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Lactobacillus reuteri prevents colitis by reducing P-selectin-associated leukocyte- and platelet-endothelial cell interactions

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Schreiber O, Petersson J, Phillipson M, Perry M, Roos S, Holm L. *Lactobacillus reuteri* prevents colitis by reducing P-selectin-associated leukocyte- and platelet-endothelial cell interactions. *Am J Physiol Gastrointest Liver Physiol* 296: G534–G542, 2009. First published January 15, 2009; doi:10.1152/ajpgi.90470.2008.—Recent findings indicate that dextran sodium sulfate (DSS)-induced colitis is associated with a prothrombogenic phenotype, with P-selectin playing a major role in platelet recruitment. It has been suggested that probiotics may ameliorate colonic inflammation. We therefore investigated how treatment with *Lactobacillus reuteri* influenced P-selectin expression, leukocyte and platelet endothelial cell interactions, and colitis severity in DSS-treated rats. Rats were divided into the following four groups: nontreated, DSS treated (5% in drinking water for 9 days), *L. reuteri*, and *L. reuteri* and DSS treated. The rats were anesthetized with Inactin (120 mg/kg ip), and the dual radiolabeled monoclonal antibody technique was used to quantify P-selectin expression. Leukocyte-endothelial and platelet-endothelial cell interactions were studied in colonic venules with intravital microscopy. Colitis severity was assessed using a disease activity index. Disease activity index increased, as did the expression of P-selectin in the entire colon after DSS treatment, but both were reduced to control levels with *L. reuteri* pretreatment. The increased platelet- and leukocyte-endothelial cell interactions after DSS treatment were abolished by pretreatment with *L. reuteri*. *L. reuteri* protects against DSS-induced colitis in rats. The protection is associated with reduced P-selectin expression and a decrease in leukocyte- and platelet-endothelial cell interactions.

dextran sulfate sodium; disease activity index

INFLAMMATORY BOWEL DISEASE (IBD) is a chronic disorder of the gastrointestinal tract and includes Crohn's disease and ulcerative colitis (UC). The exact mechanism behind the onset and perpetuation of IBD is unknown. However, it has been known for decades that the administration of antibiotics sometimes alleviates flares of the disease (10, 43). In addition to this, subgroups of IBD patients have mutations in different receptors recognizing luminal bacterial products [CARD15/NOD2, Toll-like receptor (TLR)-4], indicating that a dysfunctional interaction between the intestinal microbiota and the gut immune system may be the mechanism behind IBD (13, 42). Thus current IBD research focuses on the cross talk between bacteria and the host (7, 47).

The intestinal flora is made up of >500 bacterial species (28). It is, however, very difficult to get a complete picture of this flora since a large portion of the bacteria found here cannot be cultured on medium (19). The gut microflora is also very individual, with a great variation between individuals (58).

Probiotics are viable microorganisms with beneficial physiological or therapeutic activities, originally derived from cultured foods, especially milk products. Numerous experimental studies have shown that probiotics can ameliorate colitis (3, 17, 24, 27, 34). However, the exact anti-inflammatory mechanism remains unknown.

The inflammation in IBD is associated with an activation of platelets in the colonic microcirculation, with P-selectin playing a major role in their recruitment (30). IBD patients have higher levels of platelet aggregation responses (1, 2, 22), increased circulating P-selectin levels (15), and an elevated risk of thrombosis than do healthy controls (14). Thus heparin treatment has been suggested for IBD patients, although any possible benefits have to be weighed against the increased risk of rectal bleeding (9).

P-selectin is one of many cell adhesion molecules involved in the recruitment of leukocytes and platelets and is expressed on activated platelets and on endothelial cells. In endothelial cells, it is stored in the Weibel-Palade bodies and in platelets in α-granules. Upon activation, the P-selectin translocates to the membrane and takes part in the recruitment of leukocytes (36). Immunoblockade of either P-selectin or the P-selectin glycoprotein ligand 1 (PSGL-1) protects against dextran sulfate sodium (DSS) induced murine colitis (44, 57).

The aim of this study was to investigate the positive effects of *Lactobacillus reuteri* pretreatment on colonic mucosal inflammation, by studying its effect on the interaction between endothelium and leukocytes and platelets. P-selectin expression was measured with a dual radiolabeled monoclonal antibody (MAb) technique (35), and leukocyte- and platelet-endothelial interactions in the colonic mucosal venules were studied in vivo by intravital microscopy.

MATERIALS AND METHODS

Animals

Eighty eight male Sprague-Dawley rats (B&K) weighing 170–240 g were kept under standardized pathogen-free conditions (temperature 21–22°C, illumination 12:12-h light-dark cycle) with free access to pelleted food and water ad libitum (Ewos; Södertälje, Sweden). All experiments were approved by the Swedish Laboratory Animal Ethical Committee in Uppsala.

Rats were divided into the following four treatment groups; DSS, *L. reuteri* and DSS, nontreated, and *L. reuteri* alone (Fig. 1). All

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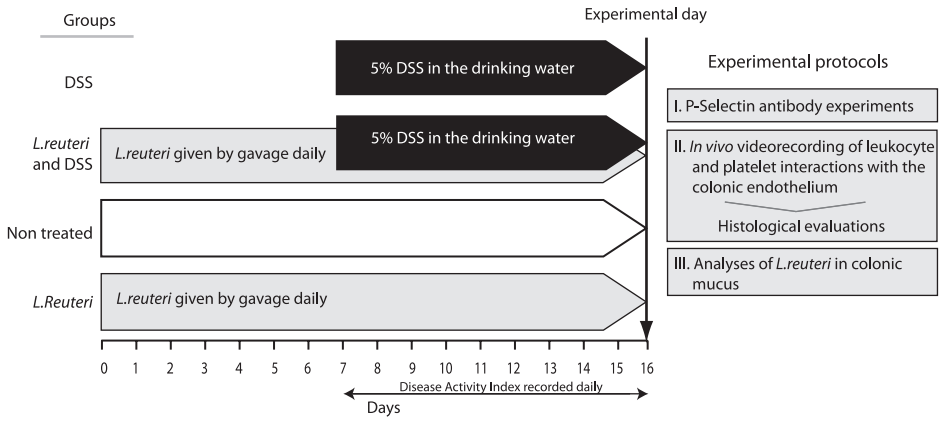


Fig. 1. Experimental protocol.

animals were anesthetized with thiobutabarbital sodium Inactin (120 mg/kg ip; Research Biochemicals, Natick, MA).

