VSL#3 Prevents Ulcerative Colitis Carcinogenesis in Mice and Cell by Regulating Inflammatory and Wnt/β-catenin Pathway

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Abstract

Background : The exact mechanism of how VSL#3 prevents ulcerative colitis (UC) carcinogenesis is still not clear. We aimed to explore the effect and mechanism of VSL#3 on UC carcinogenesis in mice and cell.

Methods: In mice, C57BL/6 mice were given azoxymethane/dextran sulfate sodium to establish the UC carcinogenesis model. The treatment groups received 5-ASA, VSL#3, 5-ASA combined with VSL#3 by gavage. The tumor load was compared in each group. TNF-α, IL-6 levels in colon tissue, the transcription activity of NF-κB and TCF-4 as well as the β-catenin distribution in the nuclei were assessed. In cell, Caco-2 cells, CCC-HIE-2 cells was co-cultured with Bifidobacterium, and was stimulated by IL-6 and TNF-α respectively. The relative luciferase activity, mRNA and protein level of β-catenin, expression of β-catenin in nucleoprotein, level of inflammatory factors, and transcriptional activity of NF-κB and TCF-4 was measured.

Results: In mice, compared with the model control group, in VSL#3 and 5-ASA+VSL#3 groups, the tumor loads significantly decreased, the TNF-α and IL-6 level, the transcription activity of NF-κB, β-catenin expression in the nuclei, and transcription activity of TCF-4 were significantly lower. In cell, the level of TNF-α and IL-6 was down-regulated, transcription activities of NF-κB and TCF-4 were decreased, and the expression level of β-catenin in nucleus was distinctly declined in the Bifidobacterium co-culture group compared with non-co-culture group.

Conclusion: VSL#3 inhibit tumor formation in UC carcinogenesis mice model by regulating inflammatory and Wnt/β-catenin Pathway. Similarly, Bifidobacterium co-culture can inhibit the activity of Wnt/β-catenin pathway in cell. Key Words: VSL#3, Ulcerative colitis carcinogenesis, Wnt/β-catenin

1. Background

As the incidence of ulcerative colitis (UC) has increased rapidly, more attention has focused on maintaining clinical remission and preventing UC carcinogenesis. Repetitive inflammation is an independent risk factor for UC carcinogenesis, therefore, control of inflammation and maintenance of remission play important roles in preventing carcinogenesis.

The intestinal microbiota plays an important role in maintaining the intestinal barrier and immune function. Many studies have indicated that imbalance of the host intestinal microbiota is required for the onset of UC, which triggers inflammation[1]. An increasing number of studies have proved the effect of probiotics in maintaining UC remission. VSL#3 is a mixture of Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. bulgaricus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis and Streptococcus salivarius, which contains 4.5 billion live bacterial colonies. VSL#3 has shown benefits in the induction and maintenance of UC remission as well as prevention of relapse in mild to moderate UC.[2–5] These studies remind us of the

potential effect of probiotics in preventing UC carcinogenesis by maintenance of remission and control of inflammation through modulating intestinal microbiota.

As a result of the long course and rare incidence of UC carcinogenesis, it is difficult to observe the effect of probiotics on UC carcinogenesis clinically. Therefore, mouse models are an effective way to study UC carcinogenesis. Dextran sulfate sodium (DSS) is a pro-inflammatory macromolecule that can destroy the intestinal barrier. In mice, it is toxic to the intestinal mucosa after being dissolved in drinking water, leading to diarrhea, bloody stools and other manifestations of colitis. The DSS-induced colitis model is widely used to simulate human UC. Azoxymethane (AOM) is a carcinogen that specifically induces colorectal cancer, and AOM-induced colorectal cancer in mice is similar to that in humans. AOM combined with DSS can induce a UC carcinogenesis model within a short time[6 – 8].

Studies in mouse models have proved the inhibitory effect of VSL#3 on UC carcinogenesis[9 – 11], but the conclusions are still controversial. One study showed that VSL#3 did not reduce colitis-associated colorectal cancer in an AOM/IL-$10^{-/-}$ model[12]. The exact mechanism of how VSL#3 prevents UC carcinogenesis is still not clear. Therefore, we used a mouse model of AOM/DSS-induced colitis-associated cancer to verify the effect of VSL#3 in preventing UC carcinogenesis and explored the specific mechanism of action, to provide a strong theoretical basis for its function.

5-Aminosalicylic acid (5-ASA) is a first-line drug for treatment of mild to moderate UC and plays an important role in controlling inflammation and maintaining remission. Many clinical trials and meta-analyses[13 – 15] have proved the dose-dependent protective effect of 5-ASA in preventing UC carcinogenesis. In clinical practice, 5-ASA is the mainstay of treatment for UC, so probiotics are rarely applied to UC patients alone, but usually combined with 5-ASA. Therefore, we included a 5-ASA monotherapy group as a positive control, and a 5-ASA combined with VSL#3 group to simulate the real clinical situation.

Taking into account the difference between animal and human, we further explored the influence of probiotics on Wnt/β-catenin pathway at human cellular level. As we mentioned above, VSL#3 was the compound consisting of three different bacterial genera, during which Bifidobacterium was an important ingredient. Since it was complex and difficult to cultivate multiple probiotics at the same time, we selected Bifidobacterium to carry out the exploration.

Our study aimed to explore the effect and mechanism of VSL#3 on UC carcinogenesis in mice and cell. We tested the hypothesis that VSL#3 can inhibit UC carcinogenesis in mice by regulating inflammatory and Wnt/β-catenin Pathway; Similarly, Bifidobacterium co-culture can inhibit Wnt/β-catenin pathway in cell.

2. Methods

2.1. Study in mice

2.1.1. Animals

Eight-week-old male C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and housed under 12 h light/dark cycle conditions (temperature 22 ± 1 °C, humidity 40–60%) in the National Cancer Center/Cancer Hospital animal facilities and were fed a standard diet for the duration of the study. All animal experiments were approved by and conducted in accordance with the recommendations of the Animal Care Ethics and Use Committee of Peking Union Medical College (XHDW-2015-0032).

2.1.2. Development of UC carcinogenesis model and treatment

All mice (n = 90) were initially housed together (5 mice per cage) for adaption 1 week prior to being randomly divided into 5 experimental groups: 5-ASA group (n = 20), VSL#3 group (n = 20), 5-ASA + VSL#3 (n = 20), model control group (n = 20), and normal control group (n = 10). For Groups 1–4, the mice were given azoxymethane (AOM; Sigma–Aldrich) 12.5 mg/kg by intraperitoneal injection, and 1 week later 2.5% dextran sulfate sodium (DSS, 36 000–50 000 Da; MP Biomedicals) in drinking water was fed for 5 days to establish the UC carcinogenesis model. This modeling method was based on previous research[6–8] and we made some changes to optimize it. This model was proved to be a stable and effective model for UC carcinogenesis in our previous studies.

The 5-ASA, VSL#3 and 5-ASA + VSL#3 groups received by gavage 5-ASA (75 mg/kg QD dissolved in drinking water; Ferring Pharmaceuticals), VSL#3 (1.5×10^9 CFU/mice QD dissolved in drinking water; Sigma–Tau Pharmaceuticals), or 5-ASA + VSL#3. The model control group was subjected to modeling without gavage intervention. The normal control group was given no modeling or gavage intervention (Fig. 1).

2.1.3. Specimen collection

After 12 weeks, the mice were anesthetized with diethyl ether and sacrificed via transcardiac perfusion and colon tissues were removed. The colons were dissected longitudinally along the main axis and washed with 0.9% saline. The diameter of each tumor was measured using a sliding caliper, and then total tumor load of each colon was calculated as the sum of the diameter of each tumor. The process of measurement and calculation was blinded by the operator. Subsequently, the whole colon was divided into sections for ELISA and histopathological examination. The remaining colon was stored at − 80 °C.

2.1.4. ELISA for tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 in colon mucosa

The levels of TNF-α and IL-6 in the colon mucosa were measured using commercial Mouse TNF-α and IL-6 ELISA Kits (eBioscience). The absorbance of the final colored product was measured at 450 nm as the primary wavelength. The results were expressed as pg/mg. Eight mice were randomly selected from each group for ELISA.

2.1.5. Immunohistochemistry of β-catenin

Paraffin sections were deparaffinized and rehydrated through dimethylbenzene and graded concentrations of ethanol solutions. After retrieval of the antigen and blocking endogenous peroxidase activity, the sections were incubated with primary antibodies at 4 °C overnight followed by Polymer Detection System reagents (PV-9000; ZSGBBIO) and DAB (ZLI-9019). Anti-β-catenin (ab32572, 1:2000; Abcam) antibodies were used as the primary antibodies. The slides were analyzed by 2 experienced pathologists in a blinded fashion.

The positively stained mucosal cells were counted in 10 randomized fields (× 400) with a light microscope (Olympus). Immunoreactivity was assessed with respect to cellular localization (membranous, cytoplasmic, paranuclear, or nuclear), intensity, and distribution. The staining intensity was scored as 0 for absent, 1 for weak, and 2 for strong staining. The staining distribution was scored as 0 for < 10%, 1 for 10–50%, and 2 for ≥ 50% positively stained areas noted. The sum of intensity and distribution scores was then used to determine the β-catenin immunoreactivity[16].

2.1.6. Electrophoretic mobility shift assay (EMSA) of nuclear factor (NF)-κB and transcription factor (TCF)-4

Transcription activity of NF-κB and TCF-4 in the colon mucosa was measured by EMSA using LightShift® Chemiluminescent EMSA Kit (Pierce). Complementary NF-κB oligonucleotides 5'-AGTTGAGGGGACTTTCCCAGGC-3' and complementary TCF-4 oligonucleotides 5'-CCCTTTGATCTTACC-3' were biotin-labeled separately using the Biotin End Labeling Kit (Pierce) and then annealed before use. Each binding reaction contained 1 binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol, pH 7.5), and 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/ μL poly (dIdC), 0.05% NP-40, 10 μg of nuclear extract, and 40 nM of biotin end-labeled target DNA. The contents were incubated at room temperature for 20 minutes. Complexes were separated on 4% nondenaturing polyacrylamide gel and were transferred to a nylon membrane. When the transfer was complete, DNA was crosslinked to the membrane at 120 mJ/cm^2 using a UV crosslinker equipped with 254 nm bulbs. The biotin end-labeled DNA was detected using streptavidin–horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to an appropriately equipped CoolImager camera.

2.2 Study in cell

2.2.1. Cell line and bacterial strain

Caco-2, which was one kind of human colorectal adenocarcinoma cell lines, was provided by the laboratory of Wang at the National Cancer Center/Cancer Hospital of the CAMS and PUMC (Beijing, China), and was cultured in minimum essential medium (MEM; Thermo Fisher Scientific, USA). CCC-HIE-2, which was human embryonic intestinal mucosa derived cell line, was purchased from the Basic Research Institute of Peking Union Medical College (Beijing, China), and was cultured in Dulbecco's modified eagle medium-high glucose (DMEM-H; Thermo Fisher Scientific, USA).

Bifidobacterium lactis was obtained from Danisco Company (Suzhou, China), and grown in MRS broth. Bacterial solution of 1 ml was added to 10 ml medium for growth of 6–14 hours at 37°C in anaerobic environment, then colony forming units were counted. After centrifugation, precipitation was resuspended and used for subsequent experiments.

With regard to groups, six groups were divided, namely, control group, Bifidobacterium group (Bifidobacterium was given according to the concentration of 2×10^5CFU/ul), IL-6 group (IL-6 was added according to the concentration of 0.1 ng/ul), Bifidobacterium plus IL-6 group (Bifidobacterium was given firstly, then IL-6 was added according to the concentration of 0.1 ng/ul), TNF-α group (TNF-α was added according to the concentration of 0.1 ng/ul), Bifidobacterium plus TNF-α group (Bifidobacterium was given firstly, then TNF-α was added according to the concentration of 0.1 ng/ul).

2.2.2. Luciferase reporter assays

Caco-2 and CCC-HIE-2 cells were cultured in 24-well plates and transfected with plasmid which expressing TOP-Flash (400 ng/well; Millipore, USA) and pRL-TK (40 ng/well; Promega, USA). Six hours later, replaced the medium with complete culture medium, then went through starvation, adding Bifidobacterium (1×10^8CFU/well) and inflammatory cytokines (IL-6 or TNF-α, 50 ng/well). Lysate was collected after cell lysis. The activity of luciferase was detected using dual reporter gene system (Promega, USA) through an automated chemiluminescence detector (BioTek, Germany).

2.2.3. Quantitative real-time polymerase chain reaction (qRT-PCR) for β-catenin

According to the instructions provided by the manufacturer (Thermo Scientific, USA), total RNA was extracted from cells, and then removed DNA, reverse transcripted to cDNA. Expression level of β-catenin was determined using SYBR Green Supermix (Takara, Japan). GAPDH expression was adopted as internal control. Sequences of primers were listed in Table 1.

Table 1
Primer sequences of β-catenin and GAPDH

Primer	Sequence(5'-3')
β-catenin (human)-Forward	AGAACCCCTTGGATATCGCC
β-catenin (human)-Reverse	TGGCCACCCATCTCATGTTC
GAPDH (human)-Forward	CTCTGCTCCTCCTGTTCGAC
GAPDH (human)-Reverse	GCGCCCAATACGACCAAATC

2.2.4. Western Blot for total and nuclear β-catenin

Total protein or nucleoprotein was extracted and concentration was measured. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. What follows was blocking

and incubation with primary antibodies (Anti-β- catenin rabbit IgG, 1:50000, Abcam. Anti-β-actin mouse IgG, 1:10000, Santa Cruz. Anti-PARP rabbit IgG, 1:1000, Cell Signaling Technologies) and secondary antibodies (Anti-mouse IgG, anti-rabbit IgG, 1:10000, Cell Signaling Technologies). Specific signals were visualized through Amersham Imager 600 (General Electric Company, USA).

2.2.5. ELISA for IL-6 and TNF-α

The levels of IL-6 and TNF-α in the cellular supernatants were measured as mentioned in 2.1.4. The results were expressed as pg/ml.

2.2.6. EMSA of NF-κB and TCF-4

Transcription activity of NF-κB and TCF-4 in the cells was tested as described in 2.1.6.

2.3. Statistical analysis

The data were presented as mean ± standard error (SE). All statistical analyses were performed using GraphPad Prism version 6.0. Statistical differences between experimental variants were assessed by two-tailed independent t-test and data from more than two groups were analyzed by one-way analysis of variance. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Results of VSL#3 on AOM/DSS mice model

3.1.1. General health of mice in each group

Mice treated with AOM/DSS showed significant symptoms of colitis including diarrhea and hematochezia, resulting in weight loss from the third day given DSS. The symptoms were alleviated after receiving ordinary drinking water. Some mice treated with AOM/DSS had bloody stools again at 9 weeks, and even anal prolapse at 10 weeks. One mouse in the 5-ASA + VSL#3 group died on day 33 and one in the model control group died on day 16 after fighting with other mice. The body weight did not differ significantly among the 5 groups at the end of 12 weeks (Fig. 2).

3.1.2. Establishment of UC carcinogenesis model

The mice were killed after 12 weeks, and the colorectal tumors in the model group and treated groups were observed macroscopically. Tumor was mainly located in the distal two-thirds of the colon. Anal tumor fusion and ring growth at the end of the rectum were observed in mice with anal prolapse (Fig. 3). The pathological analysis showed mucosal carcinoma or high-grade intraepithelial neoplasia in mice treated with AOM/DSS, which manifested as colonic gland structure disorders, deep nuclear staining, and decreased cytoplasm-to-nuclear ratio (Fig. 4).

3.1.3. Effects of VSL#3 on UC carcinogenesis

Treatment with AOM and DSS led to 100% incidence of colonic neoplasms in model group (19 mice; 1 died during the experiment due to fighting) with a mean tumor load of 0.97 ± 0.19 cm. 5-ASA and VSL#3 significantly reduced both tumor formation rate and tumor load. (Table 2, Fig. 5). The inhibitory effect of VSL#3 seems stronger than 5-ASA, but there's no significant difference between the two groups(p = 0.244). And 5-ASA + VSL#3 combination group didn't show stronger effect than VSL#3 monotherapy group. There was no colonic tumor observed in the normal control group.

