# Probiotic mixture VSL#3 prevents ulcerative colitis-associated carcinogenesis in mice and cells by regulating the inflammatory and Wnt/β-catenin pathway

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*To the Editor:* Increasing attention is being paid to preventing ulcerative colitis (UC)-associated carcinogenesis. The intestinal microbiota plays an important role in maintaining the intestinal barrier and immune function. Probiotic mixture VSL#3, that was bought from Sigma-Tau pharmaceuticals (De Simone Formulation) in May 2015, is a mixture of *Lactobacillus casei, L. plantarum, L. acidophilus, L. delbrueckii subsp. bulgaricus, Bifidobacterium longum, B. breve, B. infantis and Streptococcus salivarius* and contains 4.5 billion live bacterial colonies.[1] Previous studies have shown that VSL#3 can help induce and maintain UC remission. The effects of probiotics on UC-associated carcinogenesis are difficult to observe clinically; therefore, mouse models are often used to study this disease. Previous studies have shown that azoxymethane (AOM) combined with dextran sulfate sodium (DSS) can quickly induce a UC-associated carcinogenesis model. Studies using mouse models have demonstrated that VSL#3 inhibits UC-associated carcinogenesis, but the conclusions remain controversial. The exact mechanism by which VSL#3 prevents UC-associated carcinogenesis remains unclear. Therefore, we used a mouse model of AOM/DSS-induced UC-associated carcinogenesis to verify the effect of VSL#3 in preventing UC-associated carcinogenesis and explored the specific mechanism of action. Considering the differences between mice and humans, we further explored how probiotics influence the Wnt/β-catenin pathway at the human cellular level. Since cultivating multiple probiotics simultaneously is challenging, we used *Bifidobacterium* to explore the effects and mechanisms of VSL#3 on UC-associated carcinogenesis in mice and cells.

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 (No. XHDW-2015-0032). Mice ($n = 90$) were randomly divided into the 5-aminosalicylic acid (5-ASA) ($n = 20$), VSL#3 ($n = 20$), 5-ASA + VSL#3 ($n = 20$), model control ($n = 20$), and normal control ($n = 10$) groups. For the first four groups, the mice were intraperitoneally injected with 12.5 mg/kg AOM. One week later, they received 2.5% DSS in their drinking water for 5 days to establish the UC-associated carcinogenesis model. The 5-ASA, VSL#3, and 5-ASA + VSL#3 groups were gavaged with 5-ASA (75 mg/kg every day [quaque die, QD]), VSL#3 ($1.5 \times 10^9$ colony-forming units [CFU]/mice QD), and both 5-ASA and VSL#3, respectively. The model control group received AOM and DSS without gavage intervention. The normal control group received no treatments. After 12 weeks, the mice were euthanized, and their colons were removed. The tumor load in each colon was calculated as the sum of the diameter of each tumor. Tumor-necrosis factor (TNF)-$\alpha$ and interleukin (IL)-6 levels in the colonic mucosa were measured using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, Thermo Fisher Scientific Inc, Vienna, Austria). The transcription activities of nuclear factor (NF)-$\kappa$B and transcription factor (TCF)-4 in the colonic mucosa were

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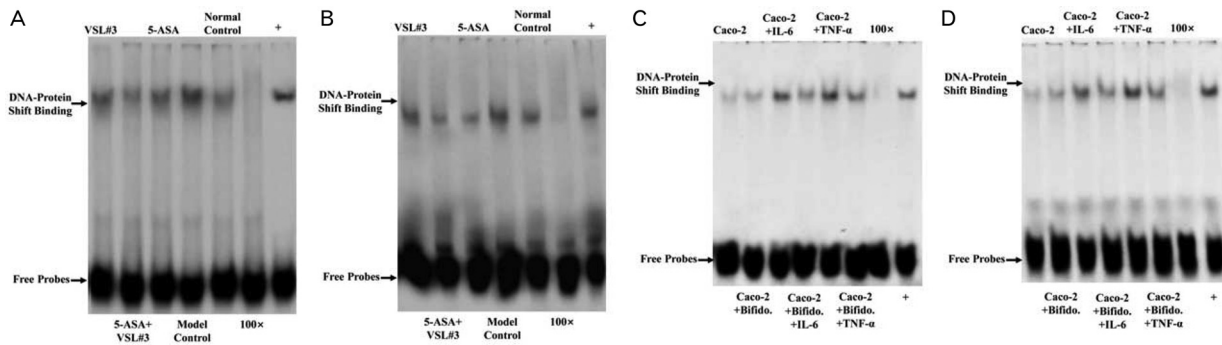E-mail: lijn2008@126.com

