VSL#3 probiotics regulate the intestinal epithelial barrier *in vivo* and *in vitro* via the p38 and ERK signaling pathways

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Abstract. Probiotics can play a role in enhancing intestinal barrier function. However, the underlying mechanisms are not fully understood. The aim of this study was to examine the effects of VSL#3 probiotics on colonic epithelium permeability, tight junction protein expression and MAPKs signaling pathways *in vivo* and *in vitro*. *In vivo*, acute colitis was induced by administration of 3.5% dextran sodium sulfate for 7 days. Rats in two groups were treated with either 15 mg VSL#3 or placebo via a gastric tube once daily after induction of colitis. Tight junction protein expression and the MAPKs signaling pathways were studied by immunohistochemistry and immunoblotting. *In vitro*, HT-29 cells were exposed to TNF-α for up to 48 h with or without pre-treatment with a p38 MAPK inhibitor, an ERK inhibitor or a JNK inhibitor. Then tight junction proteins and the phosphorylation of MAPKs were examined in the presence or absence of VSL#3. *In vivo*, VSL#3 probiotics significantly ameliorated the disease activity index from Day 4 onward. In acute colitis rats, decreased expression of the tight junction proteins were observed, whereas VSL#3 therapy prevented these changes and increased the expression of phosphorylated p38 (P-p38), and of phosphorylated ERK (P-ERK). *In vitro*, tight junction proteins, P-p38 and P-ERK in the VSL#3 group were significantly higher than in the control and TNF-α groups. The p38 MAPK inhibitor and the ERK inhibitor could effectively prevent this effect. VSL#3 probiotics protected the epithelial barrier and increased the tight junction protein expression *in vivo* and *in vitro* by activating the p38 and ERK signaling pathways.

Introduction

Probiotics are live microbial food supplements, that have a beneficial effect on the intestinal mucosa. These effects occur via several proposed mechanisms including suppression of the growth and binding of pathogenic bacteria, improvement of the barrier function of the epithelium and alteration of the immune activity of the host (1). Some studies have demonstrated that probiotics can preserve epithelial barrier function in an *in vivo* model, such as colitis (2-4). Epithelial barrier function is controlled and regulated by the tight junction (5,6). The tight junction is constituted by transmembrane proteins, such as occludin and by linker proteins, such as zonula occludens-1 (ZO-1), that affiliate with the actin cytoskeleton (7).

In the colon, the MAPK signaling pathway activation has been observed after probiotics therapy, but there is a paucity of data examining their role in regulating intestinal epithelial permeability in the presence of probiotics. The MAPK family of serine-threonine protein kinases is a highly conserved family consisting of three primary members; extracellular signal-related kinases (ERK), c-Jun amino-terminal kinases (JNK), and p38. MAPKs play an active signaling role following multiple stimuli, some of which include irradiation, osmotic stress, inflammation, growth factors, and mechanical loading (8-11).

In this study we examined whether VSL#3 probiotics protect the epithelial barrier and whether changes in tight junction protein expression and MAPK signaling pathways contribute to this effect. To address this issue, firstly rats with acute dextran sodium sulfate (DSS)-induced colitis were treated concomitantly with the probiotic mixture VSL#3 and epithelial permeability, tight junction protein expression and the MAPKs signaling pathways were studied. Subsequently, we examined this effect *in vitro* using a colon epithelial cell line (HT-29) following incubation with proinflammatory cytokines. Using inhibitors to block specific pathways, we investigated the functional relationships of these pathways in VSL#3 to tight junction proteins.

Materials and methods

In vivo experiment

Animals and induction of colitis. All rats in the study were used strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats, weighing 200-220 g, were obtained from the Experiment Animal Center, China Medical University. Rats were housed in plastic cages containing corn chip bedding and were maintained on a 12 h light:12 h dark cycle (07:00-19:00 h,

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light cycle; 19:00-07:00 h, dark cycle) with a room temperature of 22±1˚C and a humidity of 65-70%. Acute colitis was induced by administration of DSS 3.5% wt/vol in drinking water *ad libitum* for 7 days. The clinical course of colitis was monitored by a daily disease activity index consisting of the three parameters weight loss, stool consistency, and peranal bleeding, as described previously (12,13) (Table I).