Table 2
Tumor formation rate and tumor load of each group

Group	n	Tumor formation rate (%)	Tumor load (cm)	p (treatment group vs model control group)
5-ASA	20	65% (13/20)	0.43 ± 0.14	0.0269*
VSL#3	20	65% (13/20)	0.25 ± 0.07	0.0009***
5-ASA + VSL#3	19	63.2% (12/19)	0.46 ± 0.11	0.0261*
Model control	19	100% (19/19)	0.97 ± 0.19	—
Normal control	10	0	0	—
Note: data shown as mean ± SE. *p < 0.05; **p < 0.01; ***p < 0.001.				

3.1.4. Colonic TNF-α, IL-6 and IL-10 levels

Compared with the normal control group, TNF-α and IL-6 levels in the colonic tissue in the model control group were significantly higher, whereas the anti-inflammatory factor IL-10 level in the model control group was significantly lower (Tables 2–4, Figs. 6–8). Compared with the model control group, the TNF-α and IL-6 levels in the colonic tissue were significantly inhibited by 5-ASA and VSL#3 treatment (Tables 3 and 4, Figs. 6 and 7), whereas the IL-10 level was significantly up-regulated by 5-ASA and VSL#3 (Table 5, Fig. 8).

Table 4
Colon tissue IL-6 in each group

Group	n	IL-6 (pg/mg tissue)	p (vs model control group)
5-ASA	8	28.19 ± 6.80	< 0.0001***
VSL#3	8	99.71 ± 31.14	0.0044**
5-ASA + VSL#3	8	81.43 ± 26.98	0.0012**
Model control	8	254.2 ± 32.49	—
Normal control	8	25.47 ± 5.50	< 0.0001***

Table 3
Colon tissue TNF-α in each group

Group	n	TNF-α (pg /mg tissue)	p (vs model control group)
5-ASA	8	14.66 ± 0.72	0.0125 *
VSL#3	8	25.89 ± 5.25	0.0465 *
5-ASA + VSL#3	8	21.33 ± 4.55	0.0285 *
Model control	8	68.38 ± 18.73	—
Normal control	8	10.49 ± 0.30	0.008 **

Table 5
Colon tissue IL-10 in each group

Group	n	IL-10 (pg/mg tissue)	p (vs model control group)
5-ASA	8	83.70 ± 24.29	0.0438*
VSL#3	8	110.50 ± 33.98	0.0341*
5-ASA + VSL#3	8	78.61 ± 22.42	0.0470*
Model control	8	25.42 ± 7.0	—
Normal control	8	133.4 ± 12.72	< 0.0001***

3.1.5. VSL#3 suppresses transcription activity of NF-κB in colon tissue

To explore if VSL#3 affected the transcription activity of NF-κB, we extracted the nuclear protein from colonic tissue, and used EMSA to detect the transcription activity. The DNA–protein binding band of NF-κB in the 5-ASA and VSL#3 groups was weaker than that in the model control group (Fig. 9). This demonstrated the transcription activity of NF-κB declined in the VSL#3 and 5-ASA groups, which showed that VSL#3 and 5-ASA inhibited the transcription activity of NF-κB in colonic tissue.

3.1.6. VSL#3 inhibits β-catenin expression in the nucleus of colonic mucosal cells

The distribution of β-catenin in the colonic mucosal cells was shown by immunohistochemistry. In the normal control group, β-catenin was mainly expressed in the cell membrane and cytoplasm. In the model control group, it was mainly expressed in the nucleus and cytoplasm. In the 5-ASA and VSL#3 groups, β-catenin accumulated in the cytoplasm. β-Catenin expression in the nucleus of colonic mucosal cells in the 5-ASA and VSL#3 groups was significantly lower than that in the model control group. This showed that the nuclear import of β-catenin was inhibited by 5-ASA and VSL#3 (Fig. 10).

We further used the immunohistochemistry score to represent the exact level of expression in the nucleus. This showed that expression of β-catenin in the 5-ASA and VSL#3 groups was significantly lower than that in the model control group (Table 6, Fig. 11)

Table 6
Immunohistochemistry score of β-catenin in colonic mucosal cell nuclei

Group	n	β-catenin score in nuclei	p (vs model control group)
5-ASA	5	3.20 ± 0.49	0.0497*
VSL#3	5	1.60 ± 0.68	0.0051**
5-ASA + VSL#3	5	2.00 ± 0.45	0.0029**
Model control	5	4.80 ± 0.49	—
Normal control	5	0	—
Note: data shown as mean ± SE. *p < 0.05; **p < 0.01; ***p < 0.001.			

3.1.7. VSL#3 suppresses transcription activity of TCF-4 in colonic tissue

We used EMSA to measure the transcription activity of TCF-4 in colonic tissue. The DNA–protein binding band of TCF-4 in the 5-ASA and VSL#3 groups was weaker than that in the model control group (Fig. 12). This demonstrated that transcription activity of TCF-4 declined more in the 5-ASA and VSL#3 groups than in the model control group, which indicated down-regulation of the Wnt pathway.

3.2. Results of co-cultured cells with Bifidobacterium

3.2.1 Bifidobacterium inhibited the upregulation of relative luciferase activity induced by IL-6 and TNF-α

In Caco-2 cell line, compared Bifidobacterium plus IL-6 group with IL-6 group, Bifidobacterium plus TNF-α group with TNF-α group, relative luciferase activity decreased significantly (p = 0.0016, 0.0003). The declination of Bifidobacterium group compared with control group was not statistically significant (p = 0.7399), which might related to the low expression without stimulation from inflammatory factors. Furthermore, we also carried out the comparison between IL-6 group and control group, TNF-α group and control group, relative luciferase activity significantly elevated (both P < 0.001), which suggested the Wnt signal pathway activation of IL-6 and TNF-α. On the other hand, there was no difference between IL-6 group and TNF-α group (P = 0.7777) (Table 7, Fig. 13). As shown in Table 8 and Fig. 14, results in CCC-HIE-2 cell line showed similar trends.

Table 7

Effects of Bifidobacterium on relative luciferase activity stimulated by IL-6 and TNF-α in Caco-2 cells

Group	Mean ± SE	p value
Caco-2	2.9632 ± 0.1174	—
Caco-2 + Bifidobacterium	2.7514 ± 0.2167	0.7399[a]
Caco-2 + IL-6	6.6044 ± 0.2969	—
Caco-2 + Bifidobacterium + IL-6	4.0805 ± 0.4622	0.0016[b]
Caco-2 + TNF-α	6.7843 ± 0.8590	—
Caco-2 + Bifidobacterium + TNF-α	3.6025 ± 0.2542	0.0003[c]
a: compared with Caco-2 group. b: compared with Caco-2 + IL-6 group. c: compared with Caco-2 + TNF-α group. n = 4		

Table 8

Relative luciferase activity in each group in CCC-HIE-2 cells

Group	Mean ± SE	p value
CCC-HIE-2	1.0434 ± 0.0435	—
CCC-HIE-2 + Bifidobacterium	0.7543 ± 0.0393	0.0178[a]
CCC-HIE-2 + IL-6	1.4223 ± 0.1407	—
CCC-HIE-2 + Bifidobacterium + IL-6	1.0008 ± 0.0358	0.0026[b]
CCC-HIE-2 + TNF−α	1.4967 ± 0.1862	—
CCC-HIE-2 + Bifidobacterium + TNF−α	1.1049 ± 0.0057	0.0044[c]

3.2.2. Bifidobacterium has no effect on the expression level of β-catenin mRNA

Both in Caco-2 cells and CCC-HIE-2 cells, there was no statistical significance in each group, Bifidobacterium had no effect on β-catenin mRNA expression level (Table 9, Fig. 15 and Table 10, Fig. 16).

Table 9
Effects of Bifidobacterium and inflammatory factors on the level of β-catenin mRNA in Caco-2 cells

Group	Mean ± SE	p value
Caco-2	0.6475 ± 0.1523	0.1317
Caco-2 + Bifidobacterium	0.5810 ± 0.1555	
Caco-2 + IL-6	0.8947 ± 0.3299	
Caco-2 + Bifidobacterium + IL-6	0.6786 ± 0.0888	
Caco-2 + TNF-α	0.8662 ± 0.0414	
Caco-2 + Bifidobacterium + TNF-α	0.8915 ± 0.0393	

Table 10
Effects of Bifidobacterium and inflammatory factors on the level of β-catenin mRNA in CCC-HIE-2 cells

Group	Mean ± SE	P value
CCC-HIE-2	0.8772 ± 0.0466	0.1076
CCC-HIE-2 + Bifidobacterium	1.0186 ± 0.0652	
CCC-HIE-2 + IL-6	1.0399 ± 0.0272	
CCC-HIE-2 + Bifidobacterium + IL-6	1.0322 ± 0.0478	
CCC-HIE-2 + TNF−α	0.9458 ± 0.0442	
CCC-HIE-2 + Bifidobacterium + TNF−α	0.9319 ± 0.0276	

3.2.3. Bifidobacterium has no effect on the expression level of β-catenin protein

After treatment with Bifidobacterium, IL-6 and TNF-α, there was no obvious change in protein expression level of β-catenin in Caco-2 cells (Fig. 17) and CCC-HIE-2 cells (Fig. 18).

3.2.4. Bifidobacterium downregulated the level of IL-6 and TNF-α

3.2.4.1. Bifidobacterium downregulated the level of IL-6

The level of IL-6 declined obviously in Caco-2 + Bifidobacterium group, Caco-2 + Bifidobacterium + IL-6 group and Caco-2 + Bifidobacterium + TNF-α group, when compared with their corresponding group without Bifidobacterium (p = 0.0099, 0.0163, 0.0065). Besides, Caco-2 + TNF-α group showed higher IL-6 level than Caco-2 group (P = 0.0267), which implied the crosstalk between different inflammatory factors (Table 11, Fig. 19). As for CCC-HIE-2, similar results were obtained (Table 12, Fig. 20).

Table 11
The effects of Bifidobacterium on the level of IL-6 in Caco-2 cells

Group	IL-6 (Mean ± SE, pg/ml)	p value
Caco-2	1.2178 ± 0.2542	—
Caco-2 + Bifidobacterium	0.0432 ± 0.0157	0.0099[a]
Caco-2 + IL-6	3233.1833 ± 398.0008	—
Caco-2 + Bifidobacterium + IL-6	1636.8380 ± 274.0969	0.0163[b]
Caco-2 + TNF-α	2.9307 ± 0.4311	—
Caco-2 + Bifidobacterium + TNF-α	0.3895 ± 0.2305	0.0065[c]
a: compared with Caco-2 group. b: compared with Caco-2 + IL-6 group. c: compared with Caco-2 + TNF-α group. n = 4		

Table 12
The effects of Bifidobacterium on the level of IL-6 in CCC-HIE-2 cells

Group	IL-6 (Mean ± SE, pg/ml)	P value
CCC-HIE-2	1.6802 ± 0.1216	—
CCC-HIE-2 + Bifidobacterium	0.5406 ± 0.0227	< 0.001[a]
CCC-HIE-2 + IL-6	3077.0467 ± 429.7852	—
CCC-HIE-2 + Bifidobacterium + IL-6	1809.2365 ± 239.1365	0.0419[b]
CCC-HIE-2 + TNF−α	3.6714 ± 0.1395	—
CCC-HIE-2 + Bifidobacterium + TNF−α	2.5392 ± 0.0528	0.0003[c]
a: compared with CCC-HIE-2 group. b: compared with CCC-HIE-2 + IL-6 group. c: compared with CCC-HIE-2 + TNF-α group. n = 4		

3.2.4.2. Bifidobacterium downregulated the level of TNF-α

Similar to 3.2.4.1, three groups treated with Bifidobacterium showed lower level of TNF-α than those without intervention of Bifidobacterium (p = 0.0390, 0.0406, 0.0099). In addition, compared Caco-2 + IL-6 group with Caco-2 group, Caco-2 + Bifidobacterium + IL-6 group with Caco-2 + Bifidobacterium group, TNF-α level was increased (P = 0.0178, 0.0337) (Table 13, Fig. 21).Level of TNF-α displayed alike results in CCC-HIE-2 cells (Table 14, Fig. 22)

Table 13
The effects of Bifidobacterium on the level of TNF-α in Caco-2 cells

Group	TNF-α (Mean ± SE, pg/ml)	P value
Caco-2	2.3345 ± 0.4448	—
Caco-2 + Bifidobacterium	0.1440 ± 0.0316	0.0390[a]
Caco-2 + IL-6	9.3131 ± 0.8334	—
Caco-2 + Bifidobacterium + IL-6	3.9962 ± 0.7251	0.0406[b]
Caco-2 + TNF-α	2722.0430 ± 468.6997	—
Caco-2 + Bifidobacterium + TNF-α	372.9480 ± 206.6860	0.0099[c]
a: compared with Caco-2 group. b: compared with Caco-2 + IL-6 group. c: compared with Caco-2 + TNF-α group. n = 4		

Table 14
The effects of Bifidobacterium on the level of TNF−α in CCC-HIE-2 cells

Group	TNF−α (Mean ± SE, pg/ml)	p value
CCC-HIE-2	3.2799 ± 0.4620	—
CCC-HIE-2 + Bifidobacterium	0.1617 ± 0.1155	0.0225[a]
CCC-HIE-2 + IL-6	3.3954 ± 0.1155	—
CCC-HIE-2 + Bifidobacterium + IL-6	2.3560 ± 0.2310	0.0565[b]
CCC-HIE-2 + TNF-α	3405.1068 ± 299.0901	—
CCC-HIE-2 + Bifidobacterium + TNF−α	853.4675 ± 160.4092	0.0003[c]
a: compared with CCC-HIE-2 group. b: compared with CCC-HIE-2 + IL-6 group. c: compared with CCC-HIE-2 + TNF-α group. n = 4		

3.2.5. Bifidobacterium suppressed transcription activity of NF-κB

Compared Caco-2 + Bifidobacterium + IL-6 group with Caco-2 + IL-6 group, Caco-2 + Bifidobacterium + TNF-α group with Caco-2 + TNF-α group, the DNA-protein binding band of NF-κB was significantly weakened, indicating that Bifidobacterium could inhibit its transcription activity. Caco-2 + IL-6 group and Caco-2 + TNF-α group were compared with Caco-2 group, respectively. The results suggested that the binding band enhanced, implying that inflammatory factors could increase the transcription activity of NF-κB (Fig. 23). Transcription activity of NF-κB showed same trend of change in CCC-HIE-2 cells (Fig. 24).

3.2.6. Bifidobacterium reduced the level of β-catenin nucleoprotein

Compared Caco-2 + Bifidobacterium group, Caco-2 + Bifidobacterium + IL-6 group, Caco-2 + Bifidobacterium + TNF-α group with their corresponding non-co-culture group, expression level of β-catenin protein in nucleus was declined distinctly. Moreover, IL-6 and TNF-α could increase the expression of β-catenin in nucleoprotein (Fig. 25). As indicated in Fig. 26, level of β-catenin nucleoprotein was declined in CCC-HIE-2 cells.

3.2.7. Bifidobacterium suppressed transcription activity of TCF-4

Compared Caco-2 + Bifidobacterium + IL-6 group with Caco-2 + IL-6 group, Caco-2 + Bifidobacterium + TNF-α group with Caco-2 + TNF-α group, TCF-4 DNA-protein shift binding remarkably weakened. Compared with Caco-2, shift binding in Caco-2 + IL-6 group and Caco-2 + TNF-α group was stronger (Fig. 27). In CCC-HIE-2 cells, similar trends was achieved as shown in Fig. 28.

4. Discussion

We found that treatment with VSL#3 (1.5×10^9 CFU/d) significantly reduced the tumor formation rate and tumor load in a mouse model of UC carcinogenesis. This proved the protective effect of VSL#3, which was consistent with previous studies[9 – 11]. After verifying the protective function of VSL#3, we further explored its potential mechanism of action. As the main component of VSL#3, Bifidobacterium was chosen to study at the level of cell.

