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Paper II

Adhesive capability of *Lactobacillus plantarum* 299v is important for preventing bacterial translocation in endotoxemic rats

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The preventive effect of the probiotic *Lactobacillus plantarum* 299v on bacterial translocation (BT) and the role of adhesion were studied in septic rats. Five groups of rats were pretreated as follows: negative and positive control groups received regular drinking water; the oatmeal group received drinking water mixed with oatmeal; the Lp 299v group received drinking water mixed with oatmeal containing 10⁹ colony-forming units (CFU) *L. plantarum* 299v/ml; the Lp 299v-adh⁻ group received drinking water with oatmeal containing 10⁹ CFU/ml of modified *L. plantarum* 299v (*L. plantarum* 299v-adh⁻) lacking adhesive properties to enterocytes. On day 8, all rats except the negative control group were given lipopolysaccharide (LPS) intraperitoneally. After 24 h, mesenteric lymph node (MLN), liver and ileum were harvested for culture. Incidence of BT after LPS challenge was 25% and 88% in MLN and liver, respectively. BT increased to 75% in MLN and 100% in liver of endotoxemic rats pretreated with oatmeal. Pretreatment with *L. plantarum* 299v reduced BT to 0% and 12% in MLN and liver, respectively. *L. plantarum* 299v-adh⁻ did not prevent BT to MLN. Flow cytometry revealed reduced adherence of these bacteria to intestinal epithelial cells compared to *L. plantarum* 299v. Thus, *L. plantarum* 299v prevents BT in septic rats, an effect probably dependent on bacterial adherence to the intestinal mucosa. Further, our findings indicate that oatmeal (prebiotics) without probiotics does not prevent BT during sepsis.

Key words: Bacterial translocation; probiotics; prebiotics; sepsis; bacterial adhesion; *Lactobacillus plantarum*.

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Bacterial translocation (BT) is considered to be a central pathophysiological process in sepsis (1) and associated with postoperative septic complications (2). Therapies aimed at diminishing or abating BT, and thus reducing infectious morbidity, include the use of systemic anti-

biotics and gut decontamination. This, however, involves the risk of bacterial multiresistance and secondary infections. Interest has now shifted towards the possibility of stabilizing the gut microflora and thereby preventing potentially pathogenic bacteria from increasing in numbers and translocating across the intestinal barrier (3). One way to modulate the gut microflora is to administer probiotic bacteria, i.e. viable bac-

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teria, which – when ingested – alter the intestinal microflora and thereby affect the host beneficially (4). Supplementation with probiotics has proven effective in preventing pancreatic sepsis (5) and postoperative infection in liver transplant patients (6). It has previously been shown that a specific probiotic strain, *Lactobacillus plantarum* 299v, can reduce intestinal permeability induced by *Escherichia coli* (7) and abate bacterial translocation in methotrexate-induced enterocolitis (8) and liver injury (9).

The exact mechanism behind BT is still not known, but attachment of bacteria to the mucosal surface is a prerequisite in the pathogenesis of many infections originating from the gut (10). Also, for a probiotic bacterium to exert any biological effect, adherence to the mucosa seems to be of importance (11, 12). However, it remains to be clarified whether adherence of a probiotic is a prerequisite for preventing BT of the indigenous microflora.

When supplied, probiotics are sometimes given together with prebiotics. These are dietary non-digestible carbohydrates with the ability to selectively stimulate growth of one or a limited number of bacteria (4), often lactobacilli and bifidobacteria, and thereby contribute to enhancement of the intestinal barrier function. This is also the rationale for providing critically ill patients with small amounts of enteral nutrition containing dietary fiber and thus stimulating the intestinal mucosa. However, the effect of prebiotics on the indigenous microflora in a septic state is not known.