Table I. Calculation of the disease activity index.

Score	Weight loss %	Stool consistency	Peranal bleeding
0	None	Normal	None
1	1-5		
2	5-10	Pasty stools	Occult bleeding
3	10-20		
4	>20	Diarrhea	Gross bleeding

The disease activity index (DAI) was calculated as the cumulative score of the 3 parameters, weight loss, stool consistency and peranal bleeding.

Experimental protocol. Ten healthy rats without pre-treatment served as controls. In the VSL#3 group, acute colitis was induced as described above, and then 10 rats were treated once daily with VSL#3. Each sachet of VSL#3 (2.5 g) contained 450 billion freeze-dried bacteria and corn starch. VSL#3 (15 mg, containing 2.7 billion bacteria) was dissolved in 200 μl of PBS and administered via a gastric tube. The DSS group was treated with 15 mg of placebo (corn starch alone) dissolved in 200 μl of PBS in the same manner. After induction of colitis, 10 rats were euthanized and the colon was removed and snap frozen for tissue analysis.

In vivo permeability to Evans blue. In a subset of animals of each group, colonic permeability to Evans blue was measured *in vivo* (14,15). Under isoflurane-N_2O anesthesia, spontaneously breathing animals were placed in a supine position on a heating pad and a laparotomy was performed. A small polyethylene tube (G22) was inserted into the proximal colon ascendens and secured by a ligature. Via this tube the colon was gently flushed until all stool was rinsed out, and 1 ml of 1.5% Evans blue in PBS was instilled into the colon and left in place for 15 min. Then the colon was rinsed with PBS, until the peranal washout was clear. Animals were euthanized, and the colon was rapidly taken out. It was rinsed again with several milliliters of PBS, followed by 1 ml of 6 mM N-acetylcysteine to eliminate dye sticking in the colonic mucus. The colon was opened and rinsed once more with PBS. The whole colon was placed in 2 ml N,N-dimethylformamide for 12 h to extract the Evans blue dye. The dye concentration in the supernatant was measured spectrophotometrically at 610 nm and expressed as extinction per gram of colonic tissue.

Staining process of H&E. A 0.5-cm sample was selected in the descending colon (5 cm above anus), and cleaned with normal saline, fixed with 10% formalin, dehydrated, paraffin embedded, continuously sectioned, deparaffinized and rehydrated. Subsequently, hematoxylin and eosin (H&E) staining was performed followed by dehydration in a 70, 90 and 95% ethanol series, clearing in xylene, mounting in Permount or Histoclad, and the slides were checked under a microscope at x10 magnification to observe morphological change of the rats' colonic membrane.

Immunohistochemistry. Paraffin slides were deparaffinized and rehydrated, deparaffinized in xylene I, II, III for 10 min, dehydrated in 95, 90, 70% ethanol for 2 min, and then tissues sections were rinsed 3 times for 5 min each in 0.01 mol/l PBS, pre-incubated for 15 min with 0.3% H_2O_2, then pre-incubated th 15 min with 5% normal goat serum and incubated overnight at 4˚C with rabbit anti-occludin or anti-ZO-1 polyclonal antibody (rabbit anti-rat, 1:400). After rinsing with 0.01 mol/l PBS, sections were incubated for 15 min with secondary goat anti-rabbit immunoglobulin G at 37˚C, rinsed 3 times for 5 min each in 0.01 mol/l PBS, incubated for 15 minutes with tertiary antibody at 37˚C and rinsed 3x5 min in 0.01 mol/l PBS. A peroxidase reaction was performed to visualize occludin immunolabeling by incubating with 0.05% 3,3'-diaminobenzidine tetrahydrochloride for 3 min and stopping the reaction with 0.01 mol/l PBS. To assess antibody specificity, incubation with the primary antibody was omitted for some sections and no significant staining was observed in this case. Positive results showed brown or dark brown staining. The positive expressing areas and the density of occludin and ZO-1 were assessed with the Image-Pro Plus 6.0 software.

