

Original Article

Live and heat-killed probiotic: effects on chronic experimental colitis induced by dextran sulfate sodium (DSS) in rats

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Received July 14, 2015; Accepted October 28, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: Although a series of studies have shown that VSL#3 (*L. plantarum*, *L. Bulgaricus*, *L. casei* and *L. acidophilus*, *B. breve*, *B. longum* and *B. infantis* and *S. salivarius* subspecies *thermophilus*) can exert therapeutic effects on colitis, whether heat-killed VSL#3 also can exert similar effects has never been tested. The aim of the study was to investigate whether heat-killed VSL#3 exert therapeutic effects in chronic experimental colitis by inhibiting STAT3 pathway. Chronic experimental colitis was induced by dextran sulfate sodium (DSS) in rats. Rats underwent gavage once daily for seven days with heat-killed VSL#3 (0.6 g/kg/day). The disease activity index (DAI), histological score, colon length and myeloperoxidase (MPO) activity was observed. Expression of inflammatory related mediators (STAT3, P-STAT3) and cytokines (IL-6, IL-23) in colonic tissue were detected. The results showed that live and heat-killed VSL#3 have identical anti-inflammatory effects by the assessed DAI, colon length, histological score and MPO activity. Live and heat-killed VSL#3 results in reduced IL-6, IL-23, STAT3 and P-STAT3 expression in colonic tissue. Heat-killed VSL#3 have showed significant anti-inflammatory effects by suppressing STAT3 pathway.

Keywords: Probiotic, dextran sulfate sodium, chronic experimental colitis

Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of the bowel, including ulcerative colitis (UC) and Crohn's disease (CD), in recent years, with a high incidence of 29.3 per 100000 persons per year in Australia [1]. Until now, IBD remains one of the most intractable gastrointestinal diseases. Although the etiology of the disease is elusive, it has been suggested that immune system activating in response to bacterial antigen may play a key role [2].

IL-6 is an important proinflammatory cytokine produced by endothelial cells, macrophages, mast cells and other cells, and involved in T-lymphocytes activation [3]. IL-6 expression level increased can be detected in chronic inflammation of mice and play a key role in the response of the host defense against microorganisms [4]. IL-23 can promote the activation of

memory cells to produce IL-17 and play an important role in the survival and maintenance of Th17 cells, its mechanism is considered associate with STAT3 in the process of differentiation of Th17 cells, IL-23 can be mediate STAT-3 phosphorylation and induce activation of STAT3 [5]. Therefore, application of probiotic VSL#3 treatment of chronic colitis in rats is to explore the relationship of the VSL#3 and IL-6/STAT3 pathway. IL-6/STAT3 trans-signaling was activated in the intestinal mucosa of experimental colitis in rats, suggesting that signal transduction pathways play an important role in the pathogenesis of UC. Blocking this pathway may be useful in the treatment of UC.

Under normal conditions, the composition of the microbiota is stable but can be destroyed by many factors [6]. Inherent intestinal flora can be replaced by overgrowth of pathogenic microorganisms under some circumstances. The study showed that the change of intestinal

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Table 1. DAI score of fecal changes

Fecal property	Fecal occult blood	Body weight decrease (%)	Integral
Normal	Normal	0	0
		1-5	1
Relaxed	Positive fecal occult blood	> 5-10	2
		> 10-15	3
Loose stools	Naked eye fecal occult blood	> 15	4

Normal stool, shaped stool; relaxed stool, pasty, unformed stools not attached to the anus; loose stools, unshaped stools attached to the anus. Total DAI score: the score of each symptom was added.

memory the microecological in IBD patients, mainly flora diversity and the amount of beneficial bacteria were reduced [7]. In addition, the researchers found that high prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in CD and the number of *Lactobacillus*, *bifidobacterium*, *bacteroides* are reduced [8, 9]. Normalization of intestinal flora became the IBD treatment strategy.

VSL#3 is a probiotic mixture with high concentrations of bacteria and was verified on successful induction and maintenance of the remission of UC [10, 11], but live probiotic can translocate from the intestinal lumen into the internal compartment and produce systemic infection under immunocompromised condition [12]. Interestingly, some researchers have found that heat-killed probiotic may be just as effective [13, 14].

