Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis

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In this study we asked whether VSL#3, a mixture of 8 probiotic bacterial strains, protects the epithelial barrier and, if so, whether changes in tight junction protein expression and epithelial apoptosis contribute to this effect. To answer these questions, mice with acute dextran sodium sulfate (DSS)-induced colitis were treated concomitantly with the probiotic mixture VSL#3; and epithelial permeability, tight junction protein expression, epithelial apoptotic ratio, and clinical and histological inflammation were studied.

THE PATHOGENESIS of inflammatory bowel diseases (IBD) is not completely understood. One accepted theory is that an impaired epithelial barrier enhances the exposure of immune cells to antigens derived from the resident microflora of the gastrointestinal tract, leading to an inadequate immune response (16). Indeed, an increased epithelial permeability has been observed in IBD patients and, interestingly, in their healthy relatives (30, 35, 50, 60).

In healthy individuals, epithelial paracellular permeability is controlled and regulated by the apical junctional complex. The apical junctional complex consists of the tight junction and the subjacent adherens junction. The tight junction effectively regulates flux via the paracellular pathway and prevents lateral diffusion of proteins and lipids between apical and basolateral plasma membrane domains (9, 56). The tight junction complex is constituted by transmembrane proteins, like occludin and the claudin family, and by linker proteins, like zonula occludens-1 (ZO-1), that affiliate with the actin cytoskeleton (15, 25).

In individuals with IBD, redistribution and downregulation of several tight junction proteins has been observed in active disease as a molecular basis of increased epithelial permeability (19, 35, 50, 60). Although single epithelial cell apoptosis and extrusion without loss of barrier function are normal physiological events in the gastrointestinal tract (1, 36), an increased epithelial apoptotic ratio in IBD might be another cause of relevant leaks in the epithelial barrier (23–24, 27, 59–60).

Probiotics have been defined as living bacteria that, when ingested in sufficient quantity, improve the health of the host beyond their inherent basic nutrition (18). Probiotics have anti-inflammatory effects in human IBD (21–22, 38, 57) and in several animal models of IBD (2, 11, 17, 26, 28, 31, 37, 40, 42, 45, 52–53, 55).

It is hypothesized that probiotics preserve epithelial barrier function. In vitro studies on epithelial monolayers showed that probiotics improve barrier function following Escherichia coli infection or incubation with proinflammatory cytokines (3, 41, 47–48, 61). Probiotics also preserve the intestinal epithelial barrier in several in vivo models, such as the IL-10 knockout colitis (37) or models of sepsis (13, 44), and they protect against barrier dysfunction following psychological stress in rats (12, 58). Pathological bacterial translocation to mesenteric lymph nodes as a marker of impaired barrier function can also be effectively reduced by probiotic therapy (13, 40, 44, 58).

In this study we asked whether VSL#3, a mixture of 8 probiotic bacterial strains, protects the epithelial barrier and, if so, whether changes in tight junction protein expression and epithelial apoptosis contribute to this effect. To answer these questions, mice with acute dextran sodium sulfate (DSS)-induced colitis were treated concomitantly with the probiotic mixture VSL#3; and epithelial permeability, tight junction protein expression, epithelial apoptotic ratio, and clinical and histological inflammation were studied.

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Materials and Methods

Animals and induction of colitis. All animal experiments were conducted in accordance with the German Animal Welfare Law and were approved by the local district authority of Muenster, Germany. Inbred female BALB/c mice weighing 20–22 g (Charles River, Sulzfeld, Germany) were housed in pairs in standard laboratory cages with access to standard laboratory chow. Acute colitis was induced by administration of DSS (ICN Biomedicals, molecular weight 40,000) 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 (Table 1) (8, 49).