Although the exact mechanism of action of probiotics in UC carcinogenesis is still unclear, some studies have explored how intestinal microbiota participate in the occurrence and development of UC. On the one hand, when the balance of intestinal microbiota is disrupted, the intestinal barrier function is destroyed by the toxic materials produced by pathogenic bacteria. Inflammatory factors including TNF-α and IL-6 are produced and trigger the inflammatory reaction after the pathogens and toxins enter the intestinal mucosa[17]. On the other hand, the intestinal microbiota acts on the receptors of epithelia cells, dendritic cells and other antigen-presenting cells, which leads to activation and differentiation of lymphocytes and triggers Th1 or Th2 immune reactions. This finally causes the release of inflammatory mediators and

mucosal damage[18]. The recognition of intestinal mucosa by the microbiota is mediated by pattern recognition receptors (PRRs), which are upstream molecules of NF-κB, and NF-κB activation triggers the transcription of many types of inflammatory factors[19].

Previous studies[17 – 19] have shown that imbalance of the intestinal microbiota can cause the release of inflammatory factors and activation of inflammatory pathways. Repetitive inflammatory reactions are the pathological basis and initiation factor of UC carcinogenesis, and the development model of UC carcinogenesis is referred to as inflammation—intraepithelial neoplasia—cancer[20]. So, we infer that the preventive effect of VSL#3 on UC carcinogenesis may be related to inhibition of inflammatory reactions after the modulation and re-establishment of the intestinal microbiota.

Therefore, we tested the levels of inflammatory factors, including the pro-inflammatory TNF-α and IL-6 as well as the anti-inflammatory IL-10. TNF- α is the key initiator of UC carcinogenesis. In a previous UC carcinogenesis model, the degree of inflammation, tumor formation rate and tumor size in mice lacking TNF-α receptor P55 was significantly lower than in wild-type mice[21]. Furthermore, after treating wild-type mice with TNF-α antagonist, their tumor formation was obviously inhibited. IL-6 is also an important inflammatory factor participating in UC carcinogenesis. One study found the tumor load of IL-$6^{-/-}$ mice decreased more than in wild-type mice in an AOM/DSS-induced UC carcinogenesis model[22]. IL-10 is an anti-inflammatory factor and plays an important role in the inhibition of inflammation for the regulation of intestinal balance[23]. The L-$10^{-/-}$ mouse model is commonly used for inducing UC. In our study, we found that after intervention for 12 weeks, the increase in TNF-α and IL-6 caused by AOM/DSS was significantly reduced by VSL#3, and the decrease in IL-10 was significantly elevated. Similarly, co-culture groups showed lower level of TNF-α and IL-6 when compared with their corresponding non-co-culture groups. Our results proved that probiotics inhibits the activation of pro-inflammatory factors, which is the initial stage of UC carcinogenesis, and promotes anti-inflammatory factors, consequently preventing the occurrence of UC carcinogenesis. Our finding was consistent with a previous study[9], but that study did not explore the mechanism of action of probiotics to prevent UC carcinogenesis. So, we explored the next steps in its inhibitory effect on UC carcinogenesis.

NF-κB is a multidirectional transcription-modulating factor and has a critical function in the development and translation from inflammation to cancer. The classical activation pathway of NF-κB can be triggered by bacterial lipopolysaccharide and pro-inflammatory factors including IL-1β and TNF-α. After a series of signal transductions, activated NF-κB enters the cell nucleus and combines with various target genes, finally modulating expression of different cytokines including IL-1β, IL-2, IL-6, IL-8, IL-12, granulocyte–macrophage colony-stimulating factor and TNF, as well as cell adhesion molecules[24]. It has been proved that activation of the NF-κB pathway is the core component of the inflammation of UC[25]. In addition, the NF-κB pathway also plays an important role in the development of carcinogenesis; perhaps by modulating expression of Bcl2, Bcl-xl and cFLIP genes[26]. NF-κB may be a key factor participating in the inflammation–carcinogenesis transformation. In our study, we confirmed by EMSA that VSL#3 and Bifidobacterium inhibits the transcription activity of NF-κB, illustrating that inhibition of NF-κB activation

may be a potential mechanism by which probiotics prevents UC carcinogenesis. It can also explain the decreased levels of TNF-α and IL-6 in the VSL#3 group, as TNF-α and IL-6 are downstream cytokines in the NF-κB activation pathway. When NF-κB activation was suppressed, expression of TNF-α and IL-6 was also suppressed. There were some limitations to our study, as we did not explore how probiotics work on the NF-κB pathway. From a previous study[27] we can infer the potential mechanism. On the one hand, Toll-like receptor (TLR) on the intestinal mucosa, a type of PRR, was over-activated when the microbiota balance was broken, and it may mediate the recognition of intestinal microbiota. As TLR is an upstream molecule of the NF-κB pathway, the inhibitory effect of VSL#3 on NF-κB activation may be mediated by TLR. On the other hand, TNF-α was an important trigger for activating the NF-κB classic pathway, and we showed that VSL#3 and Bifidobacterium inhibits the level of TNF-α. So, we suggest that probiotics suppresses the activation of the NF-κB classic pathway by inhibition of TNF-α.

Finally, we investigated the Wnt/β-catenin pathway and found that after treatment with VSL#3 and co-cultured with Bifidobacterium, the nuclear import of β-catenin was inhibited and the transcription activation of TCF-4 was suppressed, which implied down-regulation of the Wnt/β-catenin pathway. The Wnt/β-catenin pathway is an important conduction pathway in sporadic colorectal cancer. When the pathway is activated, β-catenin cannot be degraded and accumulated in the cytoplasm. After it enters the nucleus it combines with the T cell factor/lymphoid enhancer-binding factor family, represented by TCF-4, and then acts on the promoter of downstream target genes including c-myc, cyclinD1 and VEGF. This leads to the abnormal expression of related genes to promote the development of colorectal cancer[28]. In other words, the nuclear import of β-catenin and the transcription activation of TCF-4 represent the degree of activation of the Wnt/β-catenin pathway. As well as in sporadic colorectal cancer, previous studies have shown that the Wnt/β-catenin pathway plays an important role in UC carcinogenesis. Protein chips for the tissue specimens of UC carcinogenesis patients show that β-catenin accumulation is closely related to UC carcinogenesis, which implies that there is activation of the Wnt/β-catenin pathway in UC carcinogenesis[29]. However, the initiating factor for activation of the Wnt/β-catenin pathway differs between UC carcinogenesis and sporadic colorectal cancer. Unlike in sporadic colorectal cancer, in which various gene mutations are the initiating factor[30], in UC carcinogenesis, inflammation is the initiating factor for the Wnt/β-catenin pathway[20]. In our study, we demonstrated the inhibitory effect of probiotics on the Wnt/β-catenin pathway, which explains its direct preventive effect on UC carcinogenesis.

In our study, taking into account its clinical significance, we designed the 5-ASA monotherapy group as the positive control, and 5-ASA combined with VSL#3 to simulate the real clinical situation. We confirmed the effect of full-dose 5-ASA (75 mg/kg) for preventing UC carcinogenesis, which was consistent with previous studies[13 – 15]. It was noteworthy that VSL#3 alone treatment could achieve similar, even more obvious inhibitory effect for UC carcinogenesis compared with 5-ASA. In the 5-ASA + VSL#3 group, we did not find that either agent alone had a stronger inhibitory effect on UC carcinogenesis, in relation to tumor load, level of inflammatory factors or accumulation of β-catenin. It may be that gavage of the drug combination caused interaction of the drugs, altering their concentration or even increasing stress on the mice, further influencing the effect of the drugs. Our results imply that the mouse model cannot simulate

the real situation in patients, so we can only explore the potential mechanism in a preliminary way, and provide further ideas for clinical studies in the future.

Our study had some limitations. We only observed the effect of VSL#3 for inhibition of inflammation and carcinogenesis, and did not study the specific site of action of VSL#3 on the intestinal mucosa. We did not investigate the relationship between the specific changes in the microbiota induced by VSL#3 and its inhibitory effect on UC carcinogenesis, and cells in vitro could not completely simulate human. More precise mechanistic studies will be done in the future.

5. Conclusions

Our study illustrated that VSL#3 and 5-ASA can effectively prevent UC carcinogenesis induced by AOM/DSS in mice, both by monotherapy and combined therapy. In addition, at the cellular level, Bifidobacterium could inhibit the activity of Wnt signaling pathway as well. To explain the mechanisms involved, supplementary VSL#3 and co-culture cells with Bifidobacterium may down-regulate the pro-inflammatory factors TNF-α and IL-6 and up-regulate the anti-inflammatory factor IL-10, inhibit the transcription activity of NF-κB, and finally down-regulate the Wnt/β-catenin pathway, consequently preventing the progression from inflammation to carcinogenesis. VSL#3 may be a potential therapeutic agent for prevention of UC carcinogenesis.

Abbreviations

Ulcerative colitis (UC), Dextran sulfate sodium (DSS), Azoxymethane (AOM), 5-Aminosalicylic acid (5-ASA), Tumor necrosis factor (TNF), Interleukin (IL), Electrophoretic mobility shift assay (EMSA), Nuclear factor (NF), Transcription factor (TCF), Minimum essential medium (MEM), Dulbecco's modified eagle medium-high glucose (DMEM-H), Quantitative real-time polymerase chain reaction (qRT-PCR), Polyvinylidene difluoride (PVDF), Standard error (SE), Pattern recognition receptors (PRRs), Toll-like receptor (TLR).

Declarations

Ethics approval and consent to participate

All animal care and experimental procedures in this study were approved by the Animal Care Ethics and Use Committee of Peking Union Medical College(XHDW-2015-0032).

Consent to publish

Not applicable.

Availablility of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declared that there is no Competing Interests related to this study.

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Author Contributions

LWB and WYN are the co-first author, contributed equally to this work. LJN conceived and designed the experiments; LWB, WYN and WCSE performed the experiments, analyzed and interpreted the data; LWB, WYN and LJN drafted and revised the paper; WHY, HXM, J.Y.Kao and C.Owyang gave suggests during the revision of the manuscript and assisted in the the analysis of the experiment data; MYM and ZXH offered help in the experiment operations; YH and QJM participated in the experiments. All authors approved the final version of the manuscript.

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Figures

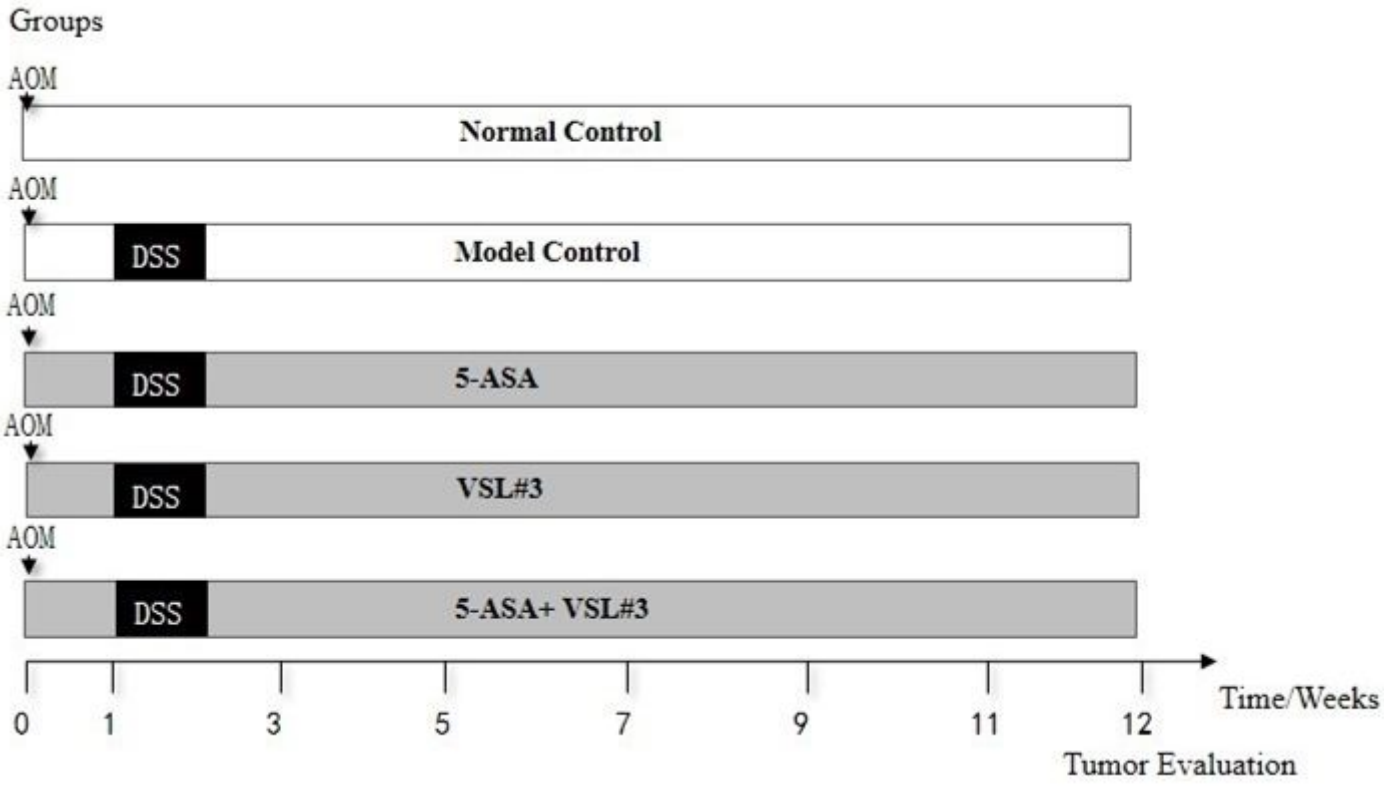

Figure 1

Experimental protocol for UC carcinogenesis model and group setting.

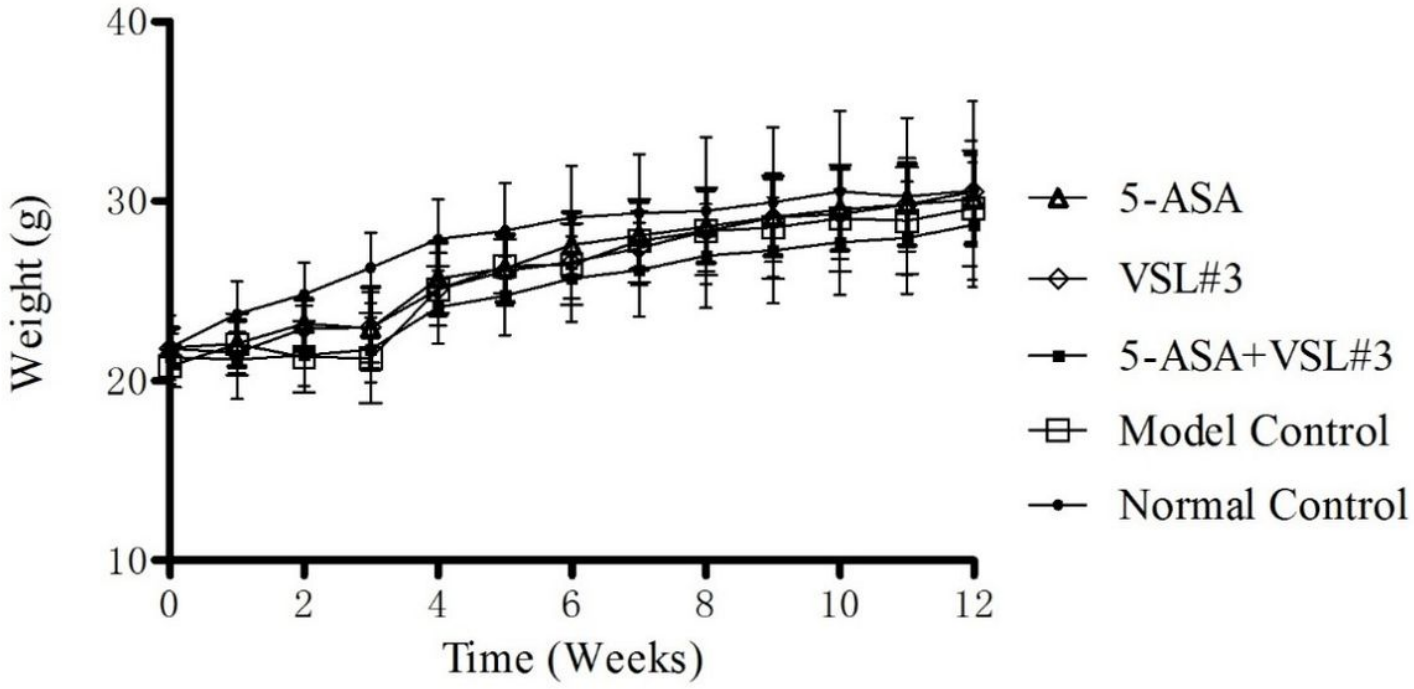

Figure 2

Body weight in each group.