The aim of the present study was to examine whether oral pretreatment with the probiotic *L. plantarum* 299v may prevent BT to MLN and liver in rats challenged with endotoxin, and whether such a preventive effect is related to adhesive capacity of *L. plantarum* 299v to the intestinal mucosa. Further, the effect of the dietary fibers of oats on BT during sepsis was studied.

MATERIAL AND METHODS

Experimental design

Male Sprague-Dawley rats (Møllegaard, Skensved, Denmark) weighing 321–423 g were used 7 days after arrival at the animal facility, where they had free access to standard rat chow (B & K Universal, Sollentuna, Sweden) and water *ad libitum*. All experiments were

approved by the local Animal Ethics Committee at Lund University. A total of 44 rats were divided into five groups and pretreated one week before the experiment as follows: negative (n=10) and positive control group (n=8) received regular drinking water; the oatmeal group (n=8) received regular drinking water mixed with an oatmeal drink containing 18.5 g oatmeal per 100 g drink (13) with an energy content of 325 kJ per 100 ml drink (14); the Lp 299v group (n=8) received regular drinking water mixed with oatmeal containing 10^9 colony-forming units (CFU) of *L. plantarum* 299v/ml; the Lp 299v-adh⁻ group (n=10) received regular drinking water mixed with oatmeal containing 10^9 CFU of *L. plantarum* 299v-adh⁻/ml. This is a strain that originates from *L. plantarum* 299v but has lost the ability to adhere to HT 29 cells (11). The two *L. plantarum* strains were grown in an oatmeal drink (14), which resulted in a decreased pH (final pH around 4) and an increased concentration of lactic acid in the drink. In both the Lp 299v and Lp 299v-adh⁻ groups, 50 ml of the oatmeal drink containing one of the bacteria was mixed with 200 ml tap water for each cage housing two rats. Thus, each pair of rats had access to 10^{10} CFU of bacteria per day. Following pretreatment, the rats were temporarily sedated with CO₂ and given an intraperitoneal injection of 1 ml sterile 0.9% saline with or without 10 mg/kg body weight lipopolysaccharide (LPS) from *Escherichia coli* serotype O111:B4 (Sigma, Stockholm, Sweden) (15). After 24 h, rats were anesthetized with diethylether and through a midline laparotomy under aseptic conditions a mesenteric lymph node (MLN) from the ileocecal junction was harvested and a tissue specimen was taken from the liver. Also, 2 cm of distal ileum was harvested, split longitudinally, and rinsed in sterile saline to remove fecal matter. All specimens were put into pre-weighed vials containing 3 ml sterile transport medium (0.9% sodium chloride, 0.1% peptone, 0.1% Tween 80, 0.02% cysteine) (16) and weighed.

Bacterial translocation

Samples from MLN and liver were placed in an ultrasonic bath (Millipore, Sundbyberg, Sweden) for 2 min and vortexed on a Chiltern (Thera-Glas, Gothenburg, Sweden) for 2 min. Viable counts were obtained by placing 1 ml of each suspended tissue sample on brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI, USA) and incubated aerobically and anaerobically (Gas Pack System, Gas Pack, Beckton Dickenson Microbiology Systems, Cockeysville, MD, USA) at 37°C for 3 days, thus yielding total aerobic and anaerobic counts, respectively. The colonies formed on each plate were counted and the result was expressed as CFU per gram tissue.

Intestinal bacteria

Distal ileum samples were placed in an ultrasonic bath and vortexed as described above. Bacterial viable counts were obtained by cultivation on violet red

bile glucose agar (VRBG; Oxoid, Hampshire, England) incubated aerobically at 37°C for 24 h (*Enterobacteriaceae* counts) and on Rogosa agar (Difco Laboratories) incubated anaerobically for 3 days (lactobacilli counts).