Experimental protocol. Healthy animals without pretreatment served as controls. In the treatment groups, acute colitis was induced as described above. Starting immediately prior to administration of DSS, animals were treated once daily during induction of colitis with VSL#3 (provided by SigmaTau, Duesseldorf, Germany). Each sachet of VSL#3 (2.5 g) contained 450 billion freeze-dried bacteria (Streptococcus thermophilus, Bifidobacterium longum, B. breve, B. infantis, Lactobacillus acidophilus, L. plantarum, L. casei, L. bulgaricus) and corn starch. VSL#3 (15 mg, containing 2.7 billion bacteria) was dissolved in 200 μl of PBS and administered via gastric tube. The placebo 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 (day 7), animals were euthanized and the colon was removed and snap frozen for tissue analysis. Because no differences were observed for immunofluorescence, Western blot, and histology in different segments of the colon in preliminary studies, all further tissue analyses were performed in the transverse colon. Stools were collected at day 7 from colitic animals and healthy controls for microbiological analysis. Stools were spread on blood agar plates and analyzed for colony formation. The number of colonies of each species was assessed semiquantitatively with a score of 0–3.

In vivo permeability to Evans blue. In a subset of animals of each group, colonic permeability to Evans blue was measured in vivo. This is a well-validated method for assessing epithelial permeability (34, 55). In isoflurane-N2O 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 (immediately adjacent to the cecum) and secured by a ligature. Via this tube the colon was gently flushed until all stool was rinsed out, and 1 ml of Evans blue 1.5% (Sigma Aldrich) 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 cut open, and 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 given as extinction per gram colonic tissue.

<table>
<thead>
<tr>
<th>Table 1. Calculation of DAI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Score</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

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

Histology. Frozen sections (3 μm) were collected on coated slides, and hematoxylin and eosin staining was performed. At least four different sections were studied for each animal. Histological inflammation was scored by two blinded investigators using a modified score introduced by Dieleman et al. (10), considering the degree of inflammation, the transmural vertical extent of inflammation, and the crypt damage score, related to the percentage of involvement of mucosal surface in each slide (Table 2).

Immunofluorescence. Frozen sections (3 μm) with histologically intact epithelium were collected on coated slides, dried overnight, fixed in acetone for 3 min, and dried for 30 min. Fixed sections were washed in PBS, and blocked in PBS with 2% bovine serum albumin (Sigma-Aldrich) for 30 min. Slides were incubated with the primary antibody for 30–60 min at room temperature [rabbit anti-occludin, rabbit anti-ZO-1, rabbit anti-claudin-1, rabbit anti-claudin-3, rabbit anti-claudin-5, rabbit anti-β-catenin, rabbit anti-caspase-3 (cleaved), rat anti-E-cadherin; Zytomed]. Slides were washed and incubated for 1 h with the appropriate secondary antibody [Alexa Fluor 488 conjugated goat anti-rabbit immunoglobulin G (Invitrogen), biotinylated anti-mouse immunoglobulin G (Vector)]. Slides with the biotinylated secondary antibody were incubated with Avidin Alexa Fluor 488 (Invitrogen) for visualization of the secondary antibody. Slides were washed, and nuclei were counterstained with DAPI (Sigma-Aldrich). Stained sections were mounted using ProLong Gold antifade (Invitrogen). Samples were imaged using a Nikon epifluorescence microscope (Eclipse, E800) connected to a Nikon FDX-35 camera.

Apoptotic ratio. For assessment of apoptotic ratio, at least four different frozen sections for each individual were stained for cleaved caspase 3. Due to the lack of colonic erosions or ulcerations in DSS mice, inflammation was constant throughout the colon segment. This ensured representative apoptotic ratios for each individual. After counterstaining with DAPI, immunofluorescence images were photographed and evaluated. The apoptotic ratio was calculated as the number of apoptotic epithelial cells per 1,000 epithelial cells. At least 10 high-power fields were analyzed per animal.