Immunoblotting. Using snap-frozen transverse colon specimens, we stripped the mucosa from the underlying submucosal tissue, homogenized and sonicated it, and transferred it into ice-cold lysis buffer with a protease inhibitor cocktail for 60 min. Lysates were centrifuged and the protein content of the supernatant was determined using the BCA protein assay kit. Depending on the antibody used, equivalent protein concentrations of 10-75 μg were loaded on each lane of SDS-polyacrylamide gels. Electrophoretically separated samples were transferred to an Immobilon Transfer membrane. Membranes were incubated with the respective primary antibodies and a corresponding peroxidase-conjugated secondary antibody. Blots were visualized by chemiluminescence using the Immobilon Western Chemiluminescent HRP substrate. After detection of the specific protein, all membranes were stripped with Restore Western Blot Stripping buffer, and an immunoblot for actin was performed to ensure equal protein loading in each lane. Densitometry was performed for each detected protein in the control, DSS and VSL#3 groups.

In vitro experiment

Cells. HT-29 cells were grown in DMEM supplemented with 10% fetal calf serum, and maintained in an atmosphere of 5% CO_2-95% O_2 at 37˚C. Cells were grown in 75 cm^2 flasks or glass chamber slides. Cells grown as polarized monolayers on filters reached confluency in 7-8 days and were used consistently within 14 days from seeding, or 5 days post-confluency.

Treatments. TNF-α was added to the basolateral side of cell monolayers and incubated for up to 48 h before harvest. Pharmacological inhibitors were used in some experiments

(SB203580, a p38MAPK inhibitor, 1 μmol/l; SP600125, a JNK inhibitor, 10 μmol/l and U0126, an ERK inhibitor, 5 μmol/l).

Immunoblotting. HT-29 cells in culture were scraped from the silastic membrane in the presence of chilled radioimmunoprecipitation assay buffer containing 4.3 mM ethylenediaminetetraacetic acid and a cocktail of protease and phosphatase inhibitors, and placed on ice to minimize cellular activity. Equal amounts of protein lysates were run on SDS-polyacrylamide (10%) reducing gels, transferred onto polyvinylidene fluoride membranes (PVDF) and non-specific binding was blocked in Tris-buffered saline containing 5% non-fat powdered milk and 0.05% Tween-20 at 4˚C. The PVDF membranes were initially probed for phosphorylated MAPKs, ZO-1 or occludin then stripped and reprobed for total MAPKs or actin. MAPK activity and ZO-1, occludin and actin concentration was calculated through densitometric analysis using Kodak 1D Image Analysis Software. Band intensities for phospho-MAPKs were normalized by respective total protein levels, and ZO-1 and occludin were normalized to actin concentration.

Statistical analysis. Data are given as means ± SE. Statistical analysis for significant differences was performed by use of one-way ANOVA, where appropriate. P-values <0.05 were considered significant.

Results

In vivo experiment

VSL#3 reduces clinical disease activity in acute DSS colitis. In contrast to the untreated control rats, rats exposed to DSS developed symptoms of acute colitis with diarrhea, rectal bleeding, wasting, and loss of body weight. This was paralleled by a dramatic rise in the disease activity index starting at Day 3 after DSS induction. Concomitant administration of VSL#3 significantly ameliorated the disease activity index from Day 4 onward (Fig. 1).

VSL#3 prevents increased colonic epithelial permeability in acute DSS colitis. We analyzed the permeability of the colonic epithelium to Evans blue *in vivo* on Day 7 of acute DSS colitis. Compared with healthy controls (0.30±0.48), a strong and significant increase of Evans blue uptake was observed into the colonic mucosa of rats in DSS group (5.60±0.52). Interestingly, this strong increase of Evans blue uptake could be completely prevented by concomitant administration of VSL#3 (0.60±0.52). This demonstrates that VSL#3 therapy ameliorates the leakiness of the colonic epithelium (Fig. 2).