Flow cytometry

The ability of the two bacterial strains to adhere to HT-29 cells was analyzed by flow cytometry. The bacteria were cultured overnight on Rogosa agar for viable counts. The strains were then cultured for 20 h in Lactobacillus Carrying Media (LCM) with 1% glucose. After harvesting, centrifugation and washing twice, 300 μ l bacteria (2×10^9 CFU/ml) was stained with 300 μ l 20 mM carboxyfluorescein diacetate (CFDA-SE, Molecular Probes Inc., Eugene, OR, USA). HT-29 cells were grown in culture flasks with Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich Ltd, Irvine, Ayrshire, UK) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. After reaching confluence, trypsin was added and 1×10^6 cells were seeded into microtiter plates, which were incubated for 2 days. The CFDA-stained lactobacilli were added to the wells at a ratio of 150 bacteria to one HT-29 cell and incubated for 1 h at 37°C. The medium was then removed and the cell/bacteria suspension fixed with paraformaldehyde. Flow cytometry analysis was performed using a Coulter® EPICS® XL Flowcytometer (Coulter Corporation, Miami, FL, USA).

Statistics

Data are expressed as median (25th–75th percentile) \log^{10} CFU/g tissue. The incidence of BT was compared using Fisher's Exact Test. To compare dif-

ferences in number of bacteria between groups, Kruskal-Wallis ANOVA by ranks was used, followed by multiple comparison with Dunn's method. $p < 0.05$ was considered significant.

RESULTS

All cultures from MLN and liver were sterile in the negative control group (Fig. 1). Intraperitoneal injection of LPS caused BT to 25% of MLN's and 88% of the livers (Fig. 1). This incidence of BT was significant in the liver compared to the negative control group ($p < 0.001$) but did not reach statistical significance in the MLN. Pretreatment with oatmeal alone increased LPS-induced BT to 75% of MLN's ($p = 0.002$ vs negative control and $p = 0.007$ vs Lp 299v) and 100% of the livers ($p < 0.001$ vs negative control and $p = 0.001$ vs Lp 299v) (Fig. 1). Thus, pretreatment with unfermented oatmeal did not have any preventive effect against BT after LPS exposure. This sharply contrasts with LPS-challenged rats pretreated with oatmeal containing *L. plantarum* 299v (group Lp 299v), in which BT to MLN's was abolished and a significant reduction in incidence of BT to livers (12.5%, $p = 0.01$ vs positive control) was seen. The incidence of BT is summarized in Table 1. There was no significant difference in bacterial counts in MLNs or livers between the pretreat-

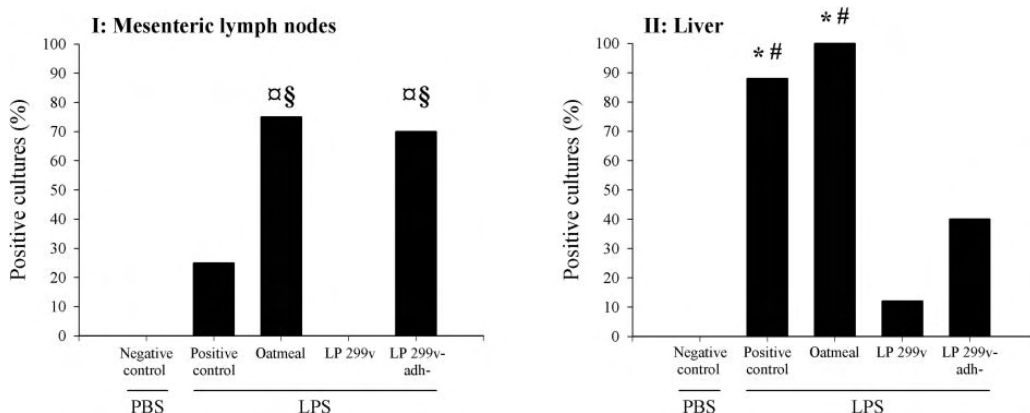


Fig. 1. Incidence of positive cultures (%) in I: mesenteric lymph nodes (□= $p < 0.01$ vs negative control and §= $p < 0.01$ vs *L. plantarum* 299v). II: liver (*= $p < 0.001$ vs negative control and #= $p < 0.05$ vs *L. plantarum* 299v). See text for description of different pretreatment and treatment groups.

