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The D-alanine content of lipoteichoic acid is crucial for *Lactobacillus plantarum*-mediated protection from visceral pain perception in a rat colorectal distension model

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Abstract The mechanisms leading to positive effects of probiotics in irritable bowel syndrome and inflammatory bowel disease have not been clarified, but the possible involvement of cell wall components is widely discussed. Reduction of the D-alanine content of lipoteichoic acid (LTA) in *Lactobacillus plantarum* (Dlt⁻ mutant) enhanced its anti-inflammatory properties in a mouse colitis model. Another *Lactobacillus* species inhibited visceral pain perception in response to colorectal distension (CRD) in rats. Therefore, we investigated if LTA modification influences the constitutive intestinal pain perception in addition to modulation of cytokine release. Male Sprague-Dawley rats were gavaged with *L. plantarum*, *L. plantarum* Dlt⁻ mutant or buffer control, respectively and the responses to CRD were tested in this non-inflammatory model. Tumour necrosis factor (TNF), interferon (IFN)-gamma and interleukin (IL)-10 release were measured in colon tissue homogenates and upon anti-CD3/CD28 activation of isolated splenocytes and mesenteric lymphocytes. Control animals showed significant bradycardia following noxious CRD, whereas only the *L. plantarum* Dlt⁻ mutant inhibited the response. The mutant also decreased the activation-induced release of TNF and IFN-gamma from mesenteric T cells and the IL-10 concentration in

colonic tissue, while increasing the activation-induced secretion of IL-10 in splenocytes and mesenteric lymphocytes and the baseline IL-10 release of splenocytes. In conclusion, D-alanine depletion of LTA in *L. plantarum* inhibited visceral pain perception in healthy, non-inflamed rats. Regardless of the non-inflammatory nature of the model decreased visceral pain perception was seen in parallel with anti-inflammatory properties.

Keywords colorectal distension, irritable bowel syndrome, *Lactobacillus plantarum*, lipoteichoic acid, probiotic, visceral pain perception.

Abbreviations CRD, colorectal distension; ECG, electrocardiogram; LTA, lipoteichoic acid; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; MLN, mesenteric lymph node.

INTRODUCTION

Abdominal pain is a predominant symptom of irritable bowel syndrome (IBS), one of the most prevalent chronic intestinal disorders in the western world. It is believed to be caused in large measure by an imbalance in the communication between the gut, the intestinal nervous system and the brain.^{1–3} First reported in 1973, IBS patients have been shown to have heightened visceral perception to colonic distension compared with normal subjects.⁴ It was also observed that previous gastro-intestinal infections, correlated with elevated pro-inflammatory cytokine levels, were able to lower the threshold for intestinal pain sensitivity^{5–7} and could result in postinflammatory IBS. Furthermore, increased infiltration of the gut mucosa and the

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Received: 6 July 2007

Accepted for publication: 19 December 2007

myenteric plexus with inflammatory cells has been seen in patients with IBS⁷⁻⁹, thus suggesting a possible correlation between immunological events and the dysregulation of intestinal functions such as the development of visceral hypersensitivity even after cessation of the inflammation.

Probiotic bacteria have been widely recognized as potent modulators of intestinal functions. Probiotics have proven to be effective in the therapy of inflammatory^{10,11} and allergic¹² conditions and also IBS itself^{13,14} by influencing the immune balance. Interestingly enough, the mechanisms by which they evoke their therapeutic effects are still controversial. Grangette *et al.* have recently suggested that the modulation of a bacterial cell wall component, lipoteichoic acid (LTA), is involved in the anti-inflammatory properties of *Lactobacillus plantarum*, possibly mediated by the up-regulation of anti-inflammatory cytokines like interleukin (IL)-10.¹⁵ However, inflammation is not the only condition in which lactobacilli have proven to be beneficial. *Lactobacillus reuteri*, a natural inhabitant of the human and rodent intestine, does protect healthy rats from visceral pain perception to noxious stimuli.¹⁶ The fact that this effect was also achieved by heat-killed and gamma-irradiated bacteria suggests the possible involvement of a bacterial cell wall associated component. Hence, we have asked the question if modification of LTA in *L. plantarum* was able to influence the outcome in the response to intestinal pain stimuli in *Sprague-Dawley* rats, in parallel with its anti-inflammatory properties.