The present study evaluated the effect of heat-killed VSL#3 on rats with chronic colitis induced by dextran sulfate sodium. Furthermore, we observed the level of the expression of IL-6/STAT3 trans-signaling related cytokines in rats with chronic colitis. This study may provide a new strategy for the treatment of chronic UC.

Materials and methods

Animals and induction of chronic DSS colitis

SD rats (weighing 180 ± 10 g) were maintained under specific pathogen free (SPF) laboratory of China Medical University (Shenyang, China). All experiments in accordance with the law concerning the protection of animals in China and were approved by the China Medical University Animals Committee. Chronic colitis were induced by four cycles; each cycle consisted of

5% DSS (Wako Pure Chemical Industries, Ltd, Osaka, Japan; molecular weight 5000 d) for 7 days followed by drinking water for 10 days. The clinical course of chronic colitis was assessed by disease activity index (DAI), including weight loss, stool consistency, and peranal bleeding (Table 1).

Heat-killed probiotics VSL#3 and mesalazine

The VSL#3 mixture combines 8 strains of lactic acid producing bacteria, including three strains of *Bifidobacterium* (*B. breve*, *B. longum* and *B. infantis*); four strains of *Lactobacillus* (*L. plantarum*, *L. bulgaricus*, *L. casei* and *L. acidophilus*) and one strain of *Streptococcus* (*S. salivarius* subsp. *thermophilus*). Each 2.5 g VSL#3 sachet contained 450 billion freeze-dried bacteria. Heat-killed VSL#3 was prepared by incubating VSL#3 at 121°C for 20 min. Mesalazine is produced by the French Ethypharm Industries.

Experimental protocol

Fifty-six SD rats were randomized into 7 groups ($n=8$). In the placebo group, 200 μ L normal saline was applied via gastric tube after induction of colitis. In the live and heat-killed VSL#3 group, rats were administrated with VSL#3 or heat-killed VSL#3 (0.6 g/kg/day) via gastric tube. The mesalazine group was administrated with mesalazine (0.4 g/kg/day) via gastric tube after induction of colitis. The VSL#3 + mesalazine group was administrated with live VSL#3 (0.6 g/kg/day) and mesalazine (0.4 g/kg/day) in the same manner.

Group L: DSS-/VSL-; Group M: DSS+/VSL-; Group N: DSS+/VSL-/NS+; Group O: DSS+/VSL-/Mesalazine+; Group P: DSS+/Live VSL+; Group Q: DSS+/Heat-killed VSL+; Group R: DSS+/Live VSL+/Mesalazine+.

Collection of digestive tissues and assessment of intestinal inflammation

Their colons were soaked in 4% formaldehyde, embedded in paraffin sections, stained with hematoxylin and eosin, and examined histologically. The remainder of each specimen was stored at -80°C. Colitis was evaluated by DAI

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Table 2. Injury score of histology

Integral	0	1	2	3	4
Inflammation	None	Mild	Moderate	Severe	-
Mucosal damage	None	Mucous layer	Submucosa	Muscularis and serosa	-
Crypt damage	None	1/3	2/3	100%	100% with epithelium loss
Pathological change range	—	0%-25%	26%-50%	51%-75%	76%-100%

Table 3. DAI and colon length in each group

Group	DAI	Colon length
Normal (L)	0.12 ± 0.354 ^{b,c}	19.13 ± 1.25 ^{b,c}
DSS (M)	7.0 ± 0.756 ^a	14.40 ± 0.77 ^{a,c}
DSS + normal saline (N)	6.5 ± 0.535 ^a	14.28 ± 0.82 ^{a,c}
Mesalazine (O)	6.25 ± 0.707 ^a	15.68 ± 0.61 ^{a,b}
Live VSL#3 (P)	6.25 ± 0.463 ^{a,b}	15.48 ± 0.69 ^{a,b}
Heat-killed VSL#3 (Q)	6.5 ± 0.535 ^a	15.48 ± 1.05 ^{a,b}
Mesalazine + VSL#3 (R)	4.5 ± 0.535 ^{a,b,c}	16.33 ± 0.65 ^{a,b}

^aP < 0.05 compared with the control group; ^bP < 0.05 compared with the DSS group; ^cP < 0.05 compared with the mesalazine group.

(Table 1), histological injury score of each rat was evaluated (Table 2).