Immunoblotting. Using snap-frozen transverse colon specimens with histologically intact epithelium, 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 [protease inhibitor cocktail 1 μl/ml buffer (Sigma-Aldrich), 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 (Igepal), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA]. Lysates were centrifuged and the protein content of the supernatant was determined by using the BCA protein assay kit (Thermo Scientific Pierce). Depending on the antibody used, equivalent protein concentrations of 10–75 μg were loaded in each lane of SDS-polyacrylamide gels. Electrophoretically separated samples were transferred to an Immobilon Transfer membrane (Millipore). Membranes were incubated with the respective primary antibodies (rabbit anti-occludin, rabbit anti-ZO-1, rabbit anti-claudin-1, rabbit anti-claudin-2, rabbit anti-claudin-3, rabbit anti-claudin-4, rabbit anti-claudin-5, rabbit anti-β-catenin, rat anti-E-cadherin; Zytomed) and a corresponding peroxidase-conjugated secondary antibody (peroxidase conjugated anti-rabbit immunoglobulin, peroxidase conjugated anti-mouse immunoglobulin; Sigma-Aldrich). Blots were visualized by chemiluminescence using Immobilon Western Chemiluminescent HRP substrate (Millipore). After detection of specific tight junction adherens junction proteins, all membranes were stripped with Restore Western Blot Stripping Buffer (Pierce), and an immunoblot for actin was performed (rabbit anti-actin, Sigma-Aldrich) to ensure equal protein loading in each lane. Densitometry was performed for each detected protein with healthy controls set as 100%. Corresponding protein expression in DSS groups on the same blot were given as percentage of healthy controls.

Statistical analysis. Data are given as means ± SE, unless indicated otherwise. All experiments were performed with triplicate or greater samples, and data shown are representative of five or more indepen-
The disease activity index starting at day 2 after DSS induction. Concomitant administration of VSL#3 significantly ameliorated the disease activity index from day 5 onward; however, VSL#3 could not completely prevent the development of colitis (Fig. 1). Although diarrhea and peranal bleeding were both significantly reduced by VSL#3 therapy, the effect on weight loss did not reach statistical significance (Table 3).

Shortening of the colon is a typical sign of inflammation in DSS colitis. In placebo-treated animals, colons were significantly shorter than in healthy controls (5.6 ± 0.5 cm; P = 0.037 vs. DSS + placebo, P = 0.002 vs. healthy controls).

DSS + placebo animals had strongly elevated histological inflammation scores that were significantly reduced by VSL#3, but, again, VSL#3 did not prevent the development of colitis (Table 4). Although inflammation and the transmural extent of inflammation were reduced significantly by VSL#3 therapy, the crypt damage score reflecting DSS-induced crypt shortening was not significantly affected by therapy with this probiotic combination (Table 4). Moreover, the surface epithelium was completely intact in all sections studied. Representative histological sections are depicted in Fig. 2.

Pretreatment with VSL#3 over a 5-day period before induction of DSS colitis had no further beneficial effect on either disease activity or the histological appearance of colonic inflammation (data not shown).

VSL#3 prevents increased colonic epithelial permeability in acute DSS colitis. Since probiotics have been reported to improve epithelial barrier function in vitro and in vivo (3, 12, 13–14, 37, 40–41, 44, 47–48, 58), we analyzed the permeability of the colonic epithelium to Evans blue in vivo on days 7 of acute DSS colitis. Compared with healthy controls, a strong and significant increase of Evans blue uptake into the colonic mucosa of DSS-treated mice was observed (Fig. 3). Interestingly, this strong increase of Evans blue uptake could be completely prevented by concomitant administration of VSL#3 (Fig. 3). This demonstrates that VSL#3 therapy ameliorates the leakiness of the colonic epithelium to a greater extent than the clinical disease activity or histological colonic inflammation.

### Table 2. Calculation of histological inflammation score

<table>
<thead>
<tr>
<th>Score</th>
<th>Inflammation</th>
<th>Extent</th>
<th>Crypt Damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>none</td>
<td>0–12</td>
<td>none</td>
</tr>
<tr>
<td>1</td>
<td>slight</td>
<td>0–12</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>moderate</td>
<td>0–12</td>
<td>basal 1/3 lost</td>
</tr>
<tr>
<td>3</td>
<td>severe</td>
<td>0–12</td>
<td>only surface epithelium intact</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0–16</td>
<td>entire crypt and epithelium lost</td>
</tr>
</tbody>
</table>

A score with a range of 0–3 was given for the criteria inflammation and transmural extent of inflammation as indicated, and a score with a range of 0–4 for crypt damage. The score of each feature was multiplied by a factor (1–4) according to the percentage of epithelial involvement. The scores of the 3 features were multiplied by a factor (1–4) according to the percentage of epithelial involvement. The values given are means ± SE.