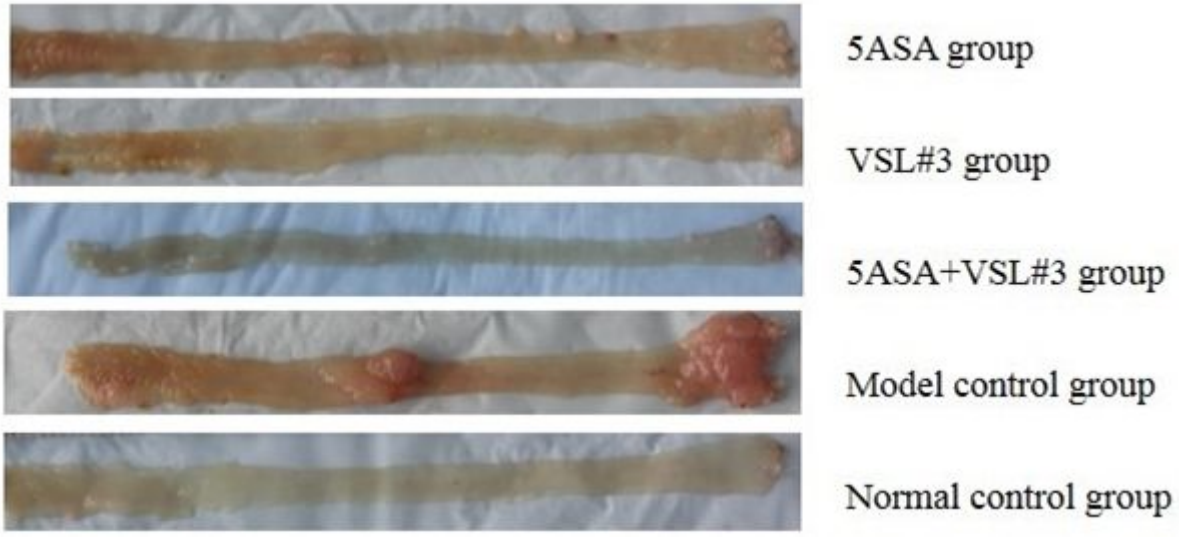

Figure 3

Representative macroscopic image of colonic tumor in each group.

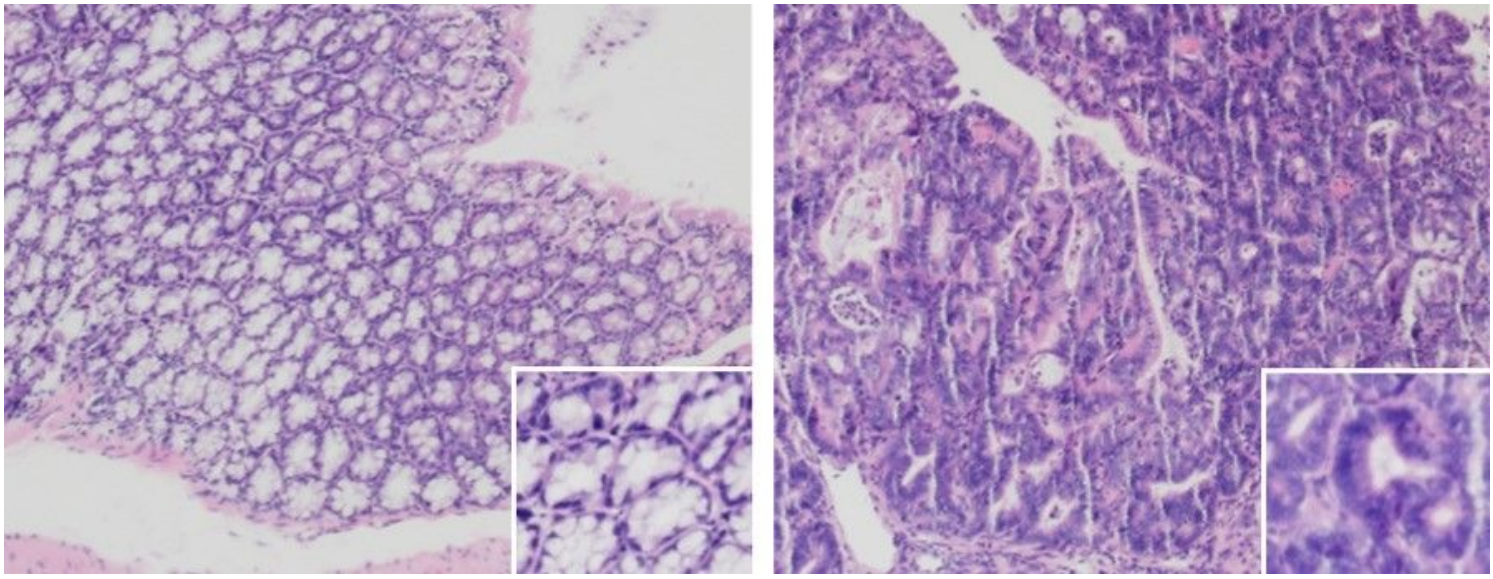

Figure 4

Representative images of hematoxylin and eosin staining of colon tissue examined under a microscope [40× and 100× (inset)]. A: Normal control group: colonic mucosal glands were normal, and the structure was regular. B: Model control group: colonic gland structural disorders, deep nuclear staining, and cytoplasm-to-nuclear ratio decreased.

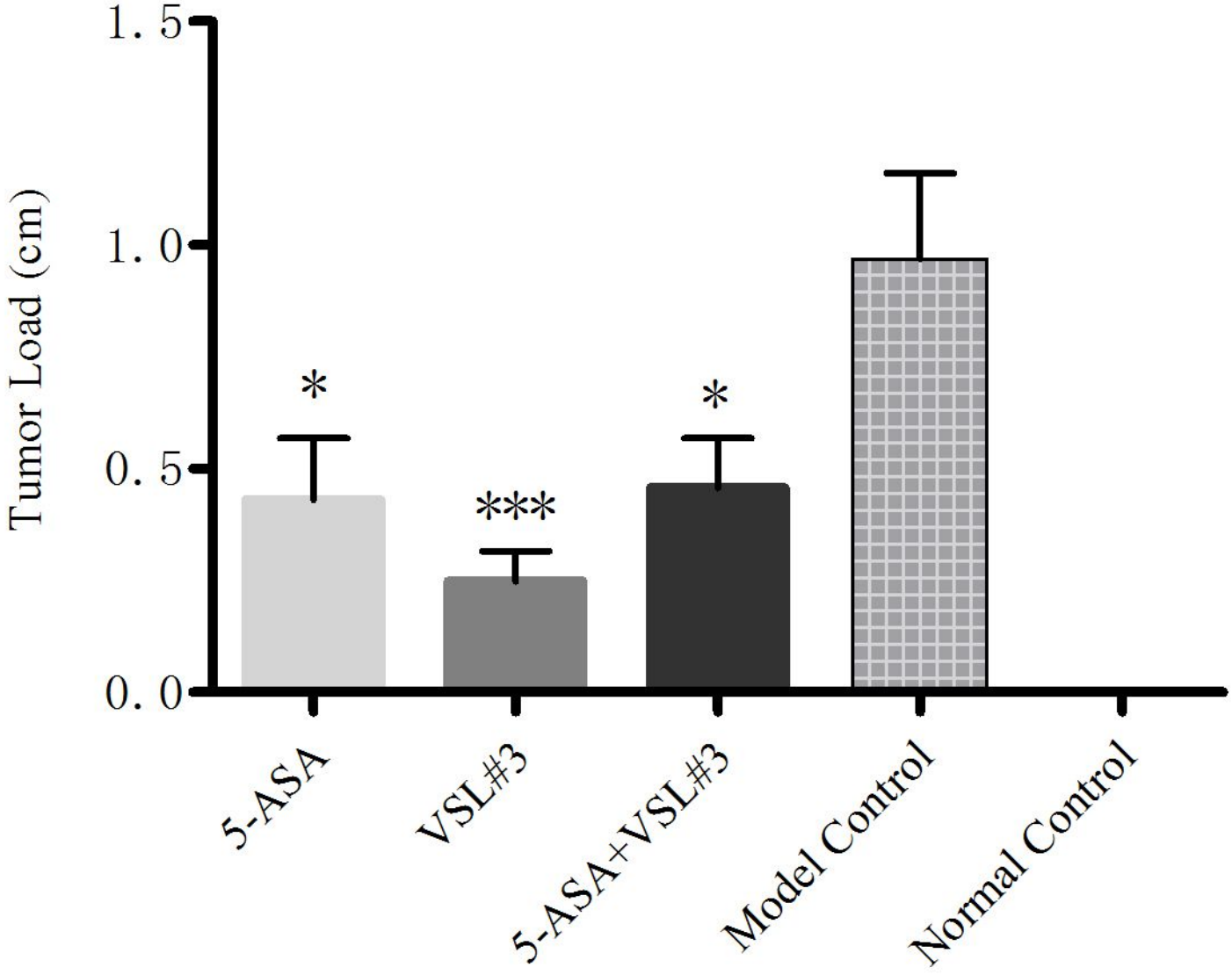

Figure 5

Tumor load in each group (*p <0.05; ** p <0.01; *** p <0.001).

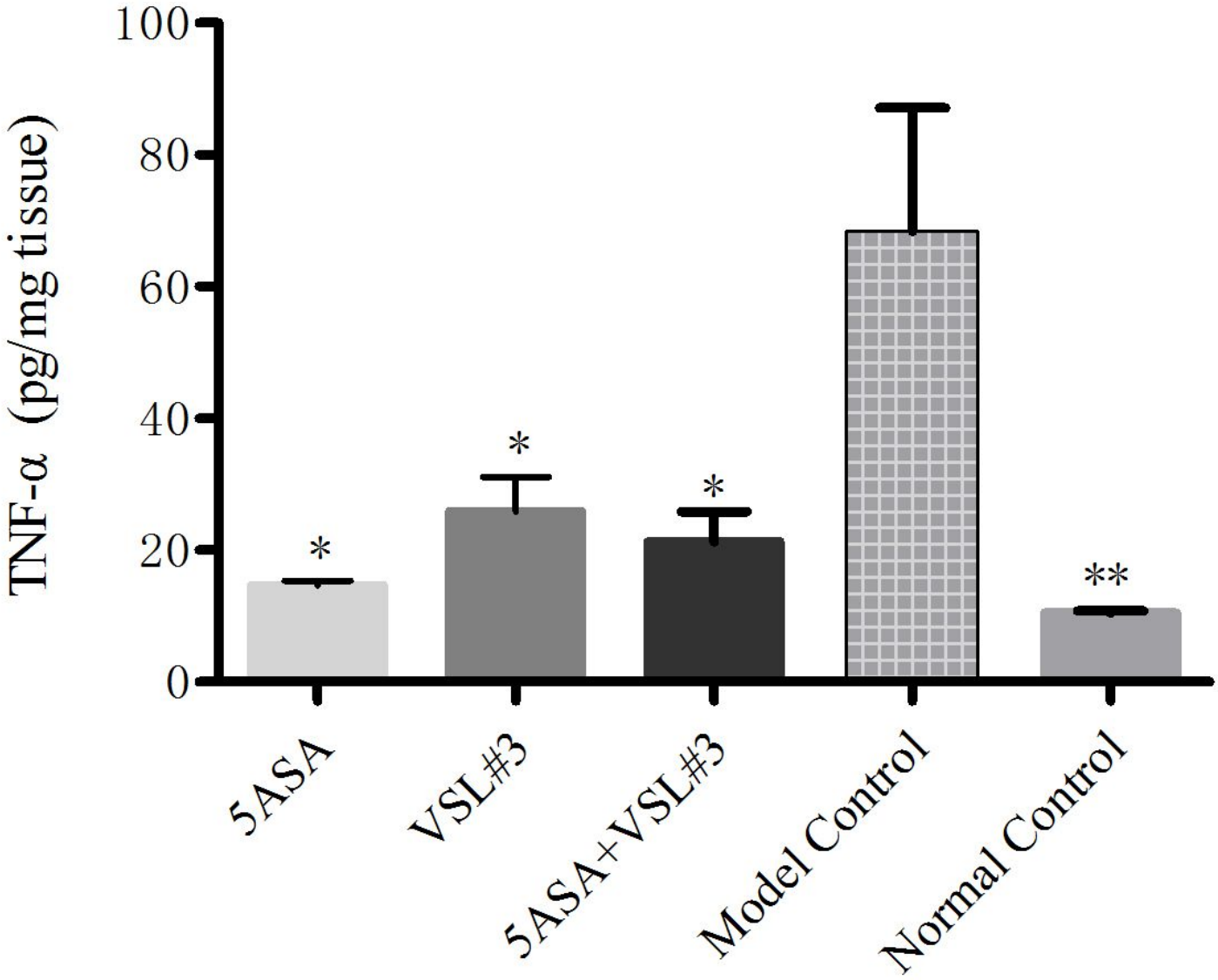

Figure 6

Colonic TNF-α level in each group (*p <0.05; **p <0.01; ***p <0.001).