Myeloperoxidase activity

We assessed leukocyte infiltration in colonic tissue by determining the levels of myeloperoxidase (MPO). MPO activity was detected with a myeloperoxidase assay kit according to the manufacturer's instructions.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Primer sequences used for cDNA amplification were as follows: mouse, β -actin, 5'-ACGTTGACATCCGTAAAGAC-3' and 5'-GAAGGTGGACAGTGAGGC-3'. IL-6, 5'-CGCAAAGCCAGAGTCATTC-3' and 5'-TGGTCTTGGTCCTTAGCC-3'. IL-23, 5'-ACCTGCTGGACTCGGACAT-3' and 5'-GGCGAGGCATCTGTTGAGT-3'. STAT3, 5'-CAAGGGCTTCTCGTTCTG-3' and 5'-GGGCTTTGTGCTTAGGAT-3'. Real-time fluorescence quantitative analysis, analysis by fluorescence quantitative analysis instrument to get the picture: running after the end of the experimental data.

Western blotting

Proteins were extracted from the rat colon tissues and quantified using a commercial protein assay. Immunoblot analysis was conducted using antibodies. The results were assessed via a chemiluminescent detection system and exposure to autoradiography film.

Statistical analysis

Data were expressed as mean ± standard deviation and compared by one-way ANOVA and *t* test. A *P*-value < 0.05 was considered statistically significant.

Results

Disease activity index, colon length and histological lesions

In the live VSL#3 group, DAIs decreased compared with the DSS group (*P* < 0.05). In the mesalazine group and heat-killed VSL#3 group, DAIs decreased compared with the DSS group (*P* > 0.05). The DAIs of live and heat-killed VSL#3 group was not significant difference compared with mesalazine group (*P* > 0.05). In the live and heat-killed VSL#3 group, colon lengths were longer than those of the DSS group (*P* < 0.05) and were not significant difference compared with mesalazine group (*P* > 0.05) (Table 3).

Histological changes of the large intestine were evaluated by HE stain (Figure 1). Extensive histological damages were observed in DSS group, including mucosa and crypt destruction. Live and heat-killed VSL#3 group had significantly less histological damage compared with DSS group (*P* < 0.05). There was no significant difference between live and heat-killed VSL#3 group (*P* > 0.05) (Figure 2).

MPO activity

The activity of MPO is shown in Figure 3. MPO activity was significantly elevated in the DSS group (14.6 ± 1.2 mU/mg) compared with the normal group (6.1 ± 0.8 mU/mg) (*P* < 0.05). Compared with DSS group, the MPO activity was significantly declined in the mesalazine group (9.3 ± 0.7 mU/mg), live VSL#3 group (9.1 ± 1.2 mU/mg), heat-killed VSL#3 (9.6 ± 0.9 mU/mg) and VSL#3 + mesalazine group (7.3 ± 0.6 mU/mg) (*P* < 0.05). MPO activity was not significant difference in heat-killed VSL#3