### Table 3. Disease activity index at day 7

<table>
<thead>
<tr>
<th>Condition</th>
<th>DSS + Placebo</th>
<th>DSS + VSL#3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight loss</td>
<td>0.2 ± 0.13</td>
<td>2.93 ± 0.20</td>
<td>2.31 ± 0.31</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0.2 ± 0.2</td>
<td>1.86 ± 0.14</td>
<td>1.08 ± 0.29</td>
</tr>
<tr>
<td>Peranal bleeding</td>
<td>0.0 ± 0.0</td>
<td>3.58 ± 0.23</td>
<td>2.0 ± 0.51</td>
</tr>
<tr>
<td>Total DAI</td>
<td>0.4 ± 0.2</td>
<td>8.36 ± 0.37</td>
<td>5.38 ± 0.89</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 for healthy control, n = 14 for dextran sodium sulfate (DSS) groups. Scores for the 3 symptoms weight loss, diarrhea, and peranal bleeding and for the composite DAI are shown for the 3 groups at day 7. VSL#3 significantly reduced scores for diarrhea, peranal bleeding, and the overall DAI, whereas the effect on weight loss did not reach significance. P values given for DSS + VSL#3 vs. DSS + placebo.
were significantly higher than healthy controls (values given for DSS did not completely prevent the development of histological inflammation. The inflammation score was significantly reduced by VSL#3 therapy, crypt damage was not changed significantly. Total inflammation score is given for the 3 groups. Although Scores for the 3 features (inflammation, extent of inflammation, crypt damage) were significantly reduced by VSL#3 therapy, crypt damage was not changed significantly. Total inflammation score was significantly reduced by ~40%. However, VSL#3 therapy did not completely prevent the development of histological inflammation. P values given for DSS + VSL#3 vs. DSS + placebo. All values of DSS groups were significantly higher than healthy controls (P < 0.001).

VSL#3 prevents changes in expression and distribution of tight junction proteins. Since epithelial tight junctions regulate paracellular permeability, we examined the effect of the probiotic mixture VSL#3 on protein expression and organization of candidate tight junction proteins by immunofluorescence microscopy and Western blotting. In untreated mice, 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 crypts, consistent with their distribution in tight junctions (Figs. 4a and 5). Claudin-1, -3, and -5 were also localized at the tight junctions, and there was a gradient with more intense staining at the surface compared with the crypt. Additionally, a subpool of claudin-1, -3, and -5 was observed further down lateral epithelial cell membranes, as shown by an intense staining of the lateral cell boundaries (Fig. 5).

In DSS-treated mice, there was a substantial loss of occludin, ZO-1, and claudin-1, -3, and -5 from 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 (Figs. 4, b–e, and 5). This was most dramatically observed for occludin and ZO-1 at the apical membrane (Figs. 4a, b–e, and 5). Moreover, a submembranous internalization of occludin was seen (Fig. 4e), confirming our previous in vitro results, in which such an internalization was observed for occludin and JAM-1 (5, 6). Unlike the apical staining, basolateral staining of claudin-1, -3, and -5 remained unchanged in DSS colitis (Fig. 5). Despite dramatic redistributions in some tight junction proteins, the localization of adherens junction proteins E-cadherin and β-catenin remained unchanged in DSS colitis.