MATERIALS AND METHODS

Animals

Healthy male *Sprague-Dawley* rats (Charles River Breeding Laboratories, Saint Constant, QC, Canada) were used for the experiments (weight 376 ± 41 g). Rats were housed in the Central Animal Facilities in micro-isolator cages equipped with filter hoods, under controlled temperature (20 °C), with a 12:12 h light-dark cycle, and free access to food and water. Prior to the experiments, animals were fasted for 18 h. All experiments were approved by the Animal Care Committee of McMaster University and all procedures conducted in accordance with the Guidelines of the Canadian Council on Animal Care.

Bacterial preparations

Bacterial cultures of *L. plantarum* NCIMB8826 (WT) and *L. plantarum* EP007 (Dlt⁻ mutant) (15) were

obtained by using a previously published protocol.¹⁵ In brief, bacteria were inoculated in fresh Mann-Rogosa-Sharpe liquid medium (MRS broth; Difco Laboratories, Detroit, MI, USA) from frozen stock and grown under anaerobic conditions in 50 mL plastic tubes, at 37 °C for 48 h. Erythromycin was added ($5 \mu\text{g mL}^{-1}$) only for the Dlt⁻ mutant. This was necessary to keep the plasmid bound mutation responsible for the D-alanylation defect, stably integrated in the genome of the *L. plantarum* Dlt⁻ mutant.¹⁵ After 2 days of culture, tubes were centrifuged at 850 g for 15 min at room temperature. Cells were washed twice with sterile phosphate-buffered saline. The pellet was re-suspended in NaHCO₃ glucose buffer (0.2 mol L^{-1} NaHCO₃ + 2% glucose) to a concentration of 1×10^8 CFU mL⁻¹ using a colorimeter (Vitek, Durham, NC, USA). Bacteria were tested for viability following 48 h of anaerobic incubation at 37 °C on MRS agar plates (WT) and MRS agar plates + $5 \mu\text{g mL}^{-1}$ erythromycin (Dlt⁻ mutant).

Treatment protocol

Rats were divided into three equal groups. In total, 36 rats were used for the distension experiments and 18 to obtain the cytokine results. Upon arrival animals were allowed to adjust to the new housing for a week followed by 1 week of handling for 10 min each day to minimize stress effects. The rats were gavaged every morning for 9 days with 0.2 mL (2×10^7 CFU) of *L. plantarum* WT, *L. plantarum* Dlt⁻ mutant or buffer control, respectively. The dose is similar to the one that has been proven to be effective in showing differences by Grangette *et al.*¹⁵ In our experience with oral gavaging of rodents, the same doses are equally effective in mice and rats.

Cardio-autonomic response to colorectal distension

Experimental protocol The methodology has been previously published.^{16,17} In brief, rats were anesthetized intra peritoneal with a mixture of ketamine hydrochloride (75 mg kg^{-1}) and xylazine (10 mg kg^{-1}). A 5-cm-long plastic balloon was inserted into the rectum and distal colon, and fixed to the tail so that the tip of the balloon was 6 cm proximal from the anus. The balloon was connected to a Teflon catheter (20 cm). The catheter was connected to a barostat system composed of a flow meter and pressure control program (Distender; G&J Electronic Inc. Toronto, Canada). The cardiac response to colorectal distension (CRD) was measured while inflating the balloon with air to

pressures of 50, 70 and 80 mmHg for 60 s. Following each distension, 10 min of rest were allowed for recovery. Only one set of distensions was applied to each rat.

Data acquisition Continuous recordings of heart rate were performed through a surface electrocardiogram (ECG), obtained through three needle electrodes applied to the left and right shoulders, and the right hind leg. The signal was amplified and recorded onto a personal computer using a commercial data acquisition program (Experimenter's Workbench; DataWave Technologies, Wayne, NJ, USA). The heart rate was measured for 60 s before, during and after every distension for a total of 180 s. To show the effect of CRD over time, the data were presented as average change from resting heart rate (=100% = rest) every 10 s during distension and two times 30 s during the postdistension period (post1 = 0–30 s post CRD; post 2 = 31–60 s post CRD). Groups were compared using the average heart rate change of all animals during the whole 60 s of distension.