Induction of Colitis

Colitis was induced by giving 5% DSS (TdB Consultancy, Uppsala, Sweden) with a molecular weight of 40 kDa ad libitum in drinking water for 9 days.

Assessment of Colitis

The severity of colitis was assessed daily using a disease activity index (DAI) ranging from zero to four. DAI is based on the scoring system of Murthy et al. (31), which assesses the following three parameters: weight loss, stool consistency, and the presence or absence of fecal blood.

Bacterial Suspensions

The bacterial cocktail consisted of the following four different strains of *L. reuteri*: two isolated from rat, R2LC (29) (a kind gift from Siv Ahrné, Lund University, Lund, Sweden) and JCM 5869 (Japan Collection of Microorganisms), and two from human sources, ATC PTA 4659 and ATCC 55730 [a kind gift from Biogaia, Stockholm, Sweden (48)]. The bacteria were cultivated separately in 200 ml Man Rogosa Sharp medium broth (Oxoid, Basingstoke, UK) at 37°C for 20 h, washed with PBS, and suspended in 2 ml freezing solution [0.82 g K_2HPO_4, 0.18 g KH_2PO_4, 0.59 g sodium citrate, 0.25 g $MgSO_4 \cdot 7H_2O$, and 172 ml glycerol (87%) in 1,000 ml]. The bacterial suspensions were mixed and stored at −70°C until used.

L. reuteri-treated rats were given a cocktail of 10^9 bacteria in 0.5 ml saline, containing an equal amount of the four different strains of *L. reuteri.* This cocktail was given daily by gavage for 16 days. The rats treated with both *L.* reuteri and DSS were given 5% DSS in the drinking water for the last 9 days of their *L. reuteri* treatment.

P-selectin Antibody Experiments: Experimental Design (Group I)

For the P-selectin experiments, 26 rats were used (see Fig. 3*D*). The right carotid artery and left jugular vein were cannulated for injection of antibodies and for blood sampling. To measure P-selectin expression, a mixture of 10 μg of ^{125}I-labeled P-selectin MAb (RMP-1) and 5 μg of ^{131}I-labeled nonbinding MAb (P-23) were injected (35). For labeling, see *Platelet Preparation and labeling* and *Expression of P-selectin activity*. After the tracer injection, the animals were heparinized (3,000 IU/kg), and blood samples were taken via the carotid artery catheter at 2.5 and 5.0 min. At 5.0 min, the animal was exsanguinated via the carotid artery with a simultaneous infusion of PBS via the jugular vein. The vena cava was then severed, and the circulation was flushed via the carotid artery with ~60 ml of buffer. The organs for study were taken out, washed, blotted dry, weighed, put into test tubes, and counted for ^{125}I and ^{131}I activity, see below. The selected organs were as follows: left and right kidneys (as control for good mixture of the injected P-selectin antibodies), stomach, duodenum, jejunum, ileum, cecum, proximal colon, transverse colon, and distal colon [divided in superficial mucosa (scraped off using a scalpel) and remaining mucosa, submucosa and, muscle].

Labeling of P-selectin with ^{125}I and ^{131}I. The MAb RMP-1 directed toward P-selectin (53) was labeled with ^{125}I (DuPont NEN, Boston, MA), whereas the isotype-matched nonbinding antibody P23 (26) was labeled with ^{131}I. (Antibodies were kindly supplied by Dr. D. Neil Granger, Louisiana State University, Shreveport, LA.) Radioiodination was performed using the iodogen method, as previously described (35). The labeled antibodies were stored in 0.5-ml aliquots at 4°C for a maximum of 3 wk.

Expression of P-selectin activity. The activity of ^{125}I and ^{131}I was determined using an LKB 1282 Compugamma (Wallac Oy, Turku, Finland). Samples were counted for sufficient time to obtain an accuracy of ± 1%. The total activity injected (and the total nanograms of antibody injected) in each experiment was calculated by counting a 5-μl sample of the injectate. The activity remaining in the injection syringe was subtracted from the total injected counts. The accumulated activity in the tissue was expressed as percent antibody bound per gram tissue and was calculated as follows:

$$\%\text{antibody bound/g} = (\%\text{ID} \times g^{-1} \text{ for } ^{125}\text{I}) - [(\%\text{ID} \times \text{g}^{-1} \text{ for } ^{131}\text{I}) \times (\%\text{ID } ^{125}\text{I in plasma})/(\%\text{ID } ^{131}\text{I in plasma})]$$

where %ID is percentage of injected dose. Nanograms of antibody bound per gram were calculated as follows:

$$\text{ng antibody bound/g} = (\text{corrected \%injected } ^{125}\text{I bound/g}) \times (\text{total ng } ^{125}\text{I antibody injected}) \times 100^{-1}$$

Intravital Videomicroscopy: Experimental Design (Group II)

For the intravital microscopy experiments, 37 rats were used (Figs. 4*B* and 5*B*). After anesthesia was induced, a tracheotomy was performed to facilitate breathing. Body temperature was maintained at 37 ± 0.5°C by a heating pad controlled by a rectal thermistor probe. The right femoral vein was cannulated for administration of Ringer solution as well as for the administration of fluorescently labeled platelets. The colon preparation has been previously described (6). Briefly, the distal colon was opened longitudinally, and a segment of the colon was draped over a truncated conical pedestal, mucosal or serosal side up. The mucosal side was used for studies of the mucosal microcirculation since the DSS-induced colitis is primarily located in the mucosa, and the serosal side was used for studies of the submucosal microcirculation as a comparison with the mucosa. A chamber with an opening in the bottom, exposing 0.8 cm^2 of the colon, was gently attached with a pliable sealant. The surface was bathed in warm

(37°C) 0.9% NaCl. The right femoral artery was cannulated for blood pressure monitoring.