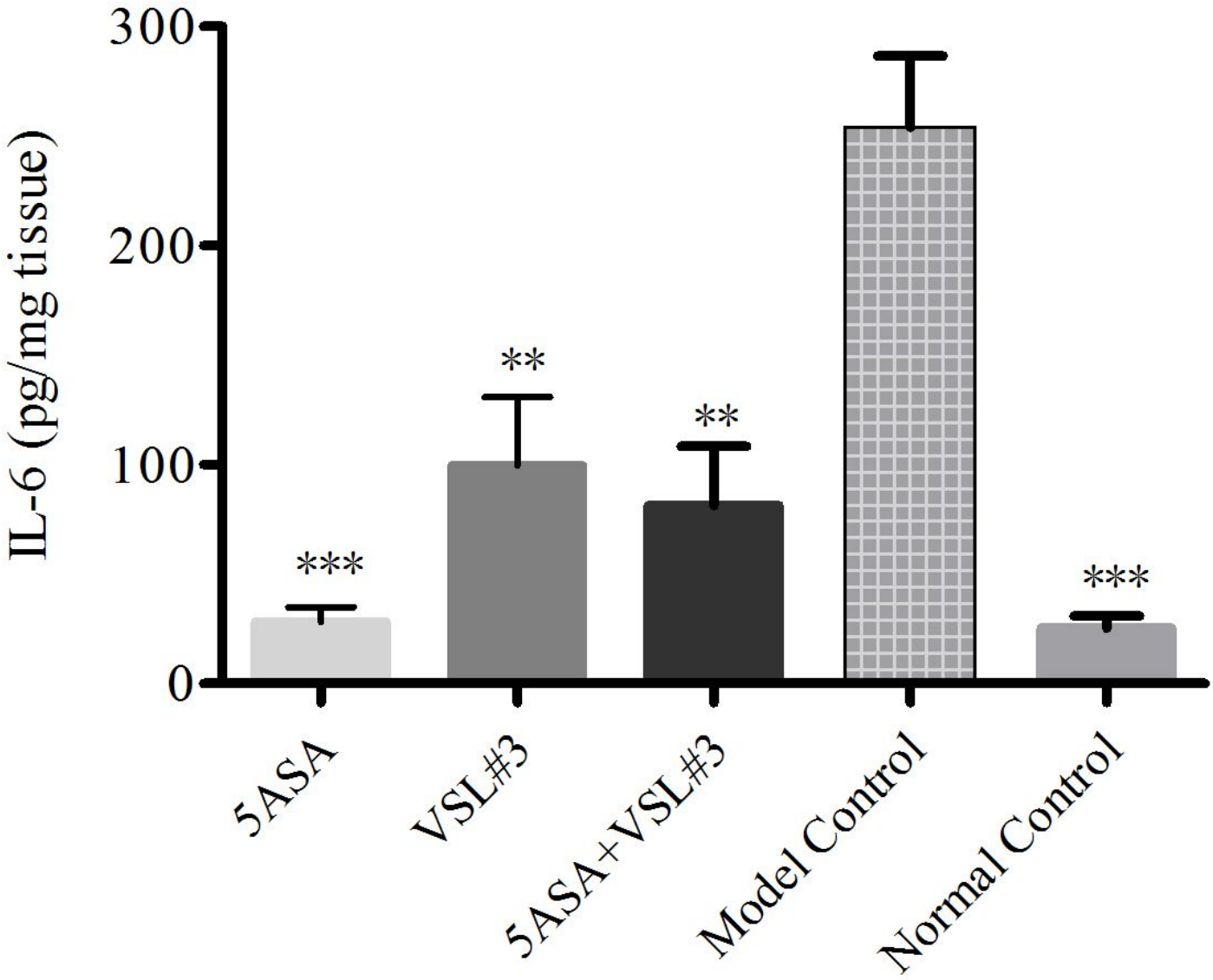

Figure 7

Colonic IL-6 level in each group (*p <0.05; **p <0.01; ***p <0.001).

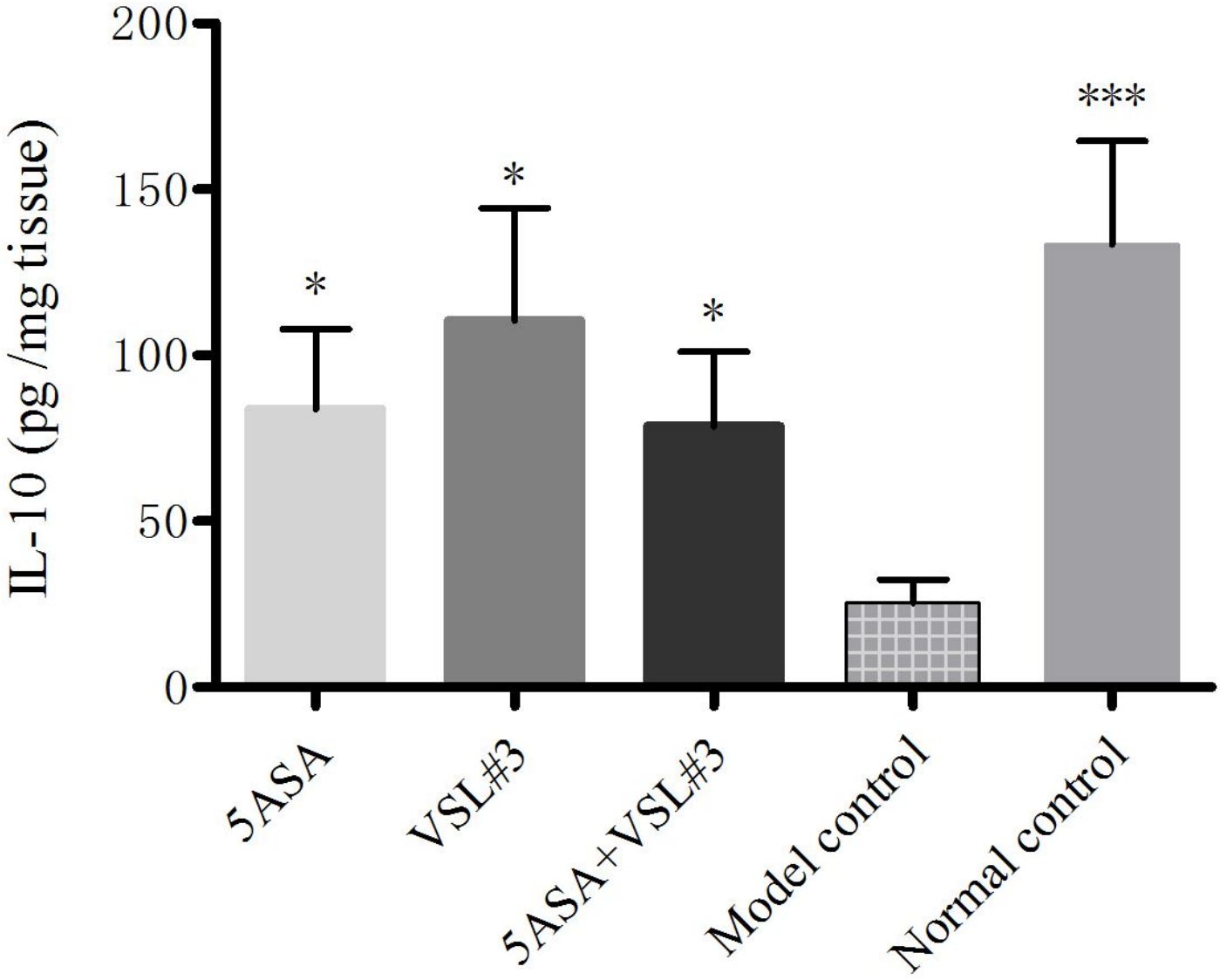

Figure 8

Colonic IL-10 level in each group (*p <0.05; **p <0.01; ***p <0.001).

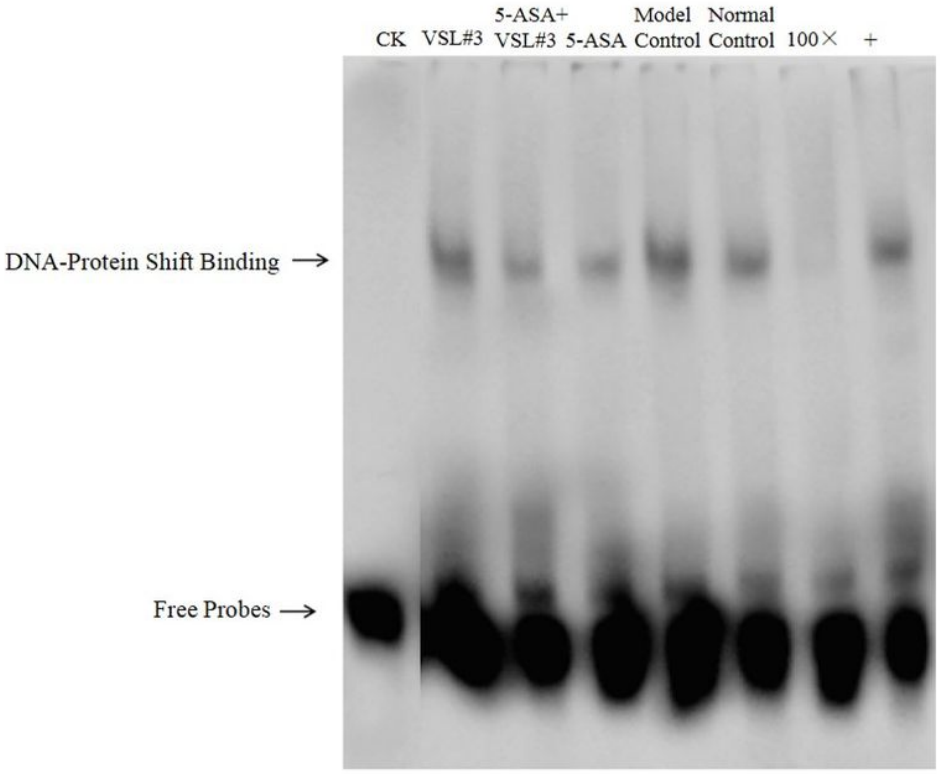

Figure 9

Transcription activity of NF-κB in colonic tissue in each group.

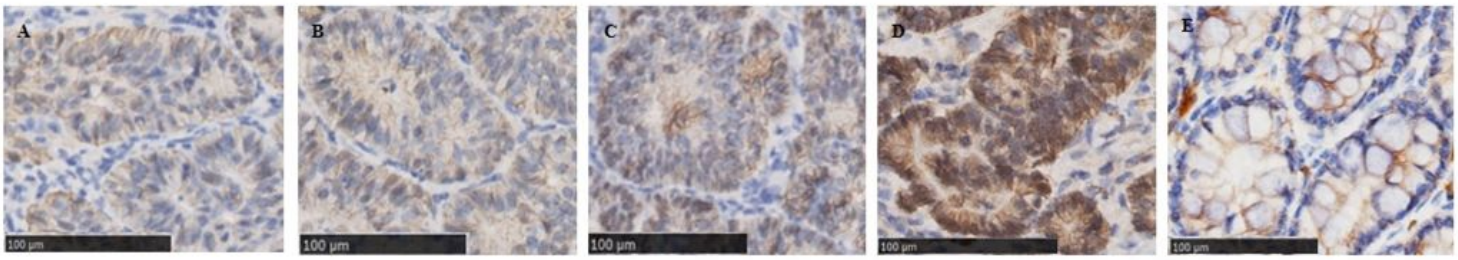

Figure 10

Immunohistochemistry of β-catenin in the nucleus of colonic mucosal cells in each group.(A: 5-ASA, B: VSL#3, C: 5-ASA+VSL#3, D: model control, E: normal control).

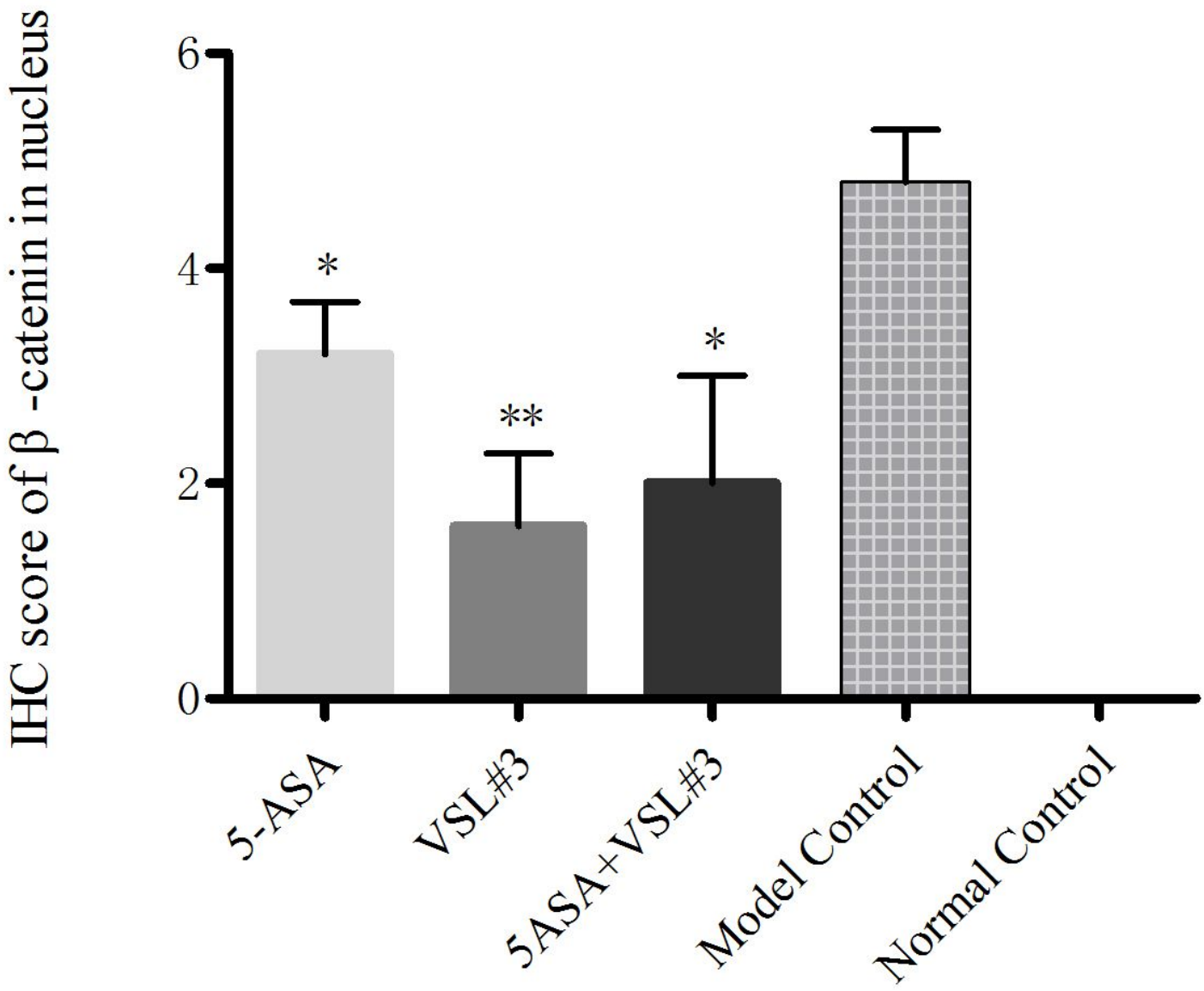

Figure 11

Immunohistochemistry score of β-catenin in colonic mucosal cell nuclei. (*p < 0.05; **p < 0.01; ***p < 0.001).

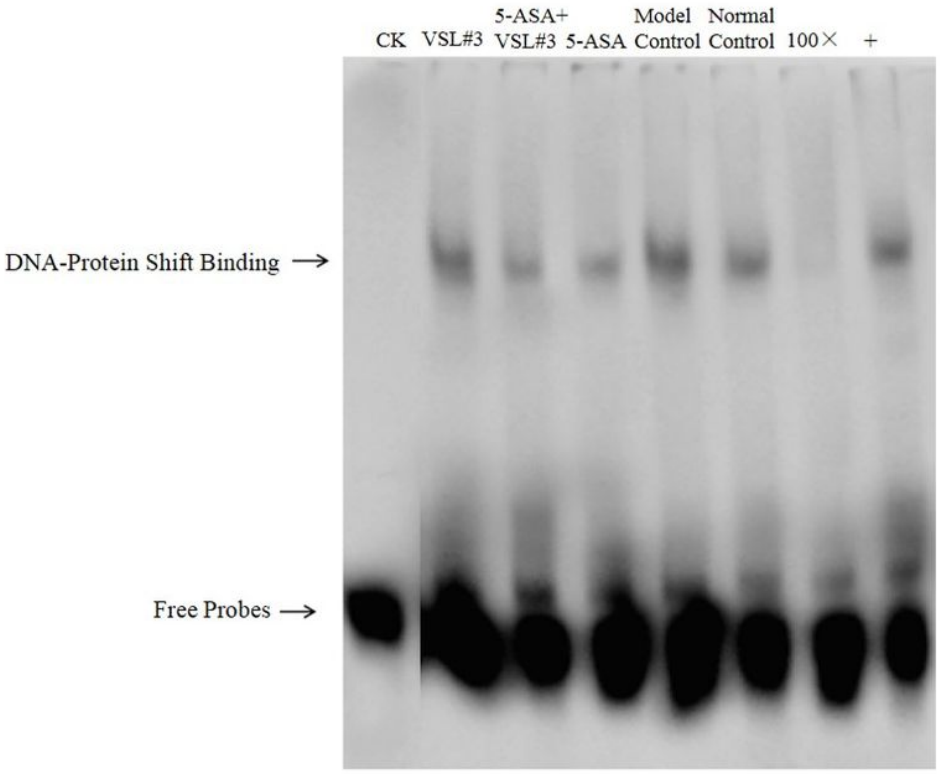

Figure 12

Transcription activity of TCF-4 in the colonic tissue.