Cytokine ELISA

Sample acquisition Samples for the cytokine analysis were obtained in a separate experiment from healthy animals which had not been distended. The rats were fasted from food 18 h prior to tissue removal. Anesthesia was given as mentioned above and the rats were killed by cervical dislocation. The abdominal cavity was opened by midline incision and the spleen and mesenteric lymph nodes (MLN) were taken and stored on ice in 10 mL of Hank's-buffered-salt solution (HBSS; Gibco, Invitrogen, Carlsbad, CA, USA). Cells were gently dispersed and filtered through a cell strainer. The cell suspension was then centrifuged at 400 g at 4 °C for 10 min. Erythrocytes were eliminated from the splenocyte suspension by treatment with RBC-lysis buffer (eBioscience, San Diego, CA, USA) for 5 min and subsequently diluted with sterile phosphate-buffered saline. The cells were washed in cold HBSS and re-suspended in medium [RPMI 1640 + 2 mmol L⁻¹ L-glutamine + 10% fetal bovine serum + 1% penicillin-streptomycin + 25 mmol L⁻¹ HEPES (all Gibco) + 55 µmol L⁻¹ 2-beta-mercaptoethanol (BDH-Chemicals, Toronto, ON, Canada)] to a concentration of 1×10^6 cells mL⁻¹. To achieve T-cell specific activation mouse anti-rat CD28 antibody (BD Pharmingen, San Jose, CA, USA) was added resulting in a final concentration of 2 µg mL⁻¹. Afterwards, the cells were placed into 96-well culture plates that had been coated over night with 10 µg mL⁻¹ of mouse anti-rat CD3 antibody (BD Pharmingen). Following 48 h of incubation at 37 °C under 5% CO₂ atmosphere, the cells were

centrifuged and the supernatant was collected and stored at -80 °C until further use in the ELISA. For the detection of spontaneous cytokine release cells were treated the same way as described above, abolishing the treatment with mouse anti-rat CD28 and placed on uncoated plates. ELISAs were carried out for IL-10 (AlpcoDiagnostics, Windham, NJ, USA), tumour necrosis factor (TNF) (eBioscience) and interferon (IFN)-gamma (eBioscience) according to the manufacturer's instructions. To measure cytokine concentrations in the tissue, samples were taken from the colon 8 cm proximal to the anus. Samples were washed, weighed, homogenized in phosphate-buffered saline with a protease inhibitor (Roche, Indianapolis, IN, USA) and centrifuged. The supernatants were then subjected to ELISA for IL-10, IFN-gamma and TNF. Myeloperoxidase was measured according to methods used previously.^{18,19}

All samples were analyzed in duplicate at the same time and detected with a micro-plate ELISA reader (Molecular Devices, Sunnyvale, CA, USA) and compared with a standard curve obtained from standard dilutions provided by the manufacturer. The results are presented in pg mL⁻¹.

Statistical analysis

All data were presented as mean ± SEM. For multi comparison, two-way ANOVA was used followed by the Bonferroni test to compare the different groups. A *P*-value ≤ 0.05 was considered statistically significant.

RESULTS

Baseline heart rate

Prior to each distension, the base line heart rate was measured in beats per min for 60 s in the anaesthetized animal. The mean resting heart rate in the control animals was 285 ± 25 beats per min. No differences were seen in resting heart rate between the treatment groups and over the duration of the experiment in each rat (data not shown).

Effect of *L. plantarum* WT and *L. plantarum* Dlt⁻ mutant on heart rate response, colorectal compliance and tissue inflammatory response

CRD was carried out with three different pressures. Differences were most obvious between the highest (80 mmHg) and the lowest (50 mmHg) applied pressure. Therefore, only these data are shown here. As previously reported,¹⁶ colorectal distension (80 mmHg)

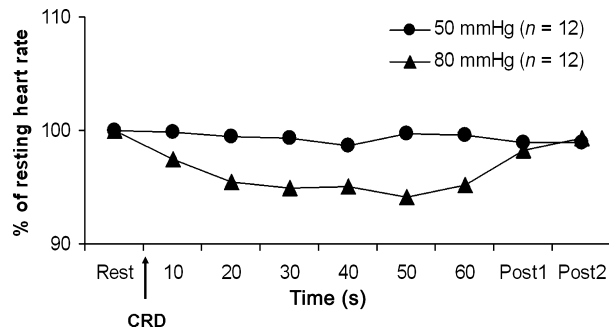


Figure 1 Time course of cardio-autonomic response to colorectal distension (CRD) of 50 (sub-threshold) and 80 mmHg (noxious) in control animals. Data are presented as mean (SEM) % change compared with resting heart rate (Rest). Post 1 and 2 are presenting an average heart rate response in the first and second 30 s post distension ($n = 12$).

caused a significant decrease in heart rate in animals of the control group with a mean maximum heart rate response of $93.1 \pm 7.7\%$ of the resting heart rate. The bradycardia lasted over the whole 60 s of distension and heart rate returned to base line level after cessation of distension (Fig. 1).