Intravital videomicroscopy. The studied area was viewed by transillumination with a xenon lamp (75 W) attached to a Leitz Ortholux microscope (Wetzlar, Germany). The images were displayed on a computer screen (iMac; Apple) via a television camera (Hamamatsu charge-coupled device camera C3077 with camera control C2400), through an analog-to-digital converter Canopus ADVC-55 (Scandinavian Photo, Stockholm, Sweden) or a digital camera JVC Tk-C148IEG (Niman Bildteknik, Stockholm, Sweden). The images were then stored on the computer for later analysis. The area under observation was linearly magnified ×25 by a Leitz water-immersion lens (sw 25/0.60).

To be able to compare results from the mucosa and submucosa, venules up to a diameter of 20 μm were studied since this was the widest venule found in the mucosa.

Leukocyte-endothelial cell interactions. Values were obtained for leukocyte adhesion and rolling as previously described (39). Leukocytes were regarded as adherent if they remained stationary for 30 s and longer and rolling if they moved with a lower velocity than red blood cells. The numbers of rolling or adherent leukocytes were measured during 1- to 2-min recordings and expressed as the numbers of rolling or adherent leukocytes per square millimeter per minute.

Platelet-endothelial cell interactions. Fluorescently labeled platelets (see below for labeling procedure), 800×10^6 platelets in 0.5 ml PBS, were infused over a period of 5 min and were then allowed to circulate for 5 min. The infused platelets accounted for ~10% of the total platelet population. The platelets were detected using a fluorescent filter (N2.1; Leitz) with an excitation of 530–560 nm and an emission >580. The number of interacting platelets was measured during ~1-min recordings in the same vessels in which the leukocytes were measured. The platelets were considered interacting if they remained stationary for ~1 s or more and were expressed as interacting platelets per square millimeter per 30 s.

Platelet preparation and labeling. Platelets were derived from anesthetized nontreated donor rats. The carotid artery was cannulated, and 5.4 ml blood were withdrawn. The blood was collected in a 10-ml syringe containing 0.6 ml Citrate-Dextrose Solution (Sigma-Aldrich, Stockholm, Sweden). A sequence of centrifugations, as described previously (11), rendered a platelet solution. A volume of the platelet solution containing 800×10^6 platelets was brought up to 1.5 ml with PBS (pH 7.4). The platelets were incubated at room temperature for 10 min with the fluorocrome carboxyfluorescein diacetate succimudyl ester (CFSE; Sigma-Aldrich, Stockholm, Sweden) at a final concentration of 90 mM. The solution was then centrifuged, the supernatant was discarded, and the pellet was resuspended in 500 μl of PBS and infused in the recipient animal over a period of 5 min.

Histology. Samples of the distal colon from *group II* (animals used for intravital microscopy) were fixed in 4% formalin, processed, embedded in wax, sectioned, and stained with hematoxylin-eosin. The percent of mucosal damage was assessed by measuring the length of mucosa that was damaged and dividing that length by the total length of the colonic section examined.

Quantification of Lactobacilli in Colonic Mucus (Group III)

The levels of lactobacilli in the colonic mucus were quantified in another 15 rats [untreated $n = 3$, DSS treated $n = 3$ (DSS for 9 days), *L. reuteri* and DSS treated $n = 3$ (bacteria for 16 days, DSS last 9 days), *L. reuteri* treated $n = 3$ (bacteria for 16 days)]. To investigate if the lactobacilli survived 2 wk after administration, a fourth group was added ($n = 3$) in which *L. reuteri* was given for 2 days only, with no bacteria administered for the following 5 days nor during the 9 days of DSS treatment. The loosely adherent mucus layer was removed under a microscope by gentle suction, and the remaining, firmly adherent mucus layer was scraped off the mucosa with a scalpel. The samples were snap-frozen in liquid nitrogen at −70°C. The total volume of the mucus layers was estimated, from measurements of mucus thickness and exposed area, a measurement that has been extensively described previously (6). The mucus samples were homogenized by pipetting and vortexing and then serially diluted. A 100-μl aliquot of each dilution was spread on Rogosa plates (Merck, Darmstadt, Germany), which were incubated at 37°C for 48 h in anaerobic atmosphere (Gaspack system, BD, Sparks, MD).

Identification and typing of isolated lactobacilli. The strain R2LC is easily detected by its strong yellow pigmentation. White and yellow lactobacilli colonies were counted separately, and 10 representative colonies from each sample were isolated for identification. The isolated lactobacilli were identified by sequencing of the first 600 bp of the 16S rRNA gene according to standard procedures (38). Isolates identified as *L. reuteri* were typed by repetitive chromosomal elements PCR by using the primer (GTG)5 according to a method described by Versalovic et al. (49). The PCR products were separated by electrophoresis at 2.5 V/cm in a 1% agarose gel in 0.5× Tris-borate-EDTA buffer. The gels were stained with ethidium bromide, and digitized images were captured under ultraviolet transillumination. The obtained rep PCR fingerprints were compared by visual inspection.

Statistics

All values are expressed as means ± SE. Parametric data was analyzed with ANOVA and nonparametric data with Mann-Whitney. Differences were considered significant at $P < 0.05$.

RESULTS

Disease Activity Index

The DAI recorded daily for 9 days in DSS-treated and *L. reuteri*- and DSS-treated animals is presented in Fig. 2*A*. The DSS-treated rats experienced a gradually increasing DAI that culminated with bloody, watery stools on the day of the experiment (1.8 ± 0.2, $n = 22$). Treatment with *L. reuteri* before and during DSS induction resulted in a markedly lower DAI (0.4 ± 0.1, $n = 15$). Interestingly, 60% of the rats treated with *L. reuteri* and DSS had a DAI of zero compared with only 5% of the DSS-treated rats. Nontreated ($n = 6$) and *L. reuteri*-treated rats ($n = 4$) did not display any disease symptoms and consequently had DAI values of zero.