TABLE 1. Incidence of bacterial translocation (%) in the different treatment groups.

Group	Incidence of BT
1. Negative control	0/10 (0%)
2. Positive control	7/8 (88%)*#
3. Oatmeal	8/8 (100%)*#
4. <i>L. plantarum</i> 299v	1/8 (12%)
5. <i>L. plantarum</i> 299v-adh ⁻	9/10 (90%)*#

BT=bacterial translocation, MLN=mesenteric lymph nodes. *P<0.001 vs negative control. #P<0.05 vs *L. plantarum* 299v.

ment groups (Table 2), but there was a tendency toward a higher number of bacteria on all the specimens from septic rats pretreated with oatmeal.

LPS increased the content of *Enterobacteriaceae* in the distal ileum (p<0.05 vs negative control) (Table 3). A higher count of *Enterobacteri-*

aceae was also found in the groups pretreated with oatmeal with or without *L. plantarum* 299v, but this did not reach statistical significance. The number of indigenous lactobacilli is relatively high in the rat and no significant changes were seen in lactobacilli count when sepsis was induced or with the different pretreatment.

To study whether any preventive effect of *L. plantarum* 299v on BT was attributable to the adherence of this bacterium to the intestinal mucosa, rats (group Lp 299v-adh⁻) were pretreated with *L. plantarum* 299v-adh⁻, a variant of *L. plantarum* 299v which has lost its ability to adhere to HT-29 cell (11). The protective effect of *L. plantarum* 299v on BT was lost when endotoxemic rats were pretreated with the adhesive-deficient *L. plantarum* 299v-adh⁻. Thus, BT to MLNs occurred in 70% of the rats (p=

TABLE 2. Viable count in mesenteric lymph nodes (MLN) and liver in the different treatment groups.

Group	n	MLN		Liver	
		Aerobe	Anaerobe	Aerobe	Anaerobe
Negative control	10	0 <i>0</i>	0 <i>0</i>	0 <i>0</i>	0 <i>0</i>
Positive control	8	0 (0-1.7) <i>2</i>	0 (0-1.4) <i>2</i>	0.8 (0-1.9) <i>4</i>	1.9 (0-2.1) <i>5</i>
Oatmeal	8	3.3 (1.6-3.7) <i>6</i>	0 (0-3.3) <i>3</i>	2.6 (1.7-3.2) <i>7</i>	2.3 (0-3.3) <i>5</i>
LP 299v	8	0 <i>0</i>	0 <i>0</i>	0 <i>1</i>	0 <i>1</i>
LP 299v-adh ⁻	10	4.1 (0-4.8) <i>6</i>	0 (0-3.5) <i>3</i>	0 <i>1</i>	0 (0-1.8) <i>4</i>

Median (25th-75th percentile), log10 CFU/g tissue. Numbers in italics indicate cultures above detection level.

TABLE 3. Intestinal microflora 24 h after lipopolysaccharide (LPS) challenge.

Group	n	Enterobacteriaceae	Lactobacilli
Negative control	6	0 <i>2</i>	6.3 (6.2 - 6.5) <i>6</i>
Positive control	8	7.1 (4.9-8.4)* <i>7</i>	5.9 (5.4-6.1) <i>8</i>
Oatmeal	8	5.3 (4.5-6.8) <i>8</i>	5.6 (0.0-6.8) <i>5</i>
LP 299v	8	5.8 (2.4-7.1) <i>6</i>	6.1 (4.9-6.8) <i>8</i>
LP 299v-adh ⁻	10	3.8 (2.3-5.0) <i>9</i>	6.2 (3.9-6.7) <i>10</i>

Median (25th-75th percentile), log10 CFU/g tissue. Numbers in italics indicate cultures above detection level. *P<0.05 vs negative control.