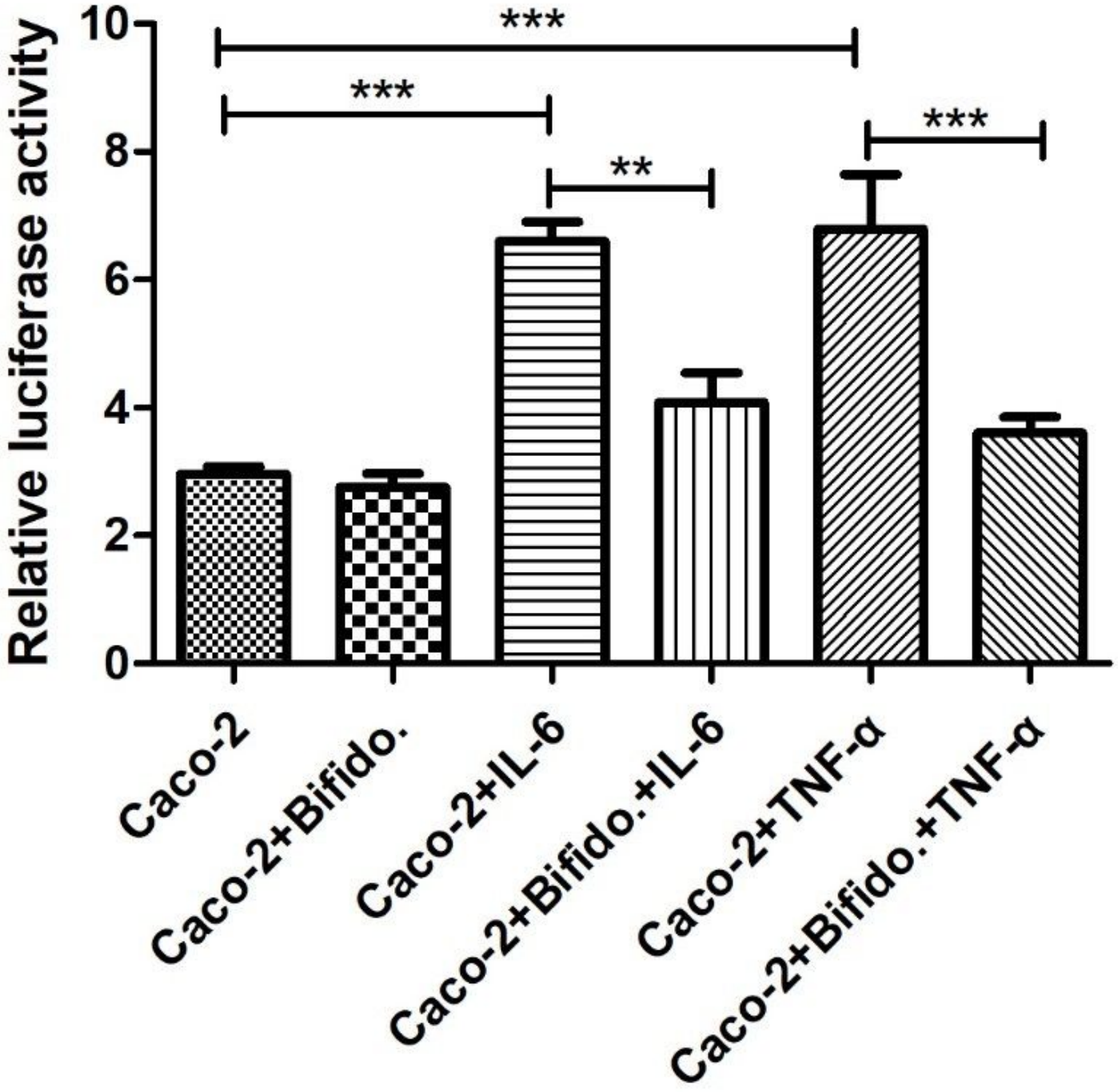

Figure 13

Relative luciferase activity of different groups in Caco-2 cells (*p<0.05； **p <0.01；***p <0.001).

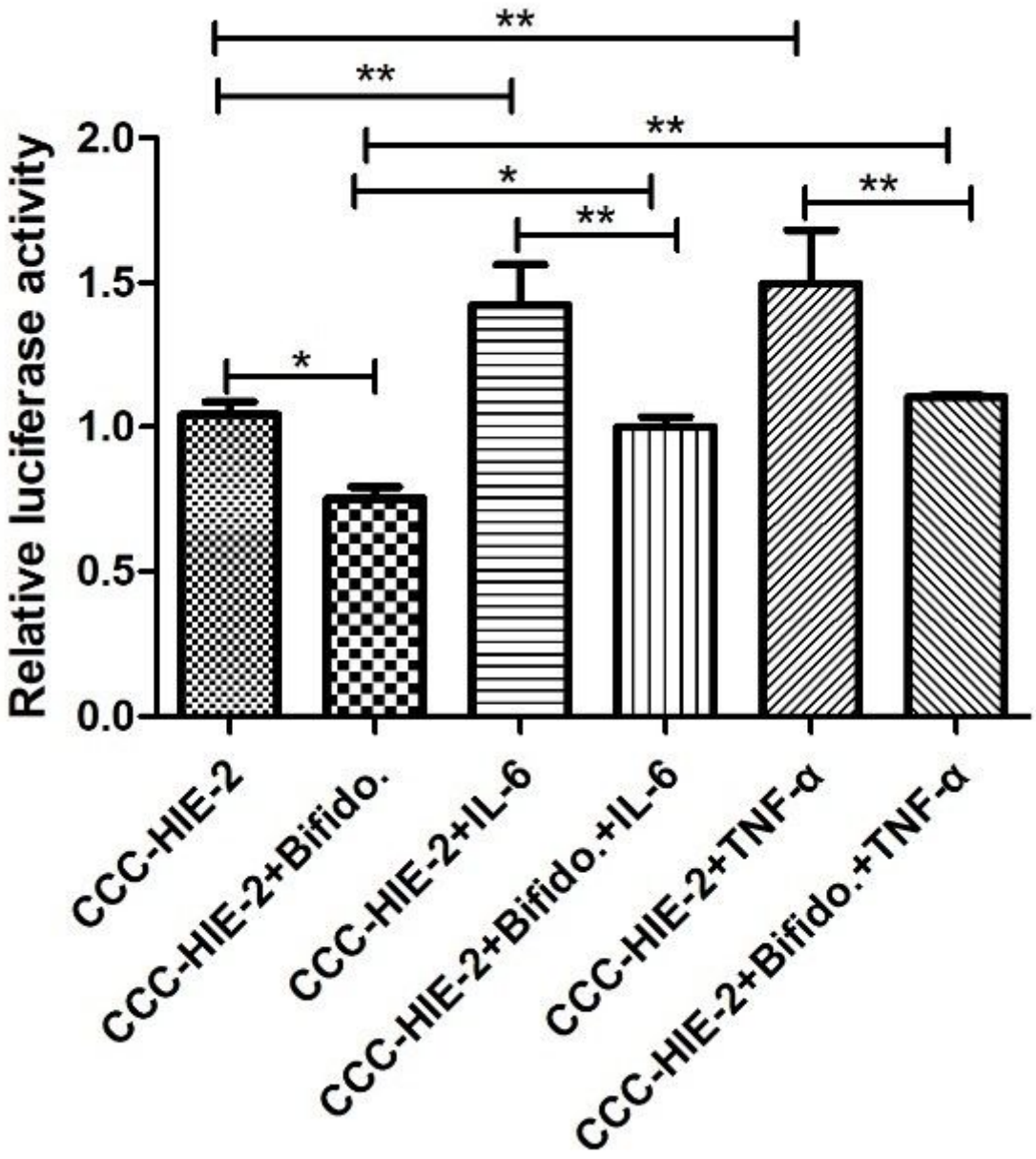

Figure 14

Relative luciferase activity in each group in CCC-HIE-2 cells.

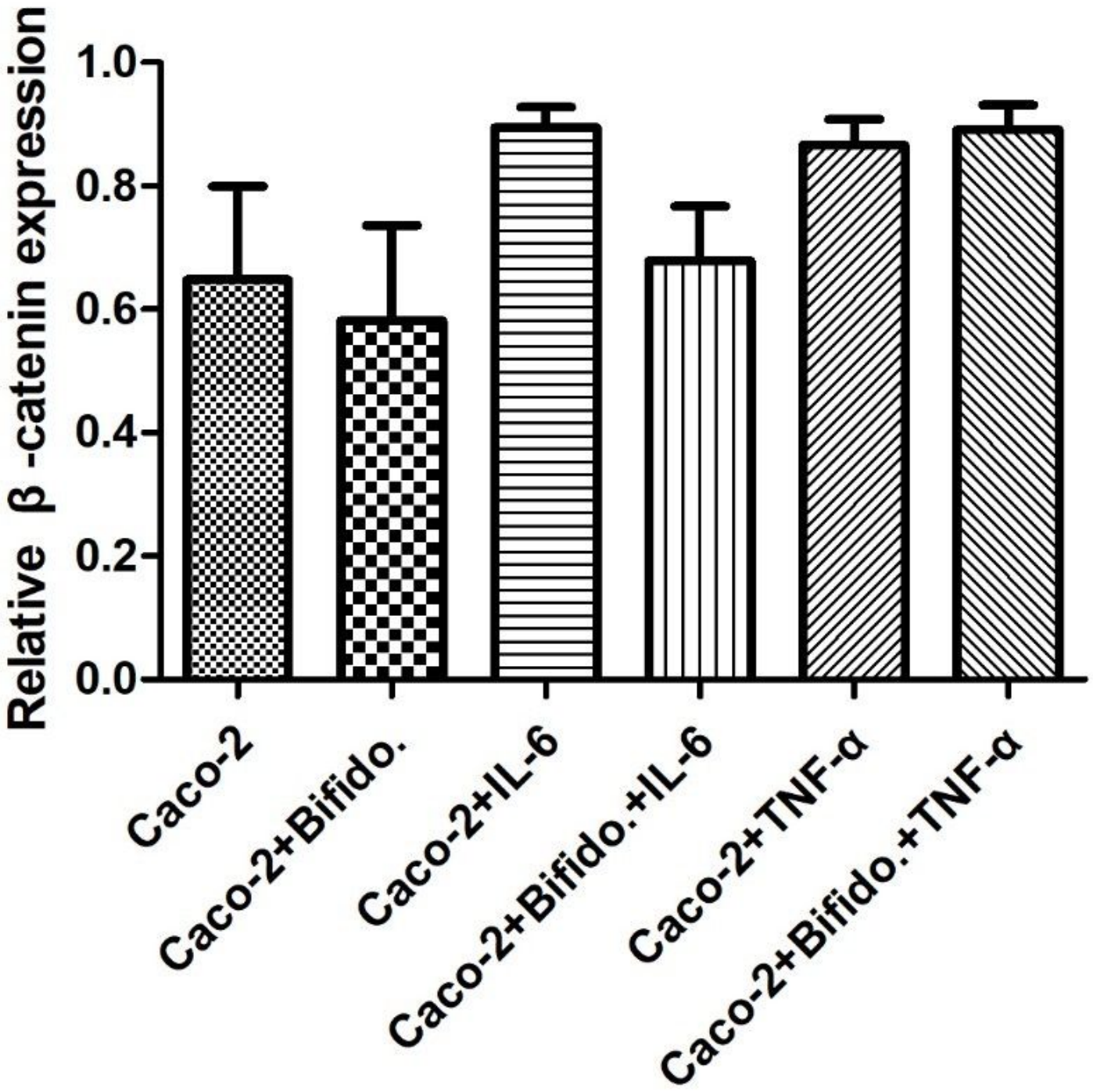

Figure 15

Effects of Bifidobacterium and inflammatory factors on the level of β-catenin mRNA in Caco-2 cells.

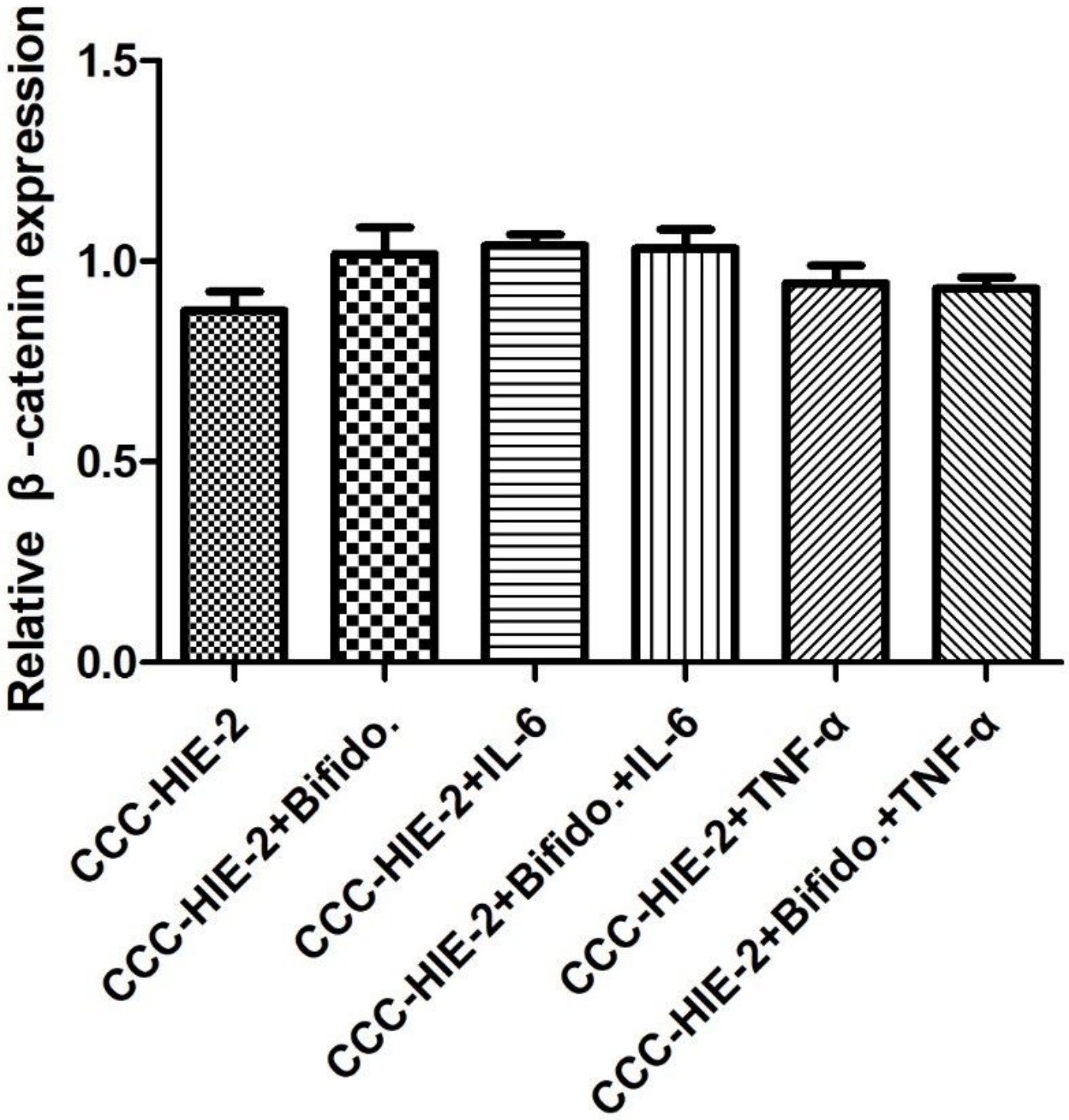

Figure 16

Effects of Bifidobacterium and inflammatory factors on the level of β-catenin mRNA in CCC-HIE-2 cells.