Histology. In the control group, colonic membrane structure was intact, without epithelial sloughing, trimmed glands, and with only a few red blood cells (RBC) appearing in the mucosa. In the DSS group, we noticed a slight damage to the epithelium, with trimmed glands, a few glands detaching, as well as some RBC and inflammatory cells appearing in the mucosa. In the VSL#3 group, the epithelium was not damaged, but a few trimmed glands, as well as a few RBC and inflammatory cells were seen in the mucosa and in the submucosal layers (Fig. 3).

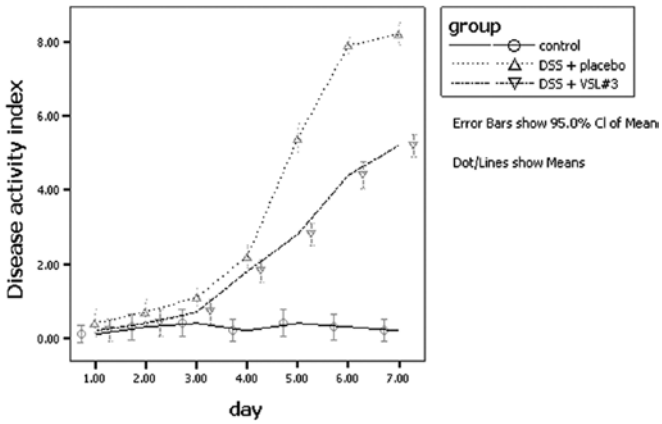

Figure 1. Disease activity index (DAI) scores of the 3 groups. Values are the means ± SE, n=10.

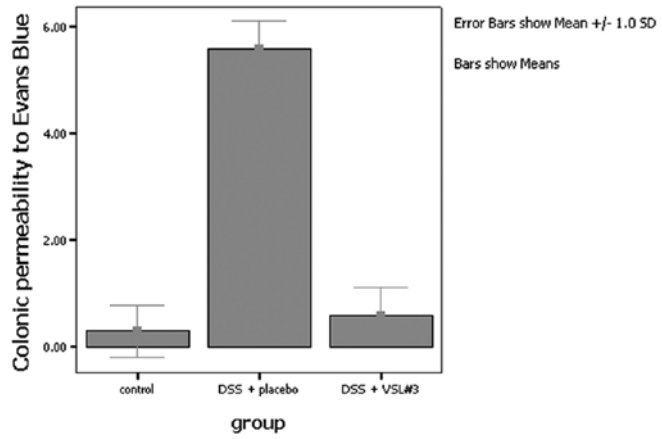

Figure 2. Colonic permeability to Evans blue. Values are means ± SE, n=10.

Immunohistochemistry of tight junction proteins. In the control group, the tight junction proteins occludin and ZO-1 were appropriately localized at the colonic epithelial apical cell-cell contacts both at the surface and in the crypts, consistent with their distribution in tight junctions. But in the DSS group, there was a substantial loss of occludin and ZO-1 from the tight junctions. This loss was manifested by discontinuities in membrane staining and a reduction in staining intensity, which in some areas led to a complete loss of staining. VSL#3 could completely prevent the loss of these two tight junction proteins (Fig. 4). The expression areas of occludin and ZO-1, the well as the density of occludin and ZO-1 in colonic tissue in DSS group were lower than in control group; while the expression areas occludin and ZO-1, as well as the density of occludin and ZO-1 in the VSL#3 group were remarkably higher than in the DSS group (Table II).