Interestingly, concomitant administration of VSL#3 could completely prevent the loss of all analyzed tight junction proteins (Figs. 4f and 5). To extend our observations of dramatic changes of tight junction protein localization, all candidate proteins were ana-

![Fig. 2. Histology. Microscopic appearance of colon specimens of the 3 groups (hematoxylin and eosin, original magnification ×40). A: healthy control. B and C: DSS + placebo. D: DSS + VSL#3. In DSS + placebo, a dense inflammatory cellular infiltrate is present in mucosa and submucosa. Crypts show a typical shortening. Focally crypts are completely lost and only surface epithelium is intact (center of B). In DSS + VSL#3, inflammation is less severe, and it does not involve the submucosa. However, crypt shortening was not affected by VSL#3 therapy.](http://ajpgi.physiology.org/)

![Fig. 3. Colonic permeability to Evans blue. To assess the permeability of the colonic epithelium at day 7 of the experiment, the colon was perfused with Evans blue in vivo for 15 min, and its uptake into the mucosa was quantified spectrophotometrically. Compared with healthy controls, there was a massively increased uptake of Evans blue in the DSS colonic epithelium at day 7.](http://ajpgi.physiology.org/)

![Table 4. Histological inflammation score](http://ajpgi.physiology.org/)

<table>
<thead>
<tr>
<th>Feature</th>
<th>Healthy Control</th>
<th>DSS + placebo</th>
<th>DSS + VSL#3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>0.36 ± 0.12</td>
<td>5.57 ± 1.02</td>
<td>2.93 ± 0.54</td>
<td>0.014</td>
</tr>
<tr>
<td>Extent</td>
<td>0.3 ± 0.17</td>
<td>5.57 ± 0.65</td>
<td>3.29 ± 0.65</td>
<td>0.029</td>
</tr>
<tr>
<td>Crypt damage</td>
<td>0.08 ± 0.06</td>
<td>3.5 ± 0.97</td>
<td>2.21 ± 0.70</td>
<td>0.271</td>
</tr>
<tr>
<td>Total Score</td>
<td>0.94 ± 0.28</td>
<td>14.64 ± 2.55</td>
<td>8.43 ± 1.82</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 7 for DSS groups, n = 9 for healthy control.
lyzed by Western blotting in the colons of control and DSS mice. Compared with untreated mice, significant reductions in total protein for occludin, ZO-1, and claudin-1 and -4 were observed in DSS-treated mice (Fig. 6). In contrast, total protein levels for claudin-2, -3, -5, and the adherens junction proteins β-catenin and E-cadherin remained unchanged. This indicates that the loss of occludin, ZO-1, and claudin-1 at colonic epithelial tight junctions of DSS mice was paralleled by quantitatively reduced protein expression levels. In contrast, the loss of claudin-3 and -5 at the tight junctions was not accompanied by a reduced quantitative expression. This might indicate a redistribution of existing proteins to the basolateral membrane.

Concomitant VSL#3 treatment completely prevented DSS-effects on tight junction protein levels (Fig. 6).

**VSL#3 prevents increased epithelial apoptosis induced by acute DSS colitis.** An increased apoptotic ratio of the colonic epithelium is a typical feature of DSS colitis. Since increased apoptosis has been shown to compromise epithelial barrier function (23–24, 27, 59–60), we evaluated the effect of VSL#3 on epithelial apoptosis in acute DSS colitis by morphology and immunostaining for cleaved caspase 3. In acute DSS-colitis, epithelial apoptosis was significantly increased, whereas VSL#3 completely prevented this increase (Fig. 7).

**VSL#3 prevents changes of bacterial distribution in the feces.** To investigate the effect of acute DSS colitis and VSL#3 on fecal flora, quantities of several bacteria were investigated semiquantitatively (scale 0–3) in the feces (Table 5). In placebo-treated DSS-colitis, *Enterococcus* species were significantly decreased compared with healthy controls (not detectable in 6 of 7 animals), whereas VSL#3 therapy prevented this decrease. There was a tendency to higher quantities of *E. coli* and *Proteus* in both DSS-treated groups, but these changes did not reach statistical significance. In placebo-treated DSS colitis, *Streptococci* and *Lactobacilli* were detected in lower quantities, whereas VSL#3 treatment prevented these changes compared with healthy controls, but again, these changes did not reach statistical significance.