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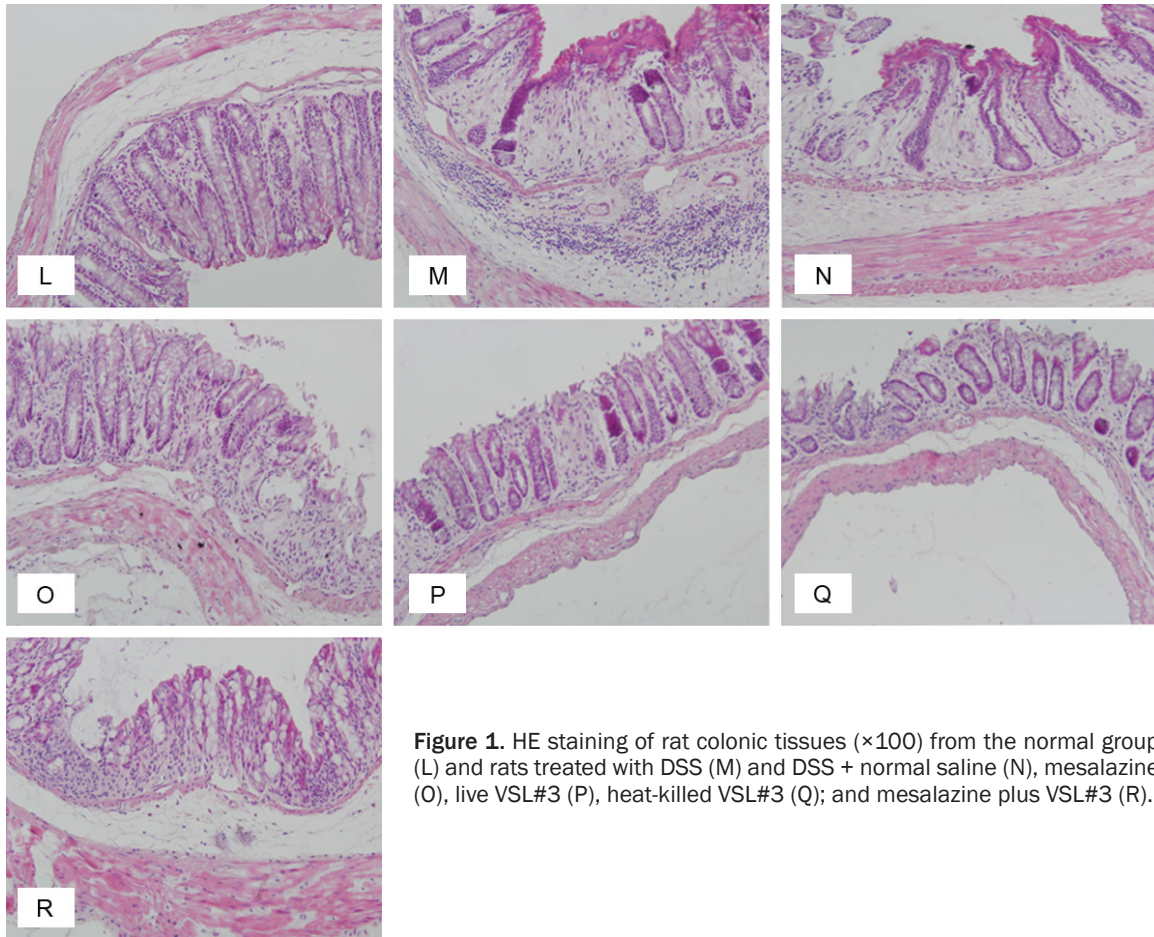


Figure 1. HE staining of rat colonic tissues (×100) from the normal group (L) and rats treated with DSS (M) and DSS + normal saline (N), mesalazine (O), live VSL#3 (P), heat-killed VSL#3 (Q); and mesalazine plus VSL#3 (R).

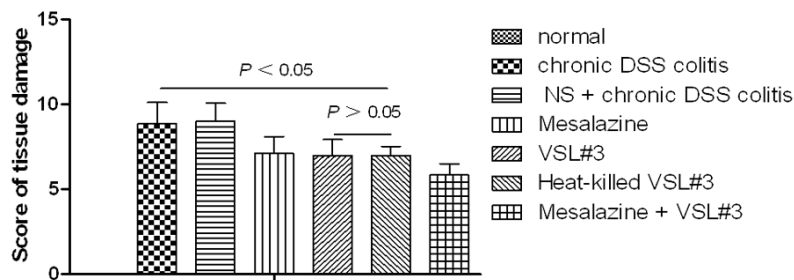


Figure 2. Injury score of histological changes.

group compared with live VSL#3 group ($P > 0.05$).

Results of real-time polymerase chain reaction (PCR)

The mRNA expression levels of IL-6, IL-23 and STAT3 were examined by real-time PCR. The mRNA levels of IL-6, IL-23 and STAT3 were significantly increased in the DSS group compared with the normal group ($P < 0.05$). The mRNA levels of these genes were significantly

decreased in live and heat-killed VSL#3 group compared with DSS group ($P < 0.05$). These were no significant differences in the mRNA levels of these genes between heat-killed VSL#3 group and VSL#3 group ($P > 0.05$) (Figure 4).

Western blotting

The protein expression levels of IL-6, IL-23 and STAT3 were examined by Western blotting. The protein levels of IL-6, IL-23 and STAT3 were significantly increased in DSS group compared with the normal group ($P < 0.05$). The protein levels of these genes were significantly decreased in live and heat-killed VSL#3 group compared with the DSS group ($P < 0.05$). These were no significant differences in the protein levels of these genes between VSL#3 group and heat-killed VSL#3 group ($P > 0.05$) (Figures 5, 6).