Alterations in the expression of tight junction proteins and in the MAPKs signaling pathways. We examined the effect of the probiotic mixture VSL#3 on the protein expression of tight junction proteins in the colons of rats of each group by Western blotting. In the control rats, the density value of the tight junction proteins, occludin and ZO-1, were 0.56±0.02 and 0.96±0.01, respectively. Compared with the control rats, the total protein

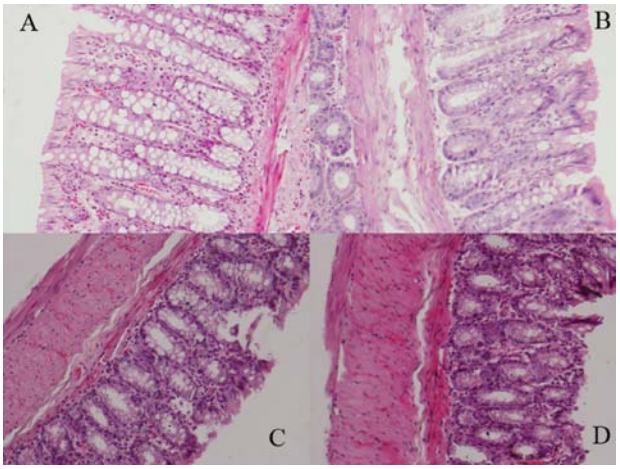

Figure 3. (A) H&E staining of the control group rat colonic tissue; (B) H&E staining of VSL#3 group rat colonic tissue; (C and D) H&E staining of DSS group rat colonic tissue. Magnification, x10.

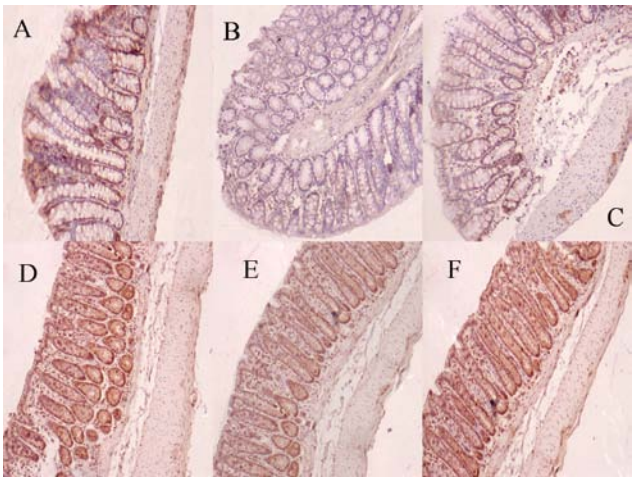

Figure 4. Immunohistochemistry for occludin of (A) control group rat colonic membrane, (B) DSS group rat colonic membrane and (C) VSL#3 group rat colonic membrane. Immunohistochemistry for ZO-1 of (D) control group rat colonic membrane, (E) DSS group rat colonic membrane and (F) VSL#3 group rat colonic membrane. Magnification, x10.

levels of occludin and ZO-1 were significantly reduced in the DSS-induced rat (0.16±0.01 and 0.58±0.01, respectively). Concomitant VSL#3 treatment completely prevented the DSS effects on tight junction protein levels (0.48±0.02 and 0.92±0.02, respectively for occluding and ZO-1).

Furthermore, we examined the effect of the probiotic mixture VSL#3 on the protein expression of p38, P-p38, ERK, P-ERK, JNK, P-JNK in the colons of rats of each group by Western blotting. The density values of p38 protein of the control, DSS and VSL#3 groups were 0.41±0.01, 0.40±0.02 and 0.40±0.03, respectively. There was no statistical difference among the groups. The density values of P-p38 protein of the control, DSS, VSL#3 groups were 0.06±0.01, 0.07±0.01 and 0.30±0.01, respectively. In contrast to rats of the control and DSS groups, increased protein expression of P-p38 was observed in the VSL#3 group. The density values of ERK protein of the control, DSS and VSL#3 groups were 0.62±0.04, 0.58±0.03 and 0.61±0.03, respectively. There was no statistical difference among the groups. The density values of P-ERK protein of the control, DSS and VSL#3 groups were 0.03±0.01, 0.04±0.01 and 0.32±0.02, respectively. In contrast to rats of the control and DSS groups, increased expression of protein P-ERK was observed in the VSL#3 group. The density values of the JNK protein of the control, DSS and VSL#3 groups were 0.52±0.01, 0.49±0.02 and 0.50±0.03, respectively. The density values of P-JNK protein of the control, DSS and VSL#3 groups were 0.05±0.01, 0.06±0.01 and 0.06±0.01, , respectively. There was no statistical difference in the density values of JNK and P-JNK protein among the groups (Fig. 5).