Histological Evaluation

The DSS-treated animals had a significantly increased mucosal damage (51.6 ± 13.2% of the mucosal length, $n = 5$) compared with control rats (10.1 ± 1.5%, $n = 3$), as seen in Fig. 2, *B* and *C*. Rats treated with either *L. reuteri* alone or *L. reuteri* plus DSS to induce colitis had mucosal damage scores similar to the control rats (*L. reuteri* 14.3 ± 6.7%, $n = 3$, *L. reuteri* and DSS 12.3 ± 3.7%, $n = 3$). The histological evaluation was based on animals from the intravital videomicroscopy experiments, since the colon of the rats used for P-selectin antibody experiments were used for counting the radioactivity.

P-Selectin expression (Group I)

During DSS-induced colitis, P-selectin was strongly upregulated throughout the colon, and the highest levels were observed in the distal colon (12.6 ± 1.8) (Fig. 3, *A–C*). Treatment with *L. reuteri* before and during DSS induction of colitis completely prevented the strong DSS-induced upregulation of P-selectin (3.8 ± 1.0) (Fig. 3). The nontreated rats and the rats

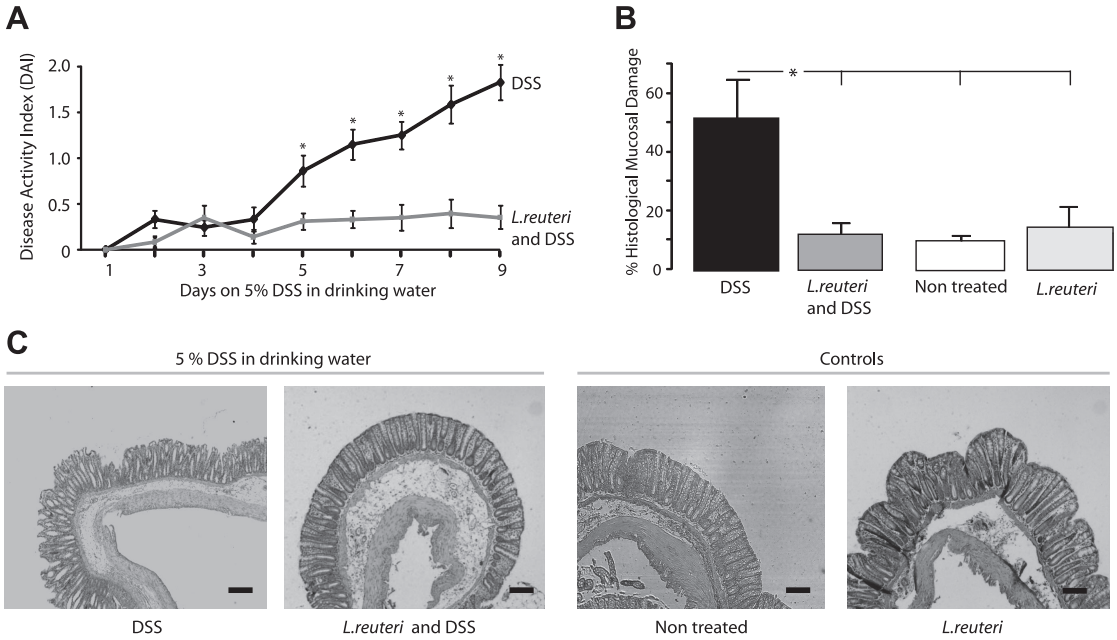
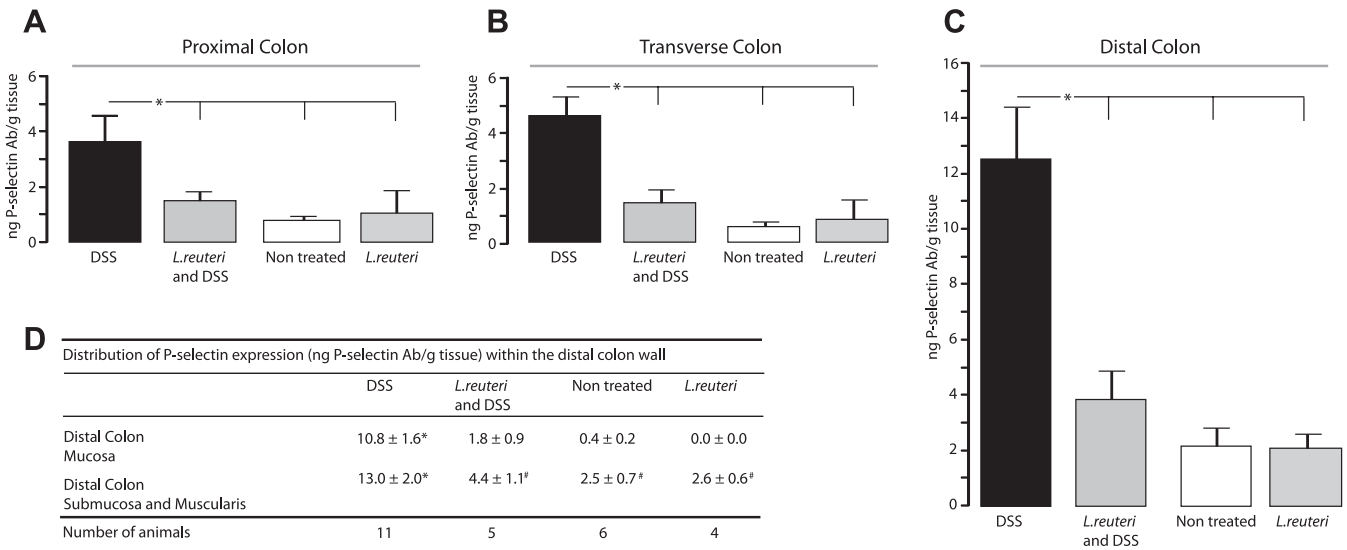