**DISCUSSION**

In this study we investigated the influence of the probiotic mixture VSL#3 on epithelial permeability, tight junction protein expression and distribution, and epithelial apoptosis in a murine model of acute colitis. We show that the impairment of epithelial barrier function in acute colitis is associated with loss and redistribution of the tight junction proteins occludin, ZO-1, claudin-1, -3, -4, and -5, and an increased epithelial apoptotic ratio. Furthermore, our results indicate that the probiotic mixture VSL#3 protects colonic epithelial barrier function in acute DSS colitis, as demonstrated by complete prevention of increased Evans blue dye uptake. Our results suggest that probiotic-induced protection of epithelial barrier function is by two mechanisms: first, prevention of changes in tight junction protein expression and distribution; second, prevention of increased epithelial apoptosis.

There is a growing body of evidence that probiotics protect the epithelial barrier in animal models of intestinal inflammation (12–14, 37, 44, 58). It has been shown recently that probiotic *E. coli Nissle* significantly reduces Evans blue uptake in DSS colitis (55). However, unlike in our study, Evans blue uptake remained significantly elevated compared with healthy controls. One might speculate that the combined use of multi-
ple probiotic strains (VSL#3 contains 8 probiotic strains) has a stronger barrier-preserving effect than *E. coli Nissle* alone. Alternatively, the dose of probiotic bacteria used in our study ranges at the upper end of doses used in mouse models (20, 26, 37, 53, 55) and is about two times higher than the recommended maximum dose of VSL#3 in human IBD patients. With the high dose used in our study, a significant reduction of epithelial permeability was observed, whereas lower doses were not as effective. This raises the question whether higher doses should also be tested in human IBD, especially since no severe side effects have been recognized so far.

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 (5–6, 39, 41, 47, 61), much remains to be understood about barrier disruptive changes under inflammatory conditions in vivo. Our results not only confirm the loss of ZO-1 in DSS colitis shown by previous studies (7, 43, 46, 55), but for the first time report an additional loss of occludin, claudin-1, -3, and -5 at apical tight junctions. Interestingly, the basolateral subpools of claudins-1, -3, and -5 and of the adherens junction proteins E-cadherin and β-catenin, which do not contribute to the sealing properties of the epithelium, remained unchanged, confirming our previous in vitro results (5).

Our results fit well with findings in active human Crohn’s disease, which is associated with reduced expression of barrier-sealing claudins (60). But although a strong upregulation of pore-forming claudin-2 was observed in Crohn’s disease (60), claudin-2 remained unchanged in our experiments.

Interestingly, we observed an internalization of occludin into the cytosol of colonic epithelial cells in acute DSS colitis. These in vivo results confirm our previous in vitro results, where we observed an internalization of occludin, claudin-1

Fig. 5. Immunofluorescence for zonula occludens-1 (ZO-1) and claudins. Representative images for ZO-1, claudin-1, -3, and -5 are shown for the 3 groups. The tight junction proteins are stained green, and nuclei are counterstained blue. ZO-1 exclusively localizes at the apical tight junction, and an intense apical fluorescence band is found in healthy animals. Claudin-1, -3, and -5 are also localized at the tight junctions at the crypt surface (arrows), but additionally they are found at the basolateral membrane. The apical staining of ZO-1 and claudins is strongly reduced in DSS + placebo animals, whereas the basolateral staining of claudins remains unchanged. In VSL#3-treated animals, apical staining is preserved (arrows). Images are representative for 5 or more animals in each group. Original magnifications: ×400.
tight junction proteins plays a role not only in DSS colitis but also in human IBD, since we detected occludin and JAM-A in subapical vesicle-like structures in actively inflamed human ulcerative colitis mucosa (6). However, the mechanisms of internalization remain to be further elucidated.

We demonstrated that VSL#3 therapy prevented the above-mentioned changes in tight junction protein distribution and expression, thus explaining the dramatically improved barrier function in DSS mice concomitantly treated with probiotics.

Most data on the effect of probiotics on tight junction expression and distribution are derived from in vitro studies on epithelial cell monolayers (41, 47, 61). In vivo, probiotic therapy increased the expression of occludin in the gut mucosa of animals with an abdominal infection (44), and the expression of ZO-1 mRNA in DSS colitis (55). Although our study confirms probiotic effects on occludin and ZO-1, we now report an increased expression of sealing Claudins as well following probiotic exposure.