**Figure 1:** Transcription activity of NF-κB in Caco-2 cells (A), Transcription activity of TCF-4 in Caco-2 cells (B), Transcription activity of NF-κB in colonic tissue (C), Transcription activity of TCF-4 in colonic tissue (D). 5-ASA: 5-Aminosalicylic acid; IL-6: Interleukin-6; NF-kB: Nuclear factor-κB; TCF-4: Transcription factor-4; TNF-a: Tumor-necrosis factor-a.

measured using an electrophoretic mobility shift assay (EMSA). The nuclear β-catenin distribution was assessed via immunohistochemistry.

Caco-2 and CCC-HIE-2 cells were co-cultured with *Bifidobacterium* and stimulated with IL-6 and TNF-a, respectively. The cells were divided into the following groups: control, *Bifidobacterium* (received $2 \times 10^5$ CFU/μL *Bifidobacterium*), IL-6 (received 0.1 ng/μL IL-6), *Bifidobacterium* plus IL-6 (received 2 x 105 CFU/μL *Bifidobacterium*, then 0.1 ng/μL IL-6), TNF-a (received 0.1 ng/μL TNF-α), and *Bifidobacterium* plus TNF-α (received $2 \times 10^5$ CFU/μL *Bifidobacterium,* then 0.1 ng/μL TNF-α). The relative luciferase activity of β-catenin was measured using luciferase reporter assays. The β-catenin mRNA levels were assessed via quantitative real-time-polymerase chain reaction. The total and nuclear β-catenin expressions were assessed via Western blotting. The inflammatory factor IL-6 and TNF-α levels were measured by ELISA, and the NF-κB and TCF-4 transcriptional activities were measured by EMSA.

All statistical analyses were performed using GraphPad Prism, version 6.0. Statistical differences between experimental variants were assessed using two-tailed independent *t* tests, and data from more than two groups were analyzed by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Compared with the model control group, mice in the VSL#3 and 5-ASA + VSL#3 groups exhibited significantly decreased tumor loads. Additionally, 5-ASA and VSL#3 treatment significantly inhibited the TNF-α and IL-6 levels in the colonic tissue compared with those of the model control group. These results were previously published by our research team.[2]

The deoxyribonucleic acid (DNA)-protein-binding band of NF-κB in the 5-ASA and VSL#3 groups was weaker than that in the model control group, demonstrating decreased transcriptional activity by NF-κB in the VSL#3 and 5-ASA groups. Hence, VSL#3 and 5-ASA inhibited NF-kB transcriptional activity in the mouse colonic tissue. Furthermore, β-catenin expression in the nuclei of colonic mucosal cells was significantly lower in the 5-ASA and VSL#3 groups than in the model control group, indicating that 5-ASA and VSL#3 inhibited the nuclear import of β-catenin. We used EMSA to measure the transcriptional activity of TCF-4 in mouse colonic tissue. The DNA-protein-binding band of TCF-4 was weaker in the 5-ASA and VSL#3 groups than in the model control group, demonstrating decreased transcriptional activity of TCF-4 in the 5-ASA and VSL#3 groups compared with that in the model control group, thus indicating downregulation of the Wnt pathway [Figure 1].

In the Caco-2 and CCC-HIE-2 cells, we compared the *Bifidobacterium* + IL-6 group with the IL-6 group and the *Bifidobacterium* + TNF-α group with the TNF-α group. The relative luciferase activity decreased significantly in the groups that received *Bifidobacterium*, demonstrating that *Bifidobacterium* inhibited the IL-6- and TNF-α-induced upregulation of the relative luciferase activity. *Bifidobacterium* did not affect the β-catenin mRNA and protein expression levels. The IL-6 levels decreased in the Caco-2 + *Bifidobacterium*, Caco-2 + *Bifidobacterium* + IL-6, and Caco-2 + *Bifidobacterium* + TNF-α groups, compared with their corresponding groups without *Bifidobacterium*. Similarly, the three groups treated with *Bifidobacterium* had lower TNF-α levels than did those without *Bifidobacterium* intervention, indicating that *Bifidobacterium* downregulated the IL-6 and TNF-α levels.