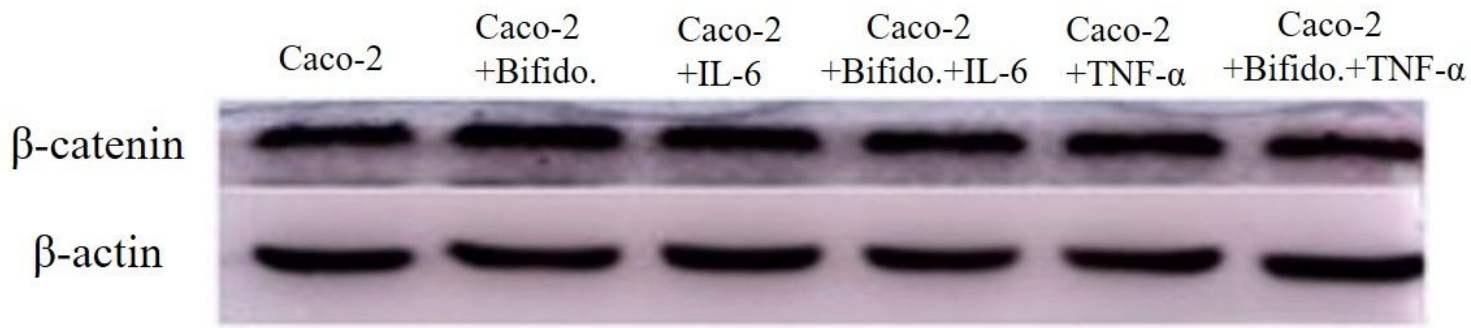

Figure 17

Bifidobacterium has no effect on the expression level of β-catenin protein in Caco-2 cells.

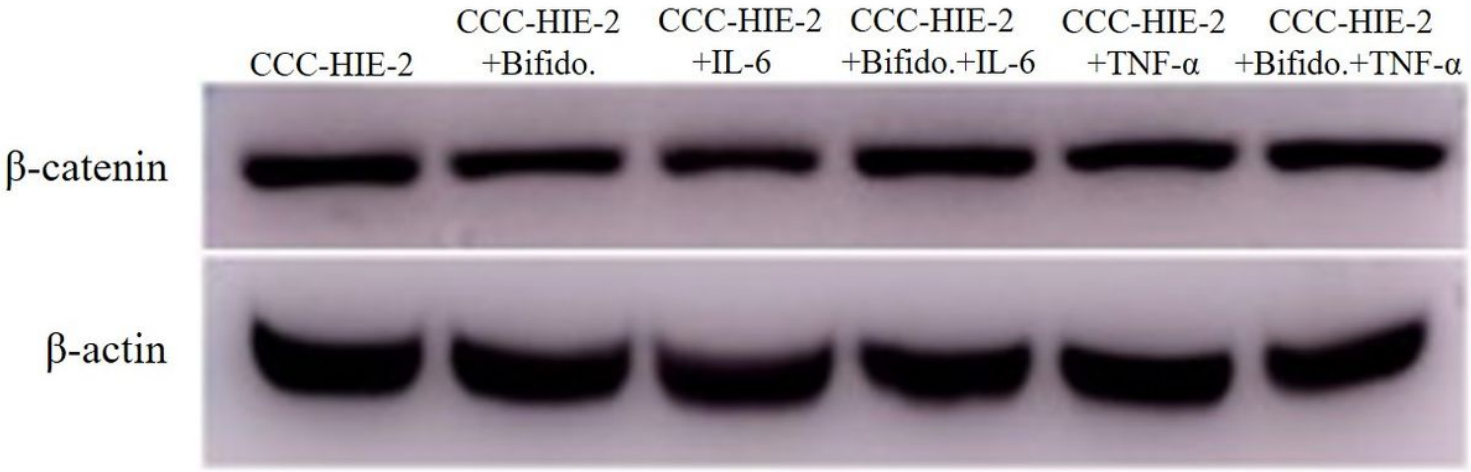

Figure 18

Effects of Bifidobacterium and inflammatory factors on the level of β-catenin protein in CCC-HIE-2 cells.

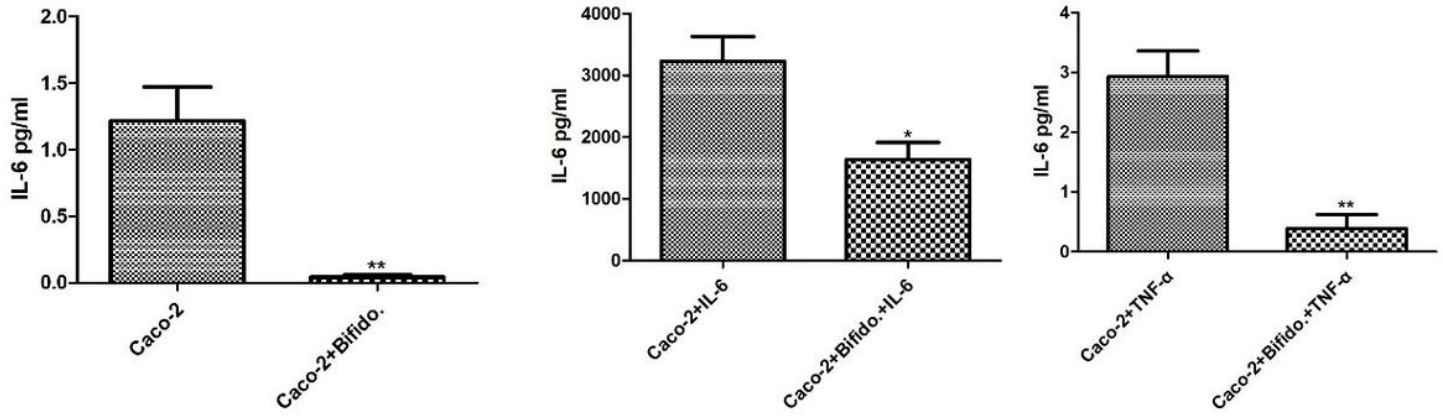

Figure 19

The effects of Bifidobacterium on the level of IL-6 in Caco-2 cells.

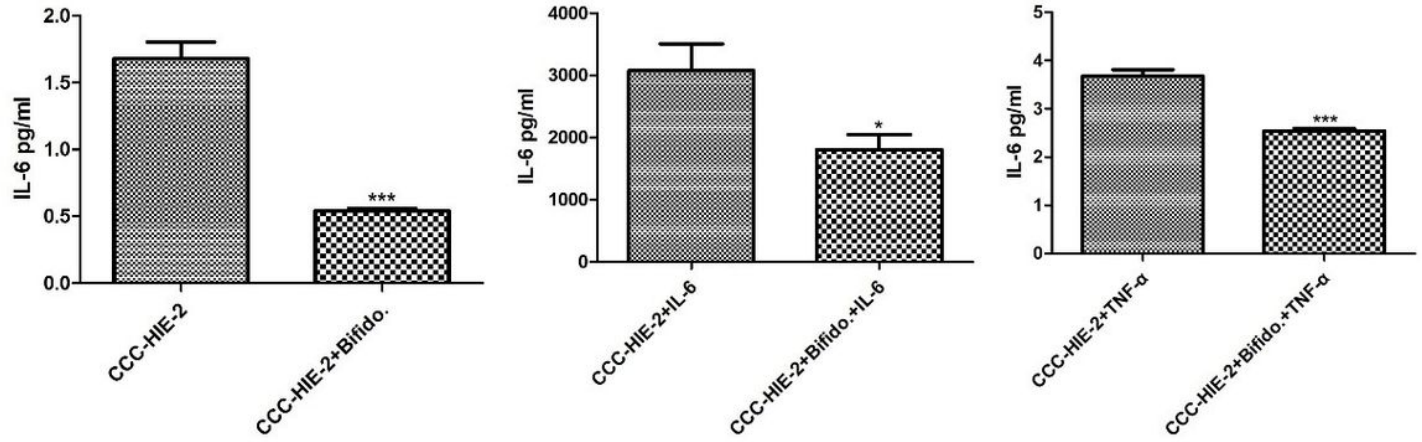

Figure 20

The effects of Bifidobacterium on the level of IL-6 in CCC-HIE-2 cells.

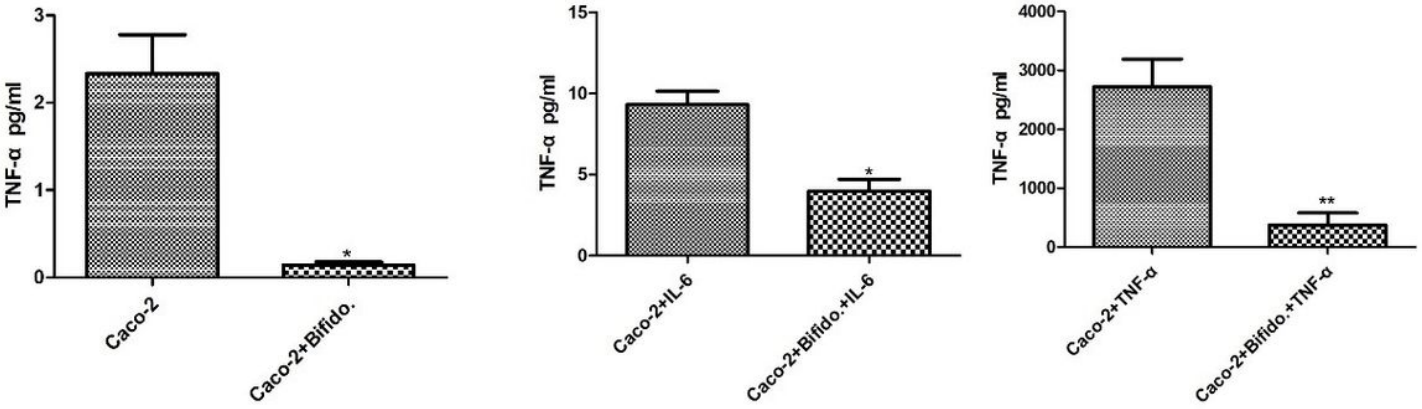

Figure 21

The effects of Bifidobacterium on the level of TNF-α in Caco-2 cells.

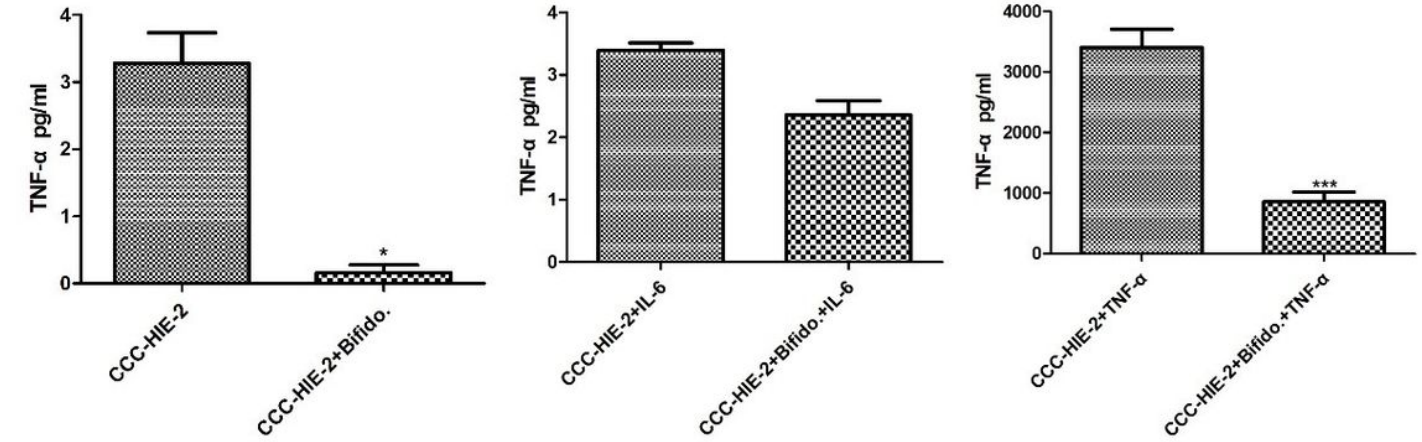

Figure 22

The effects of Bifidobacterium on the level of TNF−α in CCC-HIE-2 cells.

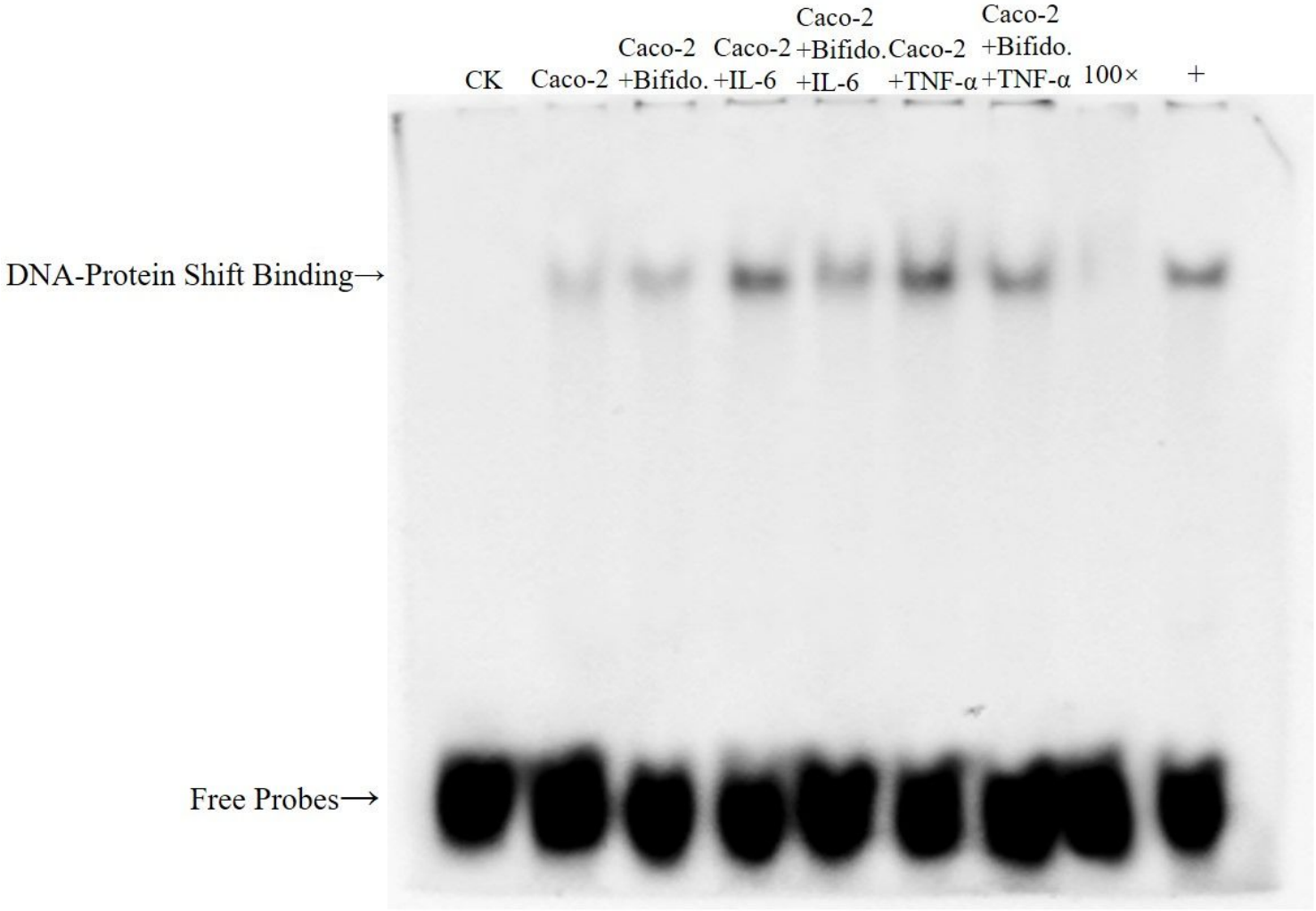

Figure 23

Bifidobacterium suppressed transcription activity of NF-κB in Caco-2 cells.