Besides changes in tight junction protein expression, an increased epithelial apoptotic rate in IBD seems to cause relevant leaks in the epithelial barrier (23–24, 27, 59–60). Because these tight junction proteins colocalized with markers of early and recycling endosomes (6), it was hypothesized that this allows a rapid redistribution to the apical cell-cell contacts with resealing of the epithelial barrier when proinflammatory stimuli are withdrawn (6, 29). Probably the internalization of tight junction proteins plays a role not only in DSS colitis but also in human IBD, since we detected occludin and JAM-A in subapical vesicle-like structures in actively inflamed human ulcerative colitis mucosa (6). However, the mechanisms of internalization remain to be further elucidated.

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### Table 5. Fecal microbiological flora

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Healthy Control</th>
<th>DSS Colitis</th>
<th>DSS Colitis + VSL#3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>2 [1–3]</td>
<td>3 [1–3]</td>
<td>3 [2–3]</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Proteus</em></td>
<td>0 [0–0]</td>
<td>0 [0–2]</td>
<td>0 [0–1]</td>
<td>0.086</td>
</tr>
<tr>
<td><em>Enterococci</em></td>
<td>1 [0–1]</td>
<td>0 [0–1]</td>
<td>1 [1–1]</td>
<td>0.001</td>
</tr>
<tr>
<td><em>Vidians group of streptococci</em></td>
<td>1 [0–2]</td>
<td>0 [0–1]</td>
<td>1 [0–1]</td>
<td>0.263</td>
</tr>
<tr>
<td><em>Nonhemolytic streptococci</em></td>
<td>1 [0–3]</td>
<td>0 [0–1]</td>
<td>1 [0–2]</td>
<td>0.139</td>
</tr>
<tr>
<td><em>Sporo–forming bacteria</em></td>
<td>0 [0–1]</td>
<td>0 [0–0]</td>
<td>0 [0–0]</td>
<td>0.115</td>
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<tr>
<td><em>Coagulase negative staphylococci</em></td>
<td>0 [0–0]</td>
<td>0 [0–0]</td>
<td>0 [0–0]</td>
<td>0.110</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0 [0–2]</td>
<td>0 [0–0]</td>
<td>0 [0–1]</td>
<td>0.504</td>
</tr>
<tr>
<td><em>Anaerobes</em></td>
<td>0 [0–2]</td>
<td>1 [0–2]</td>
<td>0 [0–2]</td>
<td>0.406</td>
</tr>
<tr>
<td><em>Lactobacilli</em></td>
<td>1 [0–1]</td>
<td>0 [0–1]</td>
<td>1 [0–1]</td>
<td>0.122</td>
</tr>
</tbody>
</table>

Data are given as median [range]. Bacterial species in stool specimens were evaluated semiquantitatively in the 3 experimental groups (n = 7 each), using a score of 0 (not detected) to 3 (maximum). In DSS colitis, *Enterococci* were significantly reduced compared to healthy controls (not detected in 6 of 7 individuals), whereas VSL#3 therapy prevented this loss of *Enterococci*. Other changes in microbiological flora did not reach significance. There was a tendency toward higher concentrations of *Lactobacilli* (P = 0.122) following VSL#3 treatment.

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**Fig. 7. Epithelial apoptosis.** Epithelial apoptotic ratio was assessed by immunofluorescence for cleaved caspase 3. As expected, animals with DSS colitis showed a significantly increased apoptotic ratio. Concomitant treatment with VSL#3 completely prevented this increase. Values are given as means ± SE, n = 5 for each group.