In vitro experiments

Effect of TNF-α, VSL#3, SB203580, SP600125 or U0126 on ZO-1 and occludin. We examined the effects of VSL#3 on tight juntion proteins (ZO-1 and occludin) and on the MAPKs signaling pathways (p38, ERK and JNK). In untreated HT-29 cells, the density value of the tight junction proteins occludin and ZO-1, were 0.48±0.02 and 0.56±0.01. Compared with untreated HT-29 cells, significant reductions in total protein for occludin and ZO-1 were observed in TNF-α-treated HT-29

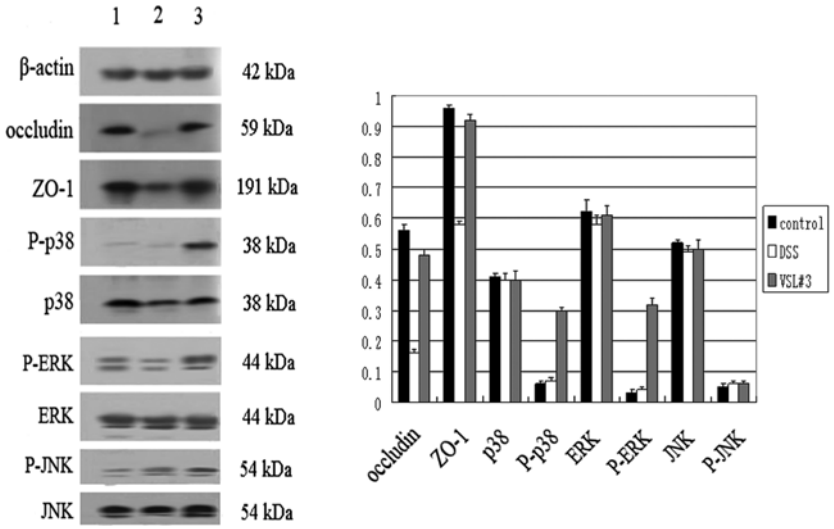

Figure 5. Western blot analyses of occludin, ZO-1, P-p38, p38, P-ERK, ERK, P-JNK and JNK *in vivo*. Lane 1, control; lane 2, DSS group; lane 3, VSL#3 group.

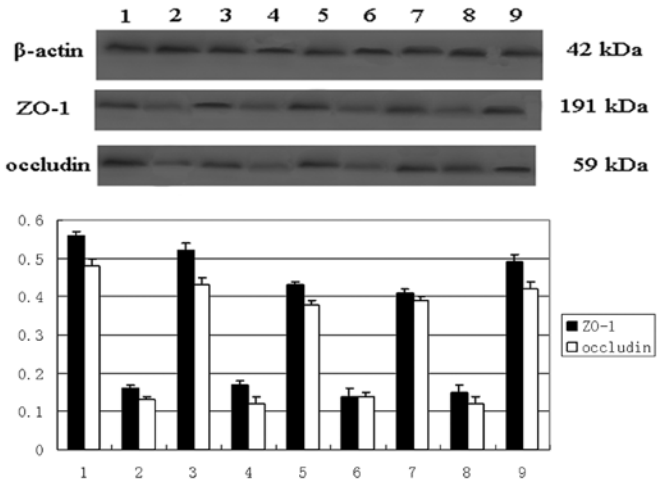

Figure 6. Western blot analyses of occludin and ZO-1 *in vitro*. Lane 1, control group; lane 2, TNF-α group; lane 3, VSL#3 group; lane 4, the p38MAPK inhibitor group; lane 5, the p38MAPK inhibitor + VSL#3 group; lane 6, the ERK inhibitor group; lane 7, the ERK inhibitor + VSL#3 group; lane 8, the JNK inhibitor group; lane 9, the JNK inhibitor + VSL#3 group.

cells (0.13±0.01 and 0.16±0.01, respectively). VSL#3 treatment prevented TNF-α effects on occludin and ZO-1 protein levels (0.43±0.02 and 0.52±0.02, respectively). However, SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK inhibitor), U0126 (a ERK inhibitor), VSL#3 + SB203580 and VSL#3 + U0126 treatments couldn't prevent the TNF-α effects on occludin and ZO-1 protein levels (Fig. 6).