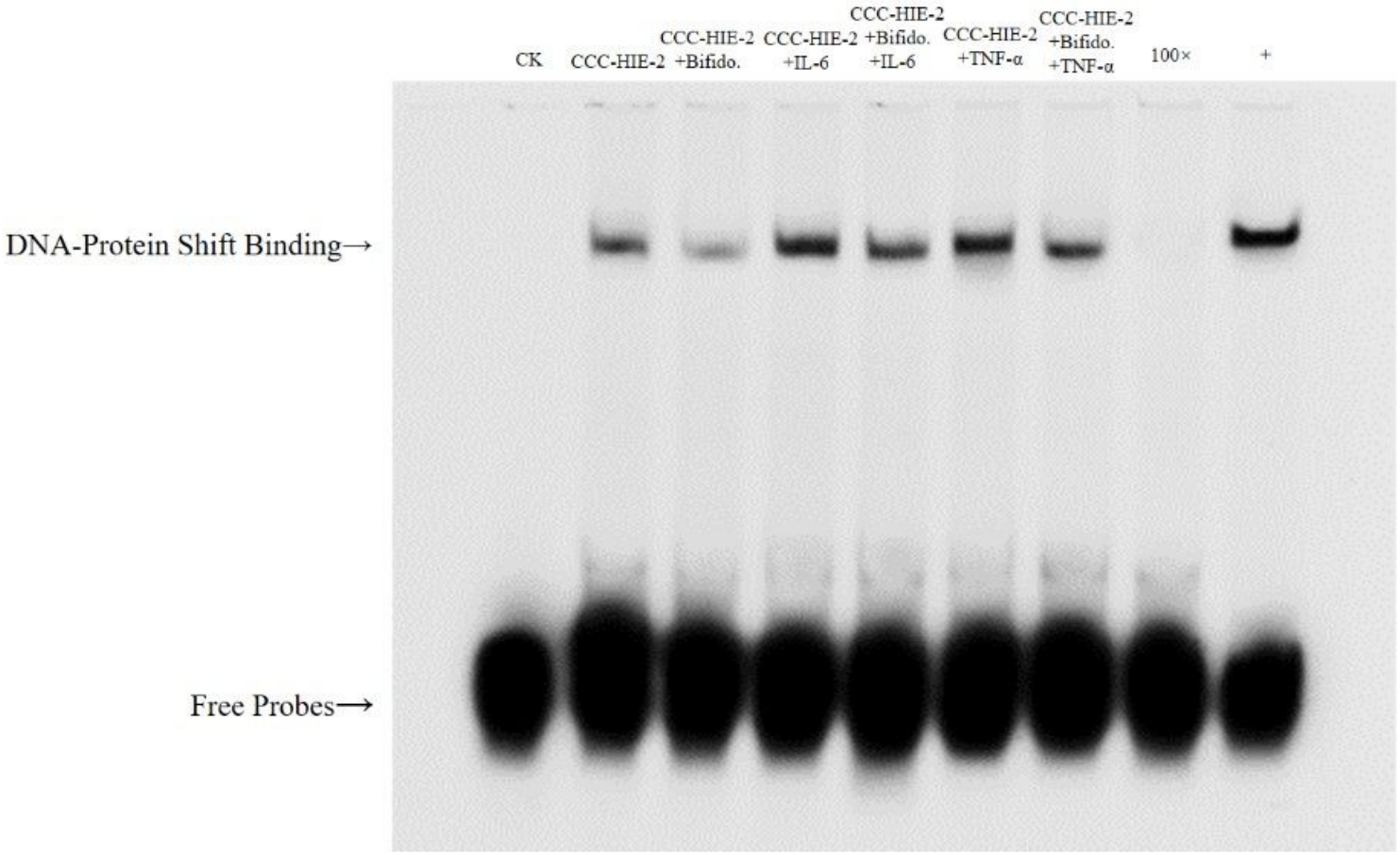

Figure 24

Bifidobacterium suppressed transcription activity of NF-κB in CCC-HIE-2 cells.

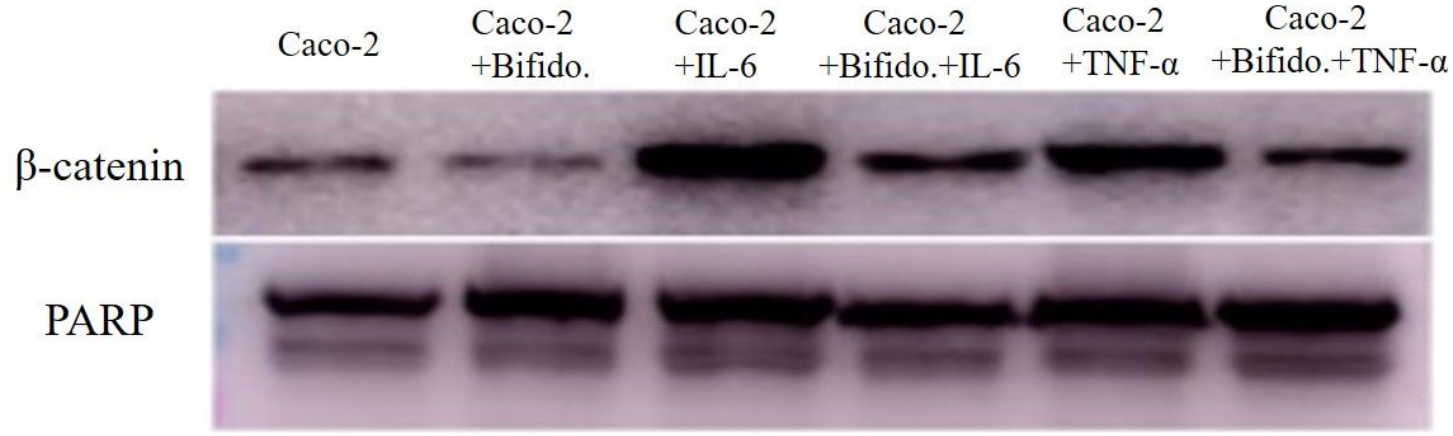

Figure 25

Bifidobacterium reduced the level of β-catenin nucleoprotein in Caco-2 cells.

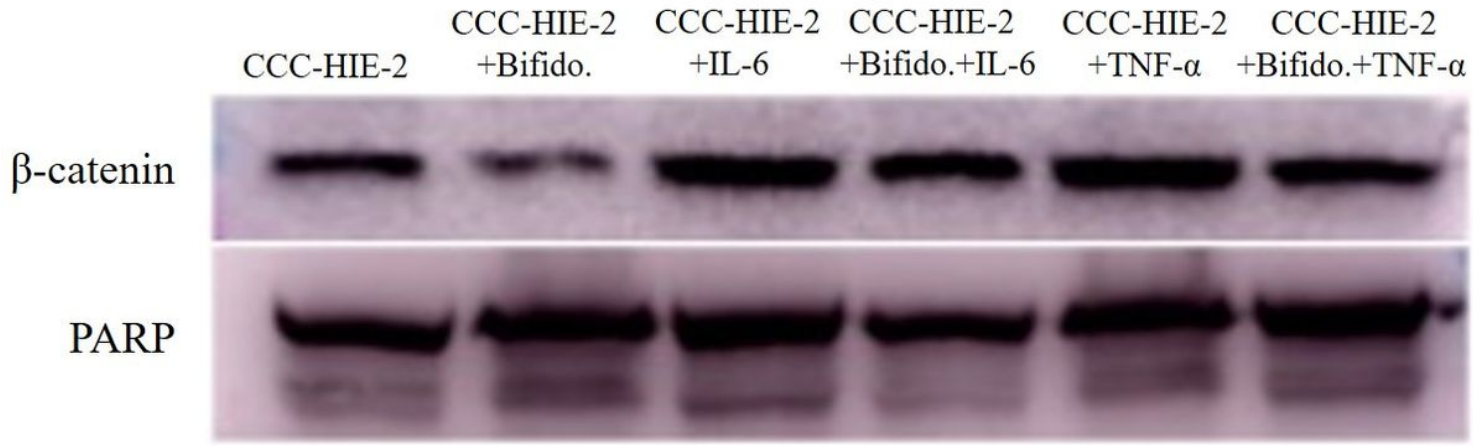

Figure 26

Bifidobacterium reduced the level of β-catenin in nucleoprotein in CCC-HIE-2 cells.

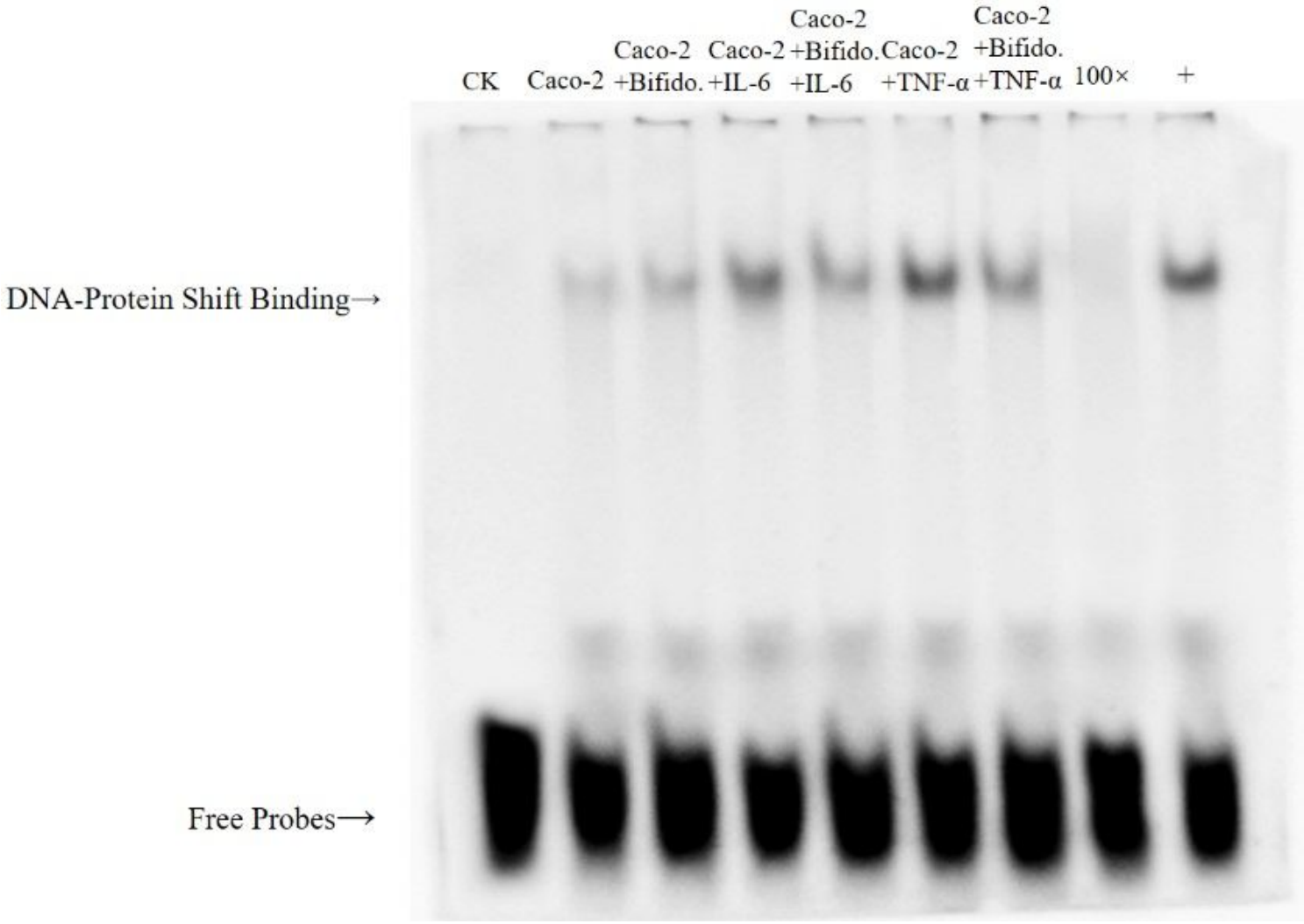

Figure 27

Bifidobacterium suppressed transcription activity of TCF-4 in Caco-2 cells.

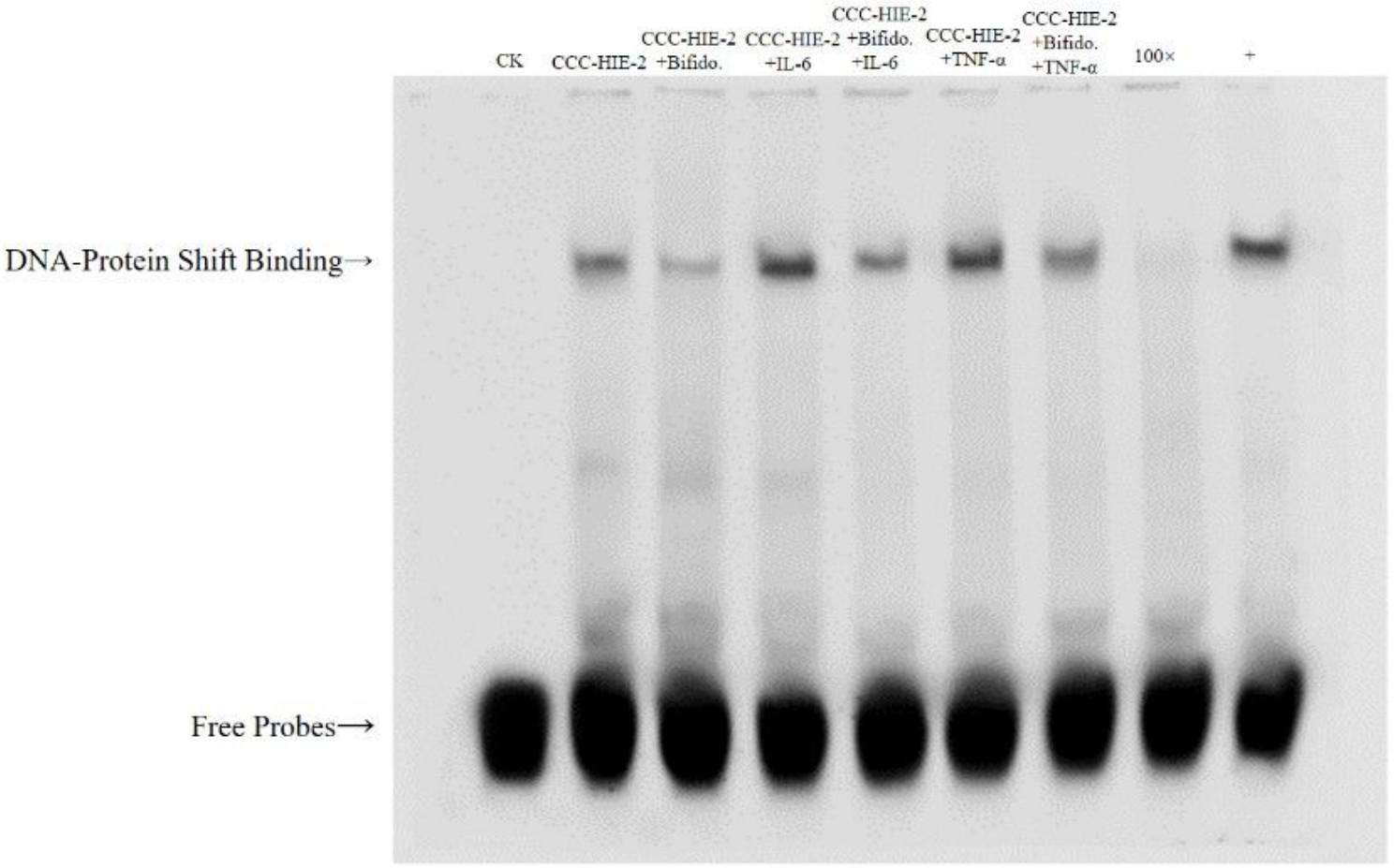

Figure 28

Bifidobacterium suppressed transcription activity of TCF-4 in CCC-HIE-2 cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelinesChecklistBMGED2000075.pdf