**Fig. 6.** A: Western blots of tight junction and adherens junction proteins. Quantitative expression of occludin, ZO-1, claudin-1, and claudin-4 was strongly reduced in placebo-treated DSS colitis. VSL#3 therapy preserved protein expression on the level of healthy controls. Other tight junction proteins and the adherens junction proteins E-cadherin and β-catenin were not affected by DSS colitis. Blots are representative of at least 5 animals, experiments were performed in triplicate or greater. To enable direct comparison and to confirm equal loading of total protein, each blot was reprobed with actin antibodies. B: Western blot densitometry. Immunoblots for actin were performed for each membrane to normalize epithelial protein loading between samples. Densitometry results of tight junction and adherens junction proteins are given in relation to healthy controls on the same blot, which were set as 100%. Expression of occludin, ZO-1, claudin-1, and claudin-4 was strongly reduced in DSS + placebo. VSL#3 therapy preserved protein expression on the level of healthy controls. Values given as means ± SE, n = 6 in each group. *P < 0.05. Differences between healthy controls and DSS + VSL#3 were not significant.
Interestingly, in our experiments VSL#3 treatment completely prevented the increase of apoptotic ratio in DSS colitis. We speculate that the prevention of increased epithelial apoptosis by probiotic therapy also contributes to the protection of the epithelial barrier.

The present study is not able to clarify which mechanism, either altered expression of tight junction proteins or increased epithelial apoptosis, is more important for barrier dysfunction in this animal model of IBD. We speculate that both mechanisms significantly contribute to the breakdown of the epithelial barrier, and certainly both mechanisms are prevented by probiotic therapy in our model.

Since DSS has a direct toxic effect on epithelial cells and can lead to erosions with a complete loss of surface epithelium, we induced a rather moderate colitis by administration of 3.5% DSS. At this concentration, distortion of crypt architecture was only mild as shown by a low crypt damage score (3.5 points of 16). We did not observe a loss of surface epithelium, thus allowing the assessment of the barrier function of an intact epithelial lining.

Although VSL#3 significantly improved DSS colitis, it did not completely prevent the development of colitis. This mirrors the clinical situation in human IBD, in which probiotics improve but do not heal the disease.

It is an interesting observation that paracellular permeability reflecting tight junction barrier integrity was completely normalized by VSL#3 therapy despite residual histological and clinical inflammation. This observation has been confirmed by a recent study (14). IL-10-deficient mice were treated with probiotics for 30 days. Although paracellular mannitol fluxes were completely normalized to the level of healthy controls, histological inflammation scores remained significantly higher than in healthy controls (14). Obviously, a mild intestinal inflammation does not necessarily lead to a breakdown of the intestinal barrier. Likewise, IBD patients can be free of symptoms although endoscopy reveals inflammatory activity.

We speculate that several factors could explain the residual inflammation despite completely normalized paracellular permeability. First, an increased transepithelial transport with a persistent uptake of antigens might lead to residual inflammation. Second, besides increased epithelial permeability, many other factors play a role in the pathogenesis of DSS-induced colitis. Cytokines, cellular reactions, and oxygen metabolites are some factors that could explain residual inflammatory activity (8, 10).

The exact pathways that mediate the barrier-preserving effect of probiotic bacteria are not yet completely clarified. The protective effects of probiotics are mediated by their own DNA rather than by their metabolites or ability to colonize the colon (4, 7, 26), and nonviable probiotics are equally effective (45). TLR-2, -4, and -9 signaling seems essential in mediating the anti-inflammatory effect of probiotics in DSS colitis (7, 26, 45).

Changes in microbial flora probably are not a major determinant of the observed anti-inflammatory effect. Recent studies indicate that parenteral administration of probiotic bacteria is effective in attenuating murine colitis (53). Hence, the ability of probiotic bacteria to colonize the intestine seems not to be a prerequisite for their anti-inflammatory action. Various studies in humans and in animal models demonstrated that oral administration of VSL#3 leads to increased numbers of *Lactobacilli* and *Bifidobacterium* in the fecal microflora (20, 37, 57), thus confirming our results.

To conclude, we demonstrated that probiotic therapy protects the epithelial barrier in a murine model of colitis by two mechanisms: first, probiotics prevent redistribution and reduced expression of sealing tight junction proteins. Second, probiotics prevent an increased apoptotic ratio. Probably both mechanisms contribute to improved barrier function. We hypothesize that protection of the epithelial barrier is a major determinant of the observed anti-inflammatory properties of VSL#3.

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