Effect of TNF-α, VSL#3, SB203580, SP600125 or U0126 on MAPKs signaling pathways. At the same time, we examined the protein expression of p38, P-p38, ERK, P-ERK, JNK and P-JNK in the HT-29 cells of each group. There was no statistical difference in the density values of p38, ERK and JNK among the groups. The density values of P-p38 protein in the VSL#3, VSL#3 + SP600125 and VSL#3 + U0126 groups were significantly higher than that in other groups. The density values of the P-ERK protein in the VSL#3, VSL#3 + SB203580, VSL#3 + SP600125 group were significantly higher than that in other groups. There was no statistical difference in the density value of P-JNK among the groups (Fig. 7).

Table II. Occludin and ZO-1 expression levels in rats' colonic membrane (means ± SE).

		Occludin		ZO-1	
Group	n	Area (μm^2)	Density	Area (μm^2)	Density
Control	10	9454.20±167.84	43253.30±2088.13	9456.50±245.26	43176.30±1728.44
DSS	10	7862.70±183.40[a]	36974.15±2103.35[a]	6785.80±177.26[a]	35688.60±2124.29[a]
VSL#3	10	9212.10±124.35[a,b]	39867.40±946.48[a,b]	8658.60±98.80[a,b]	39586.70±1468.26[a,b]

[a]P<0.05 compared with the control group; [b]P<0.05 compared with the DSS group.

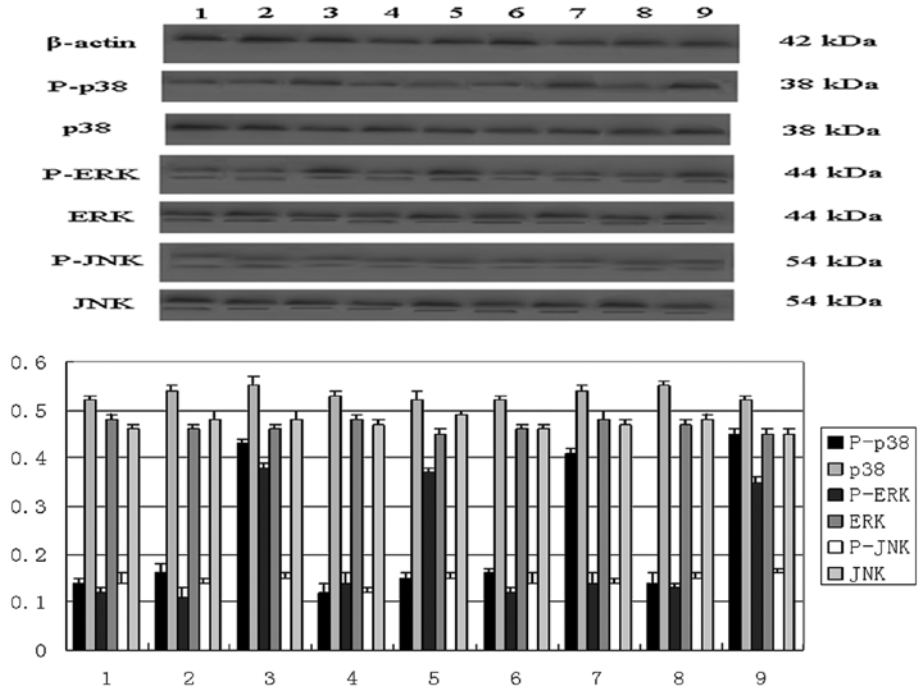

Figure 7. Western blot analyses of P-p38, p38, P-ERK, ERK, P-JNK and JNK *in vitro*. Lane 1, control group; lane 2, TNF-α group; lane 3, VSL#3 group; lane 4, p38 MAPK inhibitor group; lane 5, p38 MAPK inhibitor + VSL#3 group; lane 6, ERK inhibitor group; lane 7, ERK inhibitor + VSL#3 group; lane 8, JNK inhibitor group; lane 9, JNK inhibitor + VSL#3 group.