Western blotting results showed that compared with the Caco-2 + *Bifidobacterium*, Caco-2 + *Bifidobacterium* + IL-6, and Caco-2 + *Bifidobacterium* + TNF-α groups, β-catenin protein expression levels in the nucleus decreased sharply in their corresponding non-co-cultured groups, indicating that *Bifidobacterium* reduced the β-catenin nucleoprotein levels. Compared with the Caco-2 + *Bifidobacterium*, Caco-2 + *Bifidobacterium* + IL-6, and Caco-2 + *Bifidobacterium* + TNF-α groups, the corresponding non-co-cul-tured groups had sharply decreased nuclear β-catenin protein expression levels.

The EMSA results showed that between the Caco-2 + *Bifidobacterium* + IL-6 and Caco-2 + IL-6 groups and between the Caco-2 + *Bifidobacterium* + TNF-α and Caco-2 + TNF-α groups, the NF-κB and TCF-4 DNA-protein-binding bands were significantly weakened in the groups that did not receive *Bifidobacterium*, indicating that *Bifidobacterium* inhibited the NF-κB and TCF-4 transcription activity [Figure 1]. The CCC-HIE-2 cells yielded similar results.

This study illustrated that VSL#3 and 5-ASA effectively prevented AOM/DSS-induced UC-associated carcinogenesis in mice via both monotherapy and combined therapy. Additionally, at the cellular level, *Bifidobacterium* inhibited activity of the Wnt signaling pathway. We further explored its potential mechanism of action.

When the intestinal microbiota balance is disrupted, the intestinal barrier function is destroyed, and inflammatory factors, including TNF-α and IL-6, are produced and trigger an inflammatory reaction. Recognition of the intestinal mucosa by the microbiota is mediated by pattern-recognition receptors, which are upstream molecules of NF-κB. NF-κB activation thus triggers transcription inflammatory factors. Repetitive inflammatory reactions are the pathological basis and initiating factors of UC-associated carcinogenesis.

Treatment with VSL#3 and co-culturing with *Bifidobacterium* significantly reduced the increases in TNF-α and IL-6. Our results demonstrated that probiotics inhibited activation of proinflammatory factors, the initial stage of UC-associated carcinogenesis. NF-κB is a multidirectional transcription-modulating factor with a critical function in the development and translation from inflammation to cancer. Bacterial lipopolysaccharide and proinflammatory factors can trigger the classical NF-κB activation pathway. Activated NF-κB enters the cell nuclei and combines with various target genes, finally modulating the expressions of various cytokines and cellular adhesion molecules.[3] NF-κB may be a key factor in the inflammation-carcinogenesis transformation. Here, we confirmed via EMSA that VSL#3 and *Bifidobacterium* inhibited NF-κB transcription activity, thus illustrating that inhibiting NF-κB activation may be a potential mechanism by which probiotics prevent UC-associated carcinogenesis.

Finally, we investigated the Wnt/β-catenin pathway. When this pathway is activated, β-catenin cannot be degraded, and it accumulates 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 and promotes colorectal cancer development.[4] Our results showed that treatment with VSL#3 and co-culturing with *Bifidobacterium* inhibited the nuclear import of β-catenin and suppressed the transcription activation of TCF-4, indicating downregulation of the Wnt/β-catenin pathway.

In conclusion, VSL#3 and 5-ASA effectively prevented UC-associated carcinogenesis in mice, and supplementing VSL#3 and co-culturing cells with *Bifidobacterium* may downregulate the proinflammatory factors TNF-α and IL-6, inhibit NF-κB transcriptional activity, and finally downregulate the Wnt/β-catenin pathway, consequently preventing the progression from inflammation to carcinogenesis. VSL#3 may be a potential therapeutic agent for preventing UC-associated carcinogenesis.

### Funding

### Conflicts of interest

None.

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