0.004 vs Lp 299v) (Fig. 1), i.e. the incidence of BT was significantly higher than when rats were fed regular *L. plantarum* 299v prior to endotoxemia. An increased incidence of BT to the livers was also seen as compared to regular *L. plantarum* 299v, but this did not reach statistical significance.

L. plantarum 299v adhered to HT-29 cells after coincubation, shown as a high fluorescence intensity with flow cytometry (Fig. 2). When adhesion-deficient *L. plantarum* 299v-adh⁻ was coincubated with HT-29 cells, a much lower fluorescence intensity was found, indicating a lower rate of adherence.

DISCUSSION

Probiotics is emerging as an attractive means of preventing infectious complications after major

surgery (6) and in critical illness (5). In the present study we found that oral pretreatment with *L. plantarum* 299v of rats one week before intraperitoneal injection of LPS significantly reduced the incidence of bacteria translocating to the MLN and liver. Thus, *L. plantarum* 299v seems to prevent BT from the intestine in a septic state. This preventive effect of *L. plantarum* 299v appears to be associated with its ability to adhere to the intestinal mucosa. Further, this study indicates that pretreatment with oatmeal only, i.e. a prebiotic given without addition of a probiotic, does not prevent BT during LPS-induced sepsis.

Lactobacilli are part of the commensal microflora in both rodents and humans, and have beneficial effects on the host. There are probably differences in the effects between different strains of lactobacilli. In this study we specifi-