Discussion

In this study we investigated the influence of the probiotic mixture VSL#3 on epithelial permeability, tight junction protein expression and MAPKs signaling pathways in a rat model of acute colitis and in HT-29 cells.

In vivo studies, we showed that the impairment of epithelial barrier function in acute colitis is associated with loss of the tight junction proteins, occludin and ZO-1. Tight junctions are the major determinants of paracellular permeability. Although changes in epithelial tight junction protein expression have been studied extensively in monolayers stimulated by cytokines, challenged by bacteria, or exposed to aspirin (16-22), much remains to be understood about barrier disruptive changes under inflammatory conditions *in vivo*. Our results have indicated that the probiotic mixture VSL#3 protects colonic epithelial barrier function in acute DSS colitis, as demonstrated by effective prevention of increased Evans blue dye uptake. Therefore, we hypothesized that probiotic-induced protection of epithelial barrier function occurs via the prevention of changes in tight junction protein expression. But the mechanisms of the probiotic-induced changes in tight junction protein expression observed *in vivo* were not apparent, so we attempted to elucidate these mechanisms in *in vitro* studies.

Previous *in vitro* studies have shown that probiotics could protect intestinal epithelial cells from decrements in barrier function induced by pro-inflammatory cytokines. However, the specific mechanisms are not very clear. In our study, we explored phosphorylation of the MAPKs as a possible mechanism underlying the probiotics-induced protection of epithelial barrier function. Firstly, we cultured HT-29 cells for 7-8 days until cells had grown as polarized monolayers. Then TNF-α was added to the basolateral side of cell monolayers and incubated for up to 48 h before harvest. Using this model of the intestinal epithelium, that TNF-α reduced the tight junction protein expression. We also found that VSL#3 increased the tight junction protein expression.

In the present study we also analyzed the phosphorylation levels of ERK, JNK and p38 MAPK in the TNF-α, VSL#3, SB203580, SP600125 and U0126 groups. Phosphorylation levels of ERK and p38 MAPK were significantly higher in the VSL#3 group compared with the control and TNF-α groups, but there were no significant differences in the phosphorylation levels of JNK in the VSL#3, TNF-α and control groups. Pre-treatment of the monolayers with specific inhibitors of p38 MAPK (SB203580) or ERK (U0126) prevented the VSL#3-induced phosphorylation of each MAPK, and reduced the barrier protective function of VSL#3. We thus hypothesized that activation of p38 MAPK and ERK by VSL#3 could lead to reorganization of the tight junction complex, and to an increase in the expression levels of tight junction proteins. This is in line with our *in vivo* studies and evidence that the dynamic regulation of the tight junction is in part modulated by MAPK-dependent pathways.

We have shown that VSL#3 probiotics preserve epithelial barrier function in DSS-induced colitis. We also showed that VSL#3 probiotics reversed the effects of TNF-α on the barrier function in HT-29 cells. The protective effects of VSL#3 on barrier function apparently involved p38 MAPK and ERK. VSL#3 interacted with intestinal epithelial cells, activating the p38 MAPK and ERK signaling pathways.

In summary, our findings suggest a complex network of mechanisms involved in the beneficial effects of probiotics in the epithelial barrier dysfunction. We demonstrated that VSL#3 probiotics therapy protected the epithelial barrier in a rat model of colitis by preventing redistribution and reduced expression of tight junction proteins. At the same time, we found that VSL#3 preserved barrier function in HT-29 cells, and we explored the p38 MAPK and ERK signaling pathways as a possible mechanism. Our data extend the spectrum of the effects of probiotics on intestinal epithelial function and may further justify their use in many intestinal diseases such as inflammatory bowel disease.

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