Fig. 2. Evaluation of disease activity and histological damage. *A*: disease activity index in dextran sulfate sodium (DSS)-treated animals was significantly greater than that observed in animals treated with *Lactobacillus reuteri* plus DSS. Nontreated and *L. reuteri*-treated animals had a disease activity index of zero throughout the experiment and therefore are not included. *B*: %mucosa showing histological damage in distal colon expressed as %total length of the mucosa examined. *C*: histological slides of the distal colon showing mucosal damage only in DSS-treated animals (bar = 200 μm). *$P < 0.05$ vs. all other groups.

treated only with *L.* reuteri showed similar, very low, levels of P-selectin expression (2.2 ± 0.7 and 2.1 ± 0.5, respectively). Dividing the distal colon in the superficial mucosa and deeper layers showed that, under control conditions, P-selectin expression in the mucosa is barely detectable (3D). During DSS treatment, P-selectin in the mucosa was significantly increased to 10.8 ± 1.6 ng antibody (Ab)/g tissue, and similar values were recorded in the deeper layers of the colon. *L. reuteri* treatment significantly reduced the DSS-induced P-selectin expression in both mucosa and submucosa/muscularis to control levels. In the rest of the gastrointestinal tract, only the stomach had elevated P-selectin levels during DSS colitis (1.1 ± 0.3 ng Ab/g tissue) compared with nontreated controls (0.5 ± 0.2 ng Ab/g tissue).

Intravital Videomicroscopy (Group II)

In the intravital videomicroscopy experiments, blood pressure remained stable during the entire experimental period within the groups and did not differ between the groups [nontreated: 97 ± 4 mmHg (n = 8), DSS treated: 97 ± 4 mmHg (n = 8), *L. reuteri* and DSS treated: 99 ± 4 mmHg (n = 6)]. Because no P-selectin upregulation or any damage was seen in the group receiving only *L.* reuteri, we did not measure



D

Distribution of P-selectin expression (ng P-selectin Ab/g tissue) within the distal colon wall

	DSS	*L.reuteri* and DSS	Non treated	*L.reuteri*
Distal Colon Mucosa	10.8 ± 1.6*	1.8 ± 0.9	0.4 ± 0.2	0.0 ± 0.0
Distal Colon Submucosa and Muscularis	13.0 ± 2.0*	4.4 ± 1.1#	2.5 ± 0.7#	2.6 ± 0.6#
Number of animals	11	5	6	4

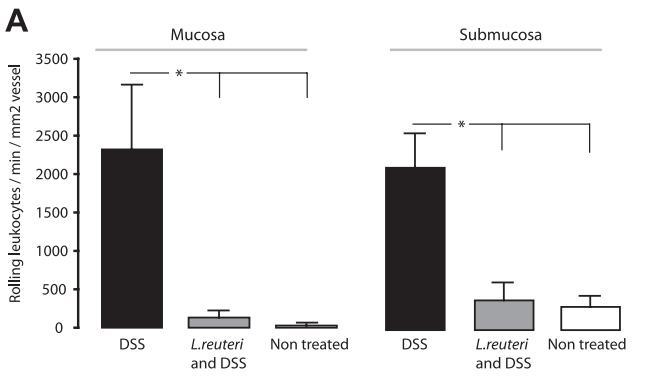
Fig. 3. P-selectin expression in proximal (*A*), transverse (*B*), and distal (*C*) colon. *D*: P-selectin expression in the distal colon divided in superficial mucosa and deeper layers (combined submucosa and muscularis). $P < 0.05$ vs. all other groups (*) and vs. distal colon mucosa within group (#).

leukocyte and platelet-endothelial cell interactions in this group but used the nontreated group as the only control group.

Leukocyte-endothelial cell interactions. In the control animals, there was a very low level of leukocyte rolling in the mucosal venules, but significantly more rolling cells in the submucosal venules, although the level is still low (Fig. 4*A*). Rolling leukocytes were seen in 5% of the mucosal vessels studied and in 55% of the submucosal venules of similar size.

The number of adherent leukocytes in the control colonic venules was very low and did not differ between the mucosal and the submucosal venules (Fig. 4*C*). Adherent leukocytes were found in 8% of the mucosal venules and in 9% of the submucosal venules studied.

Inducing colitis with DSS markedly increased both the number of rolling leukocytes in the mucosal and submucosal venules by 90 and 20 times, respectively, and also the number of adherent leukocytes in the mucosal and submucosal vessels by 7 and 20 times, respectively, with no significant difference seen between the values obtained in superficial and deeper venules. Treatment with *L. reuteri* before and during the DSS treatment prevented the increase in leukocyte rolling and adherence in the mucosal and submucosal venules (Fig. 4, *A* and *C*). Figure 5 shows images of submucosal venules in nontreated, DSS-treated, and *L. reuteri* and DSS-treated rats illustrating the difference in leukocyte-endothelial cell interactions.



B

	Mucosa			Submucosa		
	DSS	*L.reuteri* and DSS	Non treated	DSS	*L.reuteri* and DSS	Non treated
Studied vessels (n)	30	31	36	19	13	11
Animals (n)	8	6	8	6	4	5

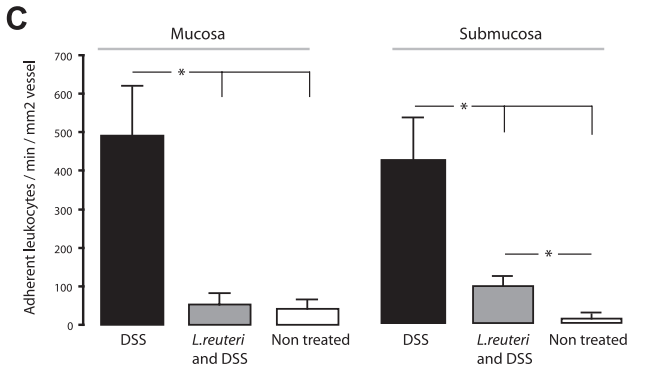


Fig. 4. Leukocyte-endothelial cell interactions in the distal colon. *A*: rolling leukocytes per min and mm^2 in mucosa and submucosal venules. *B*: no. of studied vessels and animals in the mucosa and submucosa. *C*: adherent leukocytes in mucosal and submucosal venules. *$P < 0.05$ vs. all other groups.