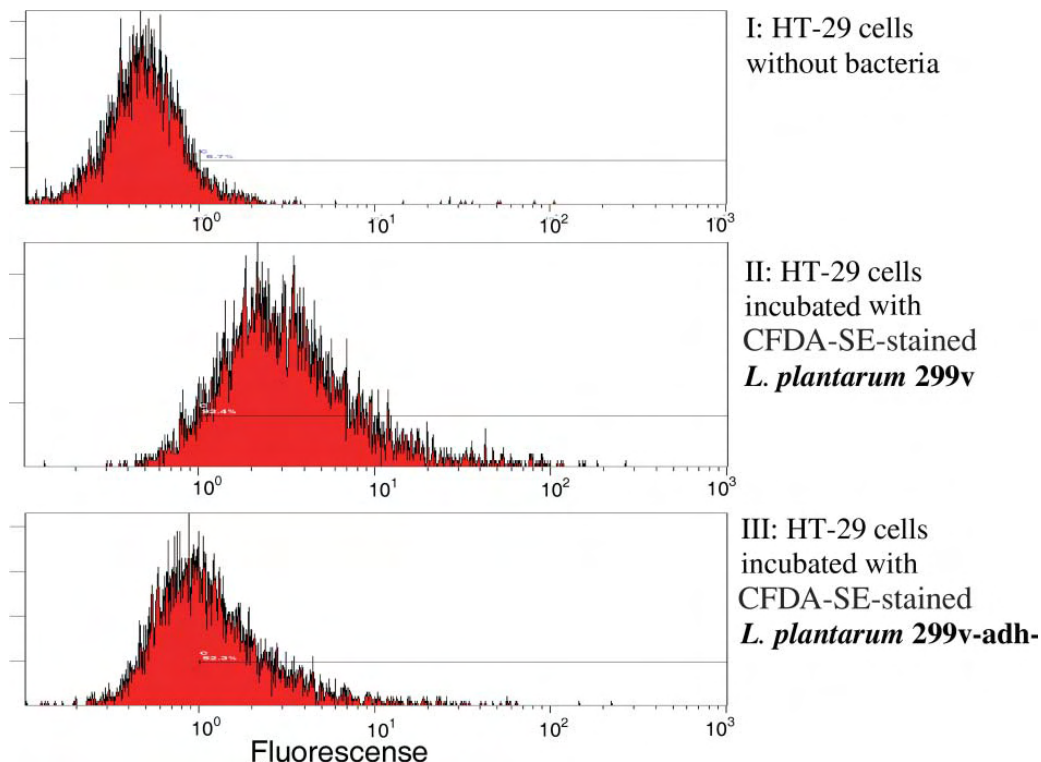


Fig. 2. Flow cytometry. *L. plantarum* 299v and *L. plantarum* 299v-adh⁻ were stained with CFDA-SE and coincubated with HT-29 cells to study adherence. Reduced fluorescence intensity with *L. plantarum* 299v-adh⁻ indicates lower adherence of these bacteria to HT-29 cells. I: HT-29 cells without bacteria. II: HT-29 cells coincubated with CFDA-SE-stained *L. plantarum* 299v. III: HT-29 cells coincubated with CFDA-SE-stained *L. plantarum* 299v-adh⁻. See text for description.

cally used *L. plantarum* 299v, which in experimental settings has been shown to enhance and interact with the gut immune system (17, 18), increase mucin production (19), compete with *E. coli* for colonization (18), reduce translocation in enterocolitis (8) and liver injury (9), and prevent *E. coli*-induced intestinal permeability (7).

The number of lactobacilli needed to achieve certain biological effects is not known, but there are no indications that there is an upper limit as to how many bacteria can be given without evoking adverse effects. In this study, rats received an average of 1.1×10^{10} CFU of *L. plantarum* 299v per day without any adverse effects such as diarrhea or impaired general condition. They drank almost twice the volume of fluids compared to the rats receiving only regular tap water, this possibly being due to the fiber content of the oatmeal in which the *L. plantarum* 299v is mixed or perhaps due to the acidity of the drink. By mixing the lactobacilli in drinking water, the supply of bacteria is more continuous, which presumably facilitates colonization on the intestinal mucosa.

In a septic state, where the mucosal barrier and immune response are compromised, translocation occurs to such an extent that viable bacteria are able to reach extraintestinal sites, as shown in the positive control group in the present study and by others (20, 21). BT to MLN in animal studies varies between 40–100% (15, 22). In this study, BT to MLN was 25% in the positive control group, which indicates the great variation in the rate of translocation between rat species and even between rats of the same species. Possible reasons for the relatively low incidence of translocation to MLN in the positive control group in this study could be sampling error or inadequate culturing technique. This is, however, unlikely, as cultures both of MLN in the oatmeal- and Lp 299v groups and cultures of liver were positive and standard culturing techniques were applied. The reason why the incidence of translocation is higher to the liver than to the MLN's is unclear, but might indicate that the hepatic reticulo-endothelial system is affected by sepsis, making it insufficient to clear the liver from translocated bacteria, as previously shown in a model with cecal ligation and puncture (23). Care was taken to harvest lymph nodes from the ileocecal junc-

tion in a standardized manner to detect live bacteria, indicating BT. Translocating bacteria, on their route from the gut to the systemic circulation, pass through the lymphatics; it is, however, impossible to determine which group of lymph nodes is "sentinel", i.e. the first to harbor translocated bacteria and it might be speculated that this influences the rate of positive culture of MLN.

Endotoxin increases the number of *Enterobacteriaceae* in the distal ileum, as shown in the positive control group in this study and by others (20). Although *L. plantarum* 299v given to the rats before induction of sepsis decreased BT, the number of *Enterobacteriaceae* in the intestine was not significantly reduced. This indicates that the ability of the lactobacilli to inhibit translocation is not through reduction of the number of potentially pathogenic bacteria present in the intestine. Instead, possible explanations might be competition for adhesion sites on the mucosa or that lactobacilli together with the prebiotic enhance immune surveillance at the mucosal level, stimulating production of sIgA by subepithelial B lymphocytes, as shown by Roller *et al.* (24). Another preventive effect of lactobacilli is the ability to strengthen the physical barrier, e.g. the mucus layer covering the mucosa (19), thus preventing direct contact between enterocytes and intestinal bacteria. However, the exact mechanism by which *L. plantarum* 299v prevents translocation is not known and further studies are warranted.