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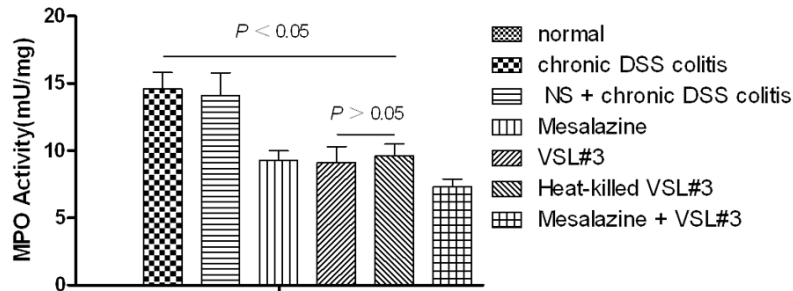


Figure 3. MPO activity of rats in each group.

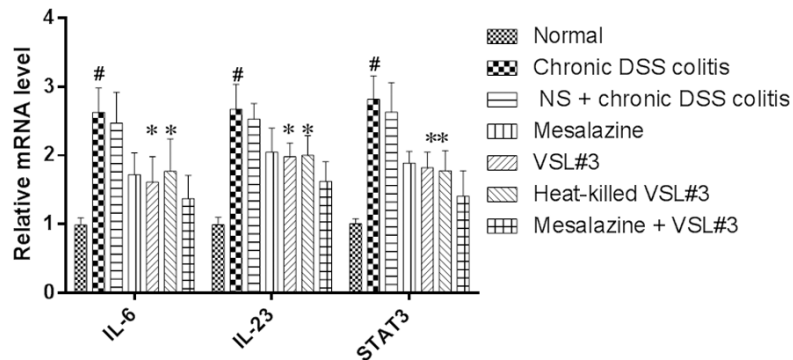


Figure 4. Relative expression of IL-6, IL-23 and STAT3 mRNA in groups of rats. (# $P < 0.05$ versus control group; * $P < 0.05$ versus DSS group).

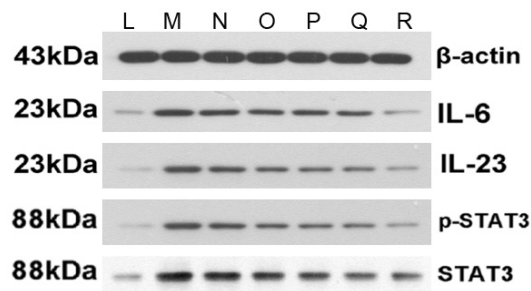


Figure 5. Western blots of IL-6, IL-23, STAT3 and P-STAT3 in each group (Lane L control group; Lane M DSS group; Lane N placebo group; Lane O Mesalazine group; Lane P VSL#3 group; Lane Q Heat-killed VSL#3 group; Lane R VSL#3 + Mesalazine group).

Discussion

By multiple criteria, we have demonstrated that the similar effects of live and heat-killed VSL#3 on chronic experimental colitis induced by DSS. Our results showed that live and heat-killed VSL#3 treatment block the increased DAI, colon injury, MPO activity, and elevate shorter colon length. In addition, our study demonstrated that these treatments reduce the expression of IL-6, IL-23, STAT3 and P-STAT3. The

results suggest that live and heat-killed VSL#3 block the STAT3 pathway so that may be a useful therapeutic approach to the treatment of UC.

Jijon et al. study have shown that VSL#3 can reduce release of proinflammatory cytokines of IL-10-KO mouse intestinal mucosa, IL-10-KO mice oral bacterial DNA extracted from VSL#3, the results showed that intestinal mucosal secretion of TNF α and IFN γ decreased intestinal mucosal tissue damage has been significantly improved. Therefore, VSL#3 or its DNA can reduce the intestinal inflammatory [15]. The DNA of VSL#3 and EcN1917 of can also ameliorate inflammation of the DSS-induced colitis in rats, and improve intestinal barrier function [16, 17]. Probiotics

can exert many mechanisms to explain their protective effects. The possible mechanism of probiotic treat experimental colitis as follows: increased the level of secretory IgA and anti-inflammatory cytokines, stimulate mucosal defense function and increase in intestinal mucus secretion, improve the permeability of the intestinal mucosa. Part of the mechanisms depends on the viability of the probiotic. However, the study found that heat-killed Shigella strains can protect colitis model in a guinea pig [13]. In addition, heat-killed body of lactobacillus brevis SBC8803 can ameliorates intestinal injury in a murine model of colitis by enhancing the intestinal barrier function [14]. Our results showed that the protective effect of the VSL#3 did not depend on its viability. This suggests that some structural components may play an important role when exerting its protective effects. Rachmilewitz was methylated and unmethylated genomic DNA extracted in the probiotic VSL#3 DNA enzyme treated probiotics and Escherichia coli (E. coli) genome research, the results show that: the genomic DNA can reduce the DSS or TNBS-induced the severity of mice with experimental colitis, but DNA modification such as methylation and DNA