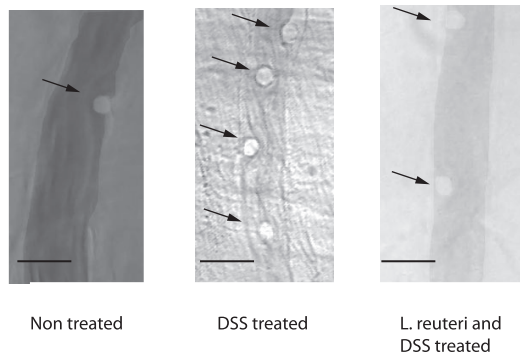
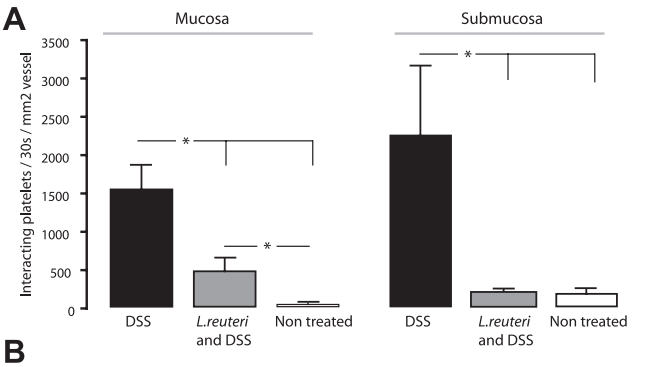


Fig. 5. Freeze frames from intravital videomicroscopy showing differences in leukocyte rolling (arrows) in submucosal venules in nontreated, DSS-treated, and *L. reuteri*- and DSS-treated rats. Bar 20 μm.

An additional observation was that the mucosal colonic venule diameter was larger ($P < 0.05$) in the two groups receiving DSS (DSS treatment 16 ± 2 μm, *L. reuteri* and DSS treatment 14 ± 1 μm) compared with the nontreated controls (12 ± 1 μm). Hence, to be able to compare the results between the different groups, leukocyte rolling and adherence were quantified per square millimeter of colonic venule, assuming cylindrical vessel shape (45).

Platelet-endothelial cell interactions. The platelets behaved differently from the larger leukocytes in that most adhered only briefly to the venular endothelium, and very few were seen to adhere for longer time periods. The platelets were considered adherent (to interact) if they halted at the endothelium for ~1 s or more.

In the colon of the nontreated animals, there were almost no interactions between platelets and mucosal endothelium, whereas a significant but still low level of interactions occurred in the submucosal venules (Fig. 6*A*), similar to the behavior observed for rolling leukocytes. After DSS induction of colitis, the



B

	Mucosa			Submucosa		
	DSS	*L.reuteri* and DSS	Non treated	DSS	*L.reuteri* and DSS	Non treated
Studied vessels (n)	21	15	19	11	13	9
Animals (n)	5	5	5	4	5	4

Fig. 6. Platelet-endothelial cell interactions in the distal colon. *A*: interacting platelets per 30 s and mm^2 vessel in mucosal and submucosal venules. *B*: no. of animals and vessels studied in the mucosa and submucosa. *$P < 0.05$.

number of adherent platelets was markedly increased, and the levels were not significantly different in the mucosal and the submucosal venules. Interactions between platelets and endothelium were seen in 80% of the mucosal venules and 100% of the submucosal venules. Treatment with *L. reuteri* before and during DSS treatment prevented the large increase in platelet-endothelial interactions in both the mucosal and submucosal colonic venules (Fig. 6*A*). However, in the mucosa, the platelet-endothelium interactions were significantly higher than in the control animals while in the submucosa the level was reduced to the control value. Figure 7 displays a series of images from a video clip of a 20-μm-diameter submucosal venule of a *L. reuteri*- and DSS-treated rat. Numerous CFSE-labeled fluorescent platelets (green) pass through the vessel, leaving a tail of green fluorescence behind, whereas one platelet transiently interacts with the endothelium before it releases. This event occurs 13 times as often in submucosal venules of DSS-treated rats.

Although the aim was to measure platelet-endothelial interactions in the same vessels as leukocyte-endothelial interactions, technical difficulties resulted in slightly fewer vessels being studied for platelet-endothelial interactions.

Analyses of Lactobacilli (Group III)

L. reuteri R2LC but none of the other three strains could be detected in the mucus samples from animals given the bacterial mix (Table 1). Strain R2LC constituted ~50% of all lactobacilli found in the mucus samples, and the number of R2LC was not affected by the DSS treatment. This strain also persisted 14 days after the last oral administration in numbers similar to those observed in the two groups where *L.* reuteri was given continuously. Furthermore, despite the rats being given the four *L. reuteri* strains for only 2 days, followed by 5 days without treatment and another 9 days with DSS treatment, they were protected against the DSS-induced colitis (DAI 0.4 ± 0.2). The total number of lactobacilli found in the colonic mucus was significantly higher in the DSS-treated rats compared with all *L. reuteri*-treated groups.

DISCUSSION

Probiotics have been shown to reduce disease severity in IBD, both in humans and in animal models (3, 17, 24, 25, 34), but relatively little is known of the mechanisms behind their anti-inflammatory actions. In this study, we show that treatment with *L. reuteri* ameliorates DSS-induced colitis in rats and prevents the upregulation of P-selectin expression as well as leukocyte and platelet interactions with the endothelium. The clinical and histological observations also show a significant reduction of the colonic inflammation, which correlates well with the results from the intravital microscopy studies and P-selectin antibody experiments.

