inhibit the epithelial-mesenchymal transition (EMT) pathway. Our current research shows that edible *L. fermentum* ZS09 can reduce the occurrence of colon cancer tumors induced by AOM and DSS by regulating the Wnt/β-catenin signaling pathway to inhibit the EMT pathway. Our experimental results show that mouse with daily intake of 10^{11} CFU *L. fermentum* ZS09 can effectively inhibit the EMT pathway by regulating the Wnt/β-catenin signaling pathway. The experimental data of pathological tissue sections, serum indexes, gene expression, and protein expression all confirmed the effect. The results of this research are of great value to the follow-up study of related experiments, and stimulate the author's

interest in the research on the mechanism of *L. fermentum* ZS09 in the intestine.

Ethical Statement

This study was approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food (20210720B, Chongqing, China) and followed the national standard of the People's Republic of China (GB/T 35892-2018) laboratory animal-guidelines for ethical review of animal welfare.

Disclosure

The authors report no conflicts of interest in this work.

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