Interestingly, the total number of lactobacilli on the intestine did not increase with feeding of *L. plantarum* 299v. In the rat, lactobacilli constitute a significant part of the indigenous microflora (25) and the addition of exogenous lactobacilli might be difficult to detect in cultures. In a liver failure model in rats, rectally administered lactobacilli increased the total number of lactobacilli in the intestine, and were able to abate liver damage and translocation, but also in that model, rectally administered *L. plantarum* 299v could not be found in a dominating number on the mucosa (25). It is noteworthy that LPS treatment in the present study did not reduce the number of lactobacilli in any of the treatment groups. Even if no quantitative differences are noted, there might be important qualitative differences between the indigenous lactobacilli and the exogenously administered *L.*

plantarum 299v shown by the observation that *L. plantarum* 299v given to the rats in the drinking water inhibited BT. This indicates that exogenously supplied bacteria may replace the indigenous lactobacilli on the intestinal mucosa.

Even though the strain of *L. plantarum* 299v used in this study is of human origin, it has been shown by randomly amplified polymorphic DNA (RAPD) to establish itself on the rat mucosa after oral intake (N Osman, personal communication) and also on the mucosa of gnotobiotic rats detected by conventional culture technique (18). The ability of *L. plantarum* 299v to adhere to the intestinal mucosa provides a possible basis for exclusion of other bacteria from adhering, one suggested mechanism by which translocation is prevented. This is further emphasized by the adhesion-deficient *L. plantarum* 299v-*adh*⁻ used herein, which did not prevent LPS-induced translocation. Flow cytometry revealed a reduced ability of *L. 299v-adh*⁻ to adhere to HT-29 cells compared to *L. plantarum* 299v. The latter have been shown to carry a mannose-specific adhesin (26) which adheres to rat intestine (18), as well as to HT-29 cells (27).

In the present study, oatmeal alone had no effect on the number of lactobacilli on the intestinal mucosa (Table 3) and was not able to prevent BT to MLN or liver (Fig. 1). However, by adding the probiotic *L. plantarum* 299v to the prebiotic, BT was inhibited. This is in contrast to some other studies where a prebiotic prevented translocation, as in a liver resection model (28). One reason for the difference might be the prebiotic used. It may also indicate that the positive effects of dietary fibers (prebiotics) can be questioned in severe clinical conditions such as sepsis where the mucosa is challenged by toxic substances, reduced blood flow, or mechanically by surgery or trauma. In such situations the trophic effect of the dietary fibers on the mucosa might be insufficient to counteract the negative effects of the trauma. Instead, the prebiotic given alone may even stimulate potentially pathogenic bacteria and thus have a negative effect by promoting BT. In contrast, the combination of prebiotics and probiotics not only strengthens the intestinal barrier but intestinal bacteria trying to translocate are prevented from doing so by competitive exclusion, increased production of sIgA, lowering of pH,

and through upregulation of MUC3 intestinal mucins. The observation in the present study that the prebiotic not only failed to prevent BT but even showed a tendency toward increased BT might be of importance in an intensive care setting, where septic patients receive part of their nutrition through enteral feeding, often with the addition of fiber. It is conceivably of importance to combine the enteral formula with both fiber and a probiotic strain in order to prevent BT in those patients.

In conclusion, our data demonstrate the capability of a probiotic bacterium, *L. plantarum* 299v, to prevent translocation of intestinal flora in a septic state and that this effect correlates with the ability of *L. plantarum* 299v to interact with the intestinal mucosa. Also, our findings raise the question whether prebiotics given in a septic state may be harmful and should only be given in conjunction with a probiotic.

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