The etiology of IBD is not yet elucidated, but the current dogma is that it might be caused by a dysregulated immune response toward the commensal flora (32). Environmental factors, such as ingestion of pathogens or chemical substances disturbing the delicate equilibrium in the intestine, as well as genetic susceptibility, have been implicated in the pathogenic mechanisms behind IBD (55). Here we have used the DSS model of colitis, which resembles UC with a diffuse mucosal inflammation covering the entire colon, resulting in diarrhea and rectal bleeding (12, 31, 33, 40).

P-selectin has been shown to be a key molecule in the pathophysiology of DSS-induced murine colitis (18, 46, 57). Biopsy specimens from IBD patients have also demonstrated an upregulation of P-selectin (15). The upregulation of P-selectin is primarily responsible for the process of leukocyte rolling (54). Immunoblockade of selectin function protects against DSS-induced murine colitis as does the immunoblockade of PSGL-1 (44, 57).

We and others (16, 20, 40) have earlier observed that the degree of inflammation in DSS-induced colitis tends to be greater in the distal than in the proximal colon. This is confirmed here in the histological sections as well as in the P-selectin expression, which was three times higher in the distal compared with the proximal and transverse colon. The in vivo studies were therefore performed in the distal colon.

Because the inflammation in DSS-induced colitis as well as in UC is confined mainly to the mucosa, we studied leukocyte- and platelet-endothelial interactions in this layer of the colon. As a comparison, we also investigated deeper layers. We found that, in the untreated control animals, leukocyte-endothelial interactions are much lower in the superficial colonic mucosa than in the deeper layers. This observation is confirmed by the antibody experiments revealing that P-selectin levels are significantly lower in the superficial mucosa than in deeper layers in all noncolitic rats. One possible explanation to this observation could be a lower venular density in the superficial mucosa, but this is less probable, since we also observed significantly less rolling leukocytes in the superficial part of the colon than in the submucosa.

It is surprising that the submucosal venules have higher levels of leukocyte-endothelial interactions, since the superficial mucosal microvasculature is closer to the inflammatory stimuli of the massive luminal bacterial content. These results do, however, agree with earlier studies from our laboratory

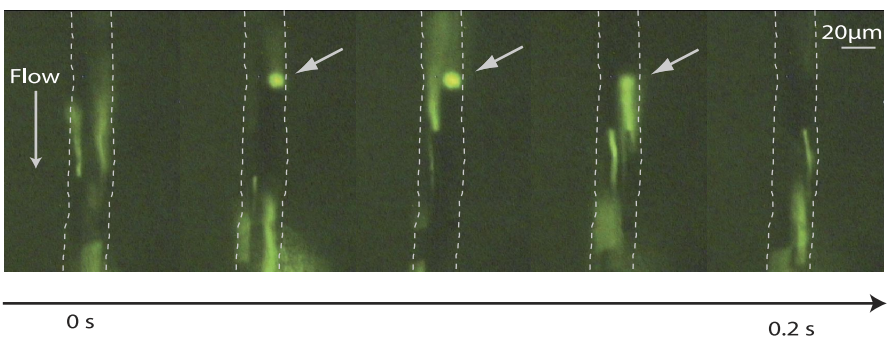


Fig. 7. A series of images from a video clip of a 20-μm-diameter submucosal venule (cross-hatched line) of a *L. reuteri*- and DSS-treated rat. Numerous carboxyfluorescin diacetate succimudyl ester (CFSE)-labeled fluorescent platelets (green) were seen passing through the vessel, leaving a tail of green fluorescence behind, whereas one platelet transiently interacted with the endothelium (arrows) before it released. This event occurred 13 times as often in submucosal venules of DSS-treated rats.

Table 1. *Detection of L. reuteri strains and total lactobacilli in colonic mucus*

	L. reuteri Treated	*L. reuteri* and DSS Treated	2 Days *L. reuteri* and DSS Treated	DSS Treated	Nontreated
R2LC ATCC 55730	5.5±0.3	4.8±0.3	5.4±0.7	ND	ND
JCM 5869 ATC PTA 4659	ND	ND	ND	ND	ND
Total lactobacilli	5.8±0.3*	5.3±0.1*	6.0±0.6*	7.4±0.6	6.5±0.5

Values are means ± SE. Units are log colony-forming units/ml. *L. reuteri*, *Lactobacillus reuteri*; DSS, dextran sulfate sodium; ND, not detected. *$P < 0.05$ vs. DSS treated.

showing that the superficial gastric and duodenal mucosal microcirculation have much lower density of intercellular adhesion molecule-1 and P-selectin and also less leukocyte-endothelial interactions than occur in the deeper layers of the gut wall (39). This was true even during stimulated upregulation with endotoxin. Thus the results from the present and our previous study imply that the circulation lining the gut lumen can by virtue of a deficiency of P-selectin resist leukocyte rolling, which might be an important property given the very inflammatory nature of the gut contents. However, the difference in P-selectin levels and leukocyte rolling in the different layers of the colonic wall was totally abolished after DSS-induced colitis, indicating that the anti-inflammatory property of the superficial colonic microcirculation was lost following DSS treatment. However, *L. reuteri* clearly inhibited the P-selectin upregulation by DSS and also reduced leukocyte-endothelial interactions. Furthermore, *L. reuteri* restored a low P-selectin expression as well as leukocyte-endothelial interactions in the superficial mucosal microcirculation compared with the deeper layers of the colon.

Platelets have been implicated in the pathogenesis of colitis in numerous studies (1, 2, 14, 15, 23). Mori et al. (30) showed that the inflammation in IBD is associated with an activation of platelets in the colonic microcirculation, with P-selectin playing a major role in their recruitment. IBD patients have higher levels of platelet aggregation responses (1, 2), increased circulating P-selectin levels (15), and an elevated risk of thrombosis compared with healthy controls (14). This evidence of platelet involvement led to clinical studies of heparin as a treatment option for IBD; however, any possible benefit has to be weighed against the increased risk of rectal bleeding (9). A colonic release formulation of parnaparin, a substance belonging to the heparin group, has shown some preliminary efficacy and safety in moderately ill UC patients (37).