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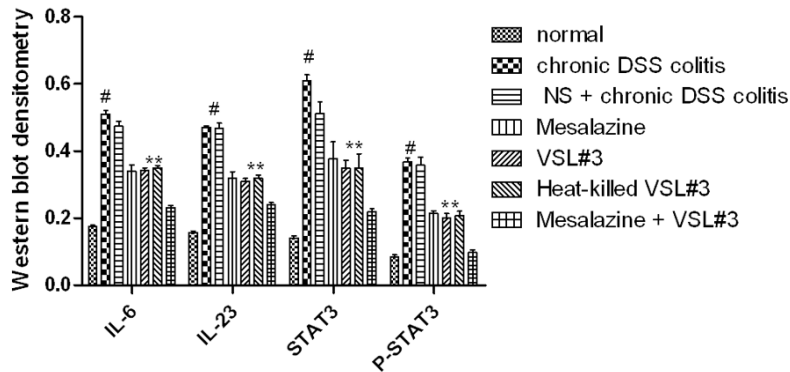


Figure 6. Western blot densitometry of the proteins IL-6, IL-23, STAT3 and P-STAT3 in colon tissue samples from groups of rats ($^{\#}P < 0.05$ versus control group; $^{**}P < 0.05$ versus DSS group).

enzyme treated probiotics had no significant effect, these results support the therapeutic effects of probiotics by the DNA-mediated, non-activated and activity of bacteria have the similar therapeutic effect [17]. Therefore, heat-killed bacteria may have more broad application prospects in future.

IL-6 is a multi-performance cytokines and was produced by lamina propria. IL-6 level higher than the normal controls intestinal mucosa of patients with UC, IL-6 levels decrease after treatment to the serum with the disease alleviate [18, 19]. IL-6 combined with soluble IL-6 receptors in the formation of IL-6/sIL-6R complexes, activation of cell surface gp130, induced signal transducers and transcriptional activation of STAT3 activation [20]. IL-6 is STAT3 activation factor, recent studies have further proved IL-6/STAT3 signal pathway plays an important role in chronic colitis [21].

IL-6, IL-23 and TGF- β 1 plays an important role in the promotion of Th17 cell differentiation in rats. TGF- β 1 and IL-6 co-existence is a necessary condition for Th17 cell differentiation starts IL-17A. When the lack of IL-6 in the environment, the separate existence of TGF- β 1 did not induce the differentiation of naive T cells to Th17 cells but promote the differentiation of CD4 $^{+}$ CD25 $^{+}$ Treg cells. IL-23 could not induce naive T cell differentiation of Th17 cells [22]. In order to investigate the relationship between STAT3 signal transduction pathways and chronic experimental colitis in rats, as well as the mechanism of action of the probiotic VSL#3, this study detected the mRNA and protein levels for IL-23, IL-6, STAT3 with P-STAT3.

The probiotic VSL#3 has a protective effect for DSS-induced chronic colitis model in rats. In summary, the live and heat-killed VSL#3 have a certain effect for chronic experimental colitis, the treatment effect with traditional therapy mesalazine was no significant difference. The possible mechanism that inhibit IL-6, IL-23, STAT3, and P-STAT3 secretion, and then inhibiting the generation of Th17 cells, thus inhibiting the development

of inflammation, improve immune disorders. This study shows that the non-active bacteria with the similar therapeutic effect compared with mesalazine and active bacteria. Therefore, heat-killed VSL#3 may be a useful probiotic for the treatment of human ulcerative colitis.

Acknowledgements

This study was funded by Natural Science Foundation of Liaoning (No. 2015020515) and Science and technology program of Shenyang (No. f13-316-1-12).

Disclosure of conflict of interest

None.

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