In the study presented here, almost no platelets were seen interacting with the superficial mucosal microcirculation in nontreated animals, whereas the submucosal microcirculation had slightly, although significantly, increased platelet-endothelial cell interactions. This difference in platelet-endothelial interactions was totally abolished by DSS treatment, which increased platelet-endothelial interactions by 50 and 15 times in the mucosal and submucosal microcirculation, respectively, compared with nontreated controls. Pretreatment with *L. reuteri* completely prevented the increase in platelet-endothelial interactions in the submucosa and dramatically reduced these events in the mucosa. These results indicate that *L. reuteri* can downregulate platelet activity during experimental colitis, and, since there is no increased risk of rectal bleeding, it might thus be a safer treatment option for IBD than heparin.

When comparing our results with earlier reported observations from Granger and colleagues (30, 52) in the mouse, we find an almost 10 times higher frequency of interacting platelets. The platelets we observe are also very transiently interacting mainly with the endothelium, whereas others have reported leukocyte-platelet interactions (4, 22, 23, 51, 52). One possible explanation for the discrepancy between our study and others could be the fact that we have studied postcapillary mucosal venules up to a diameter of 20 μm, while others have investigated platelet behavior in venules of bigger size and only in the deeper gut wall (~40 μm in diameter). In agreement with this, we did see platelet-leukocyte interactions in bigger vessels in the submucosa in a few DSS-treated animals as well as in one control rat but not in any rats treated with *L. reuteri*. We have chosen the size of the vessels based on the dimensions of the venules found in the superficial mucosa to be able to compare venules from different layers of the colon. Mucosal colonic venules are much smaller than the venules visible from the serosal side of the colon, which other groups have studied (30, 52). One must also take into consideration that the present study was conducted in a rat model while others have used mice.

Vowinkel et al. (52) discovered a large upregulation of PSGL-1 on the vascular endothelium of DSS-treated mice and concluded that platelets, which upregulate P-selectin during DSS-induced colitis, can bind to the endothelium directly through P-selectin-PSGL-1 interactions. Activated platelets have been shown to transiently interact with the endothelium, depositing the chemokine "regulated upon activation, normal T cell-expressed and secreted" also known as CCL5, on the endothelium, increasing leukocyte adhesion (50). Vowinkel et al. (52) reported recently that mice depleted of platelets, by administration of an antiplatelet serum intraperitoneally during the last 24 h of a 6-day DSS induction period, had less leukocyte rolling and adherence than DSS-treated mice with platelets. Thus the transient platelet-endothelial interactions that we observe might induce a cascade of events resulting in increased leukocyte-endothelial interactions.

Microbes produce, non-strain-specific, pathogen-associated molecular patterns that are recognized by the host's innate immune system, which consists of pattern recognition receptors (PRRs). Among PRRs are TLRs, which signal infection, neutralize microbes, and activate proinflammatory pathways (56). Studies in mice deficient in different TLRs have shown that DNA from a commercially available probiotic cocktail (VSL#3) or *Escherischia coli* strain Nissle 1917 mediated anti-inflammatory effects via TLRs (41). These studies show that microbes, through PRRs, can modulate the host's immune system and that mutations in crucial receptors can result in

increased susceptibility to developing IBD. *L. reuteri* may exert its anti-inflammatory effect through these receptors, which needs to be investigated further. Probiotics have also been shown to modulate inflammatory cytokines by lowering tumor necrosis factor-α, interleukin-8, and nuclear factor-κB secretion when cocultured with colonic biopsies from patients with UC (8).

It has been reported clinically that supplementation with *L reuteri*-enriched yoghurt administered to IBD patients had an anti-inflammatory effect, thus ameliorating the disease (25). However, lactobacilli given as an oral supplement generally do not colonize the gut and thus need to be given continuously. In our analysis of bacterial content in the mucus lining the colon, we could only detect one of the four strains administered to the rats. Thus, *L. reuteri* R2LC, one of the strains isolated from rat, was detected in the colonic mucus even 2 wk after the last administration of the bacteria, indicating that this strain colonized the mucus. We also found that this strain constituted 50% of all lactobacilli colonizing the mucus. Thus the R2LC strain must have driven some of the lactobacilli normally colonizing the mucus out of the mucus.

The DSS-treated rats that received the four strains of lactobacilli for only 2 days followed by a waiting period of 5 days then 9 days DSS treatment were as effectively protected from colitis as the group that received *L.* reuteri throughout the entire protocol, including during DSS treatment. The detectable levels of R2LC were similar to the levels observed in the two groups where *L.* reuteri was given continuously. Although not conclusive, the finding that one of the strains did colonize the rat colonic mucus, and was present in the same numbers 2 wk later, is interesting given the preventive effect of that strain on DSS-induced colitis.

The observation that the total amount of lactobacilli was increased in DSS-induced colitis might be because of an increased susceptibility of the mucus to bacterial colonization as a result of a colitis-associated defect in barrier function. Contrary to a recently presented study in mice in which the total number of lactobacilli in fecal samples decreased following DSS treatment (21), our results show an increased colonization with lactobacilli consistent with a previous study in rats (5). One explanation for the discrepancy between species is that we have quantified bacteria exclusively in the colonic mucus as opposed to in fecal samples used in the mouse study.

In summary, we have shown that a continuous supplementation with the probiotic bacteria *L. reuteri* almost totally prevented DSS-induced colitis in rats. Daily administration of *L. reuteri* inhibits the P-selection upregulation that normally occurs in the colonic vasculature during colitis, leading to decreased leukocyte rolling and adherence. In addition, *L. reuteri* also downregulates platelet-endothelial cell interactions seen in inflamed colonic venules. Pretreatment with this probiotic resulted in markedly reduced colonic inflammation. These findings highlight the anti-inflammatory potential of *L. reuteri* and provide additional indications that probiotics may serve as a preventive treatment for IBD.

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