Colorectal compliance was assessed upon colorectal distension and no changes were seen between pre and postcomparisons in any group. Myeloperoxidase levels postdistension were also not changed when compared with before distension. Feeding *L. plantarum* WT resulted in a somewhat decreased response to CRD but the change was not significant when compared with controls. Animals that received *L. plantarum* Dlt⁻ mutant responded with complete inhibition of pain response to CRD with 80 mmHg when compared with animals of the control group (Fig. 2).

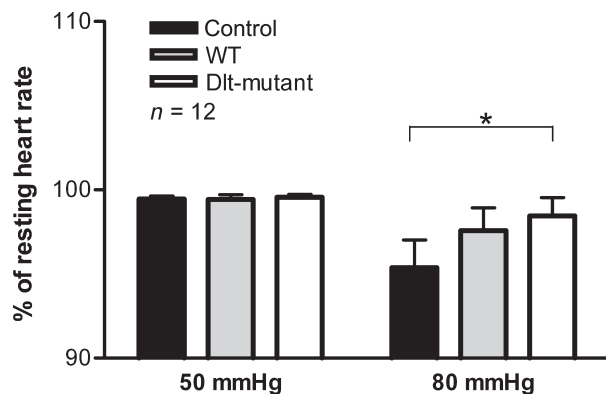


Figure 2 Effect of *Lactobacillus plantarum* on cardio-autonomic response to CRD at 50 and 80 mmHg. Data are presented as mean (SEM) % from resting heart rate (black bar = control, grey bar = *L. plantarum* wild type, white bar = *L. plantarum* Dlt⁻ mutant). * $P < 0.05$ vs control ($n = 12$).

No changes in tissue TNF were observed (data not shown). IFN-gamma was not detectable in the colon tissue (data not shown). IL-10 levels were markedly reduced (478.5 ± 54.6 for control vs 261.5 ± 54.4 pg g⁻¹ tissue for Dlt⁻ mutant, $P < 0.05$). Plasma cytokine levels were not assessed as they are too low for consistent results to be obtained.

***L. plantarum* Dlt⁻ mutant decreased TNF and IFN-gamma release by splenocytes and mesenteric lymphocytes upon T-cell specific activation but had no influence on spontaneous release**

TNF and IFN-gamma concentration were detected by ELISA in supernatant of splenocytes and mesenteric lymphocytes. Neither feeding of *L. plantarum* WT nor of *L. plantarum* Dlt⁻ mutant caused any changes in spontaneous secretion of TNF and IFN-gamma by these cells. Upon specific activation of T cells with anti-CD3/anti-CD28 for 48 h secretion increased significantly in all three groups (Fig. 3) but there was significantly less increase of TNF and IFN-gamma secretion in cells from animals that received *L. plantarum* Dlt⁻ mutant compared with cells from control animals (Fig. 3B). In mesenteric lymphocytes, a difference was seen for both TNF (Fig. 3A) and IFN-gamma (Fig. 3B), whereas in splenocytes, only the activation-induced release of IFN-gamma decreased (data not shown).

***L. plantarum* Dlt⁻ mutant increased spontaneous secretion of IL-10 in splenocytes and activation induced IL-10 secretion in T cells from the spleen and MLN**

The IL-10 ELISA showed a trend to higher spontaneous release of IL-10 in splenocytes and MLN from *L. plantarum* Dlt⁻ mutant fed rats compared with controls and animals that received *L. plantarum* WT (Fig. 4A,B). For splenocytes, the difference was significant (Fig. 4A). Following T-cell specific activation, the amount of secreted IL-10 increased in all three groups (Fig. 4A,B). Again the IL-10 amounts released by cells from animals treated with Dlt⁻ mutant were significantly higher than those from control and WT fed animals (Fig. 4A,B).

DISCUSSION

We have previously shown that feeding of *L. reuteri* to normal healthy male *Sprague-Dawley* rats resulted in inhibition of visceral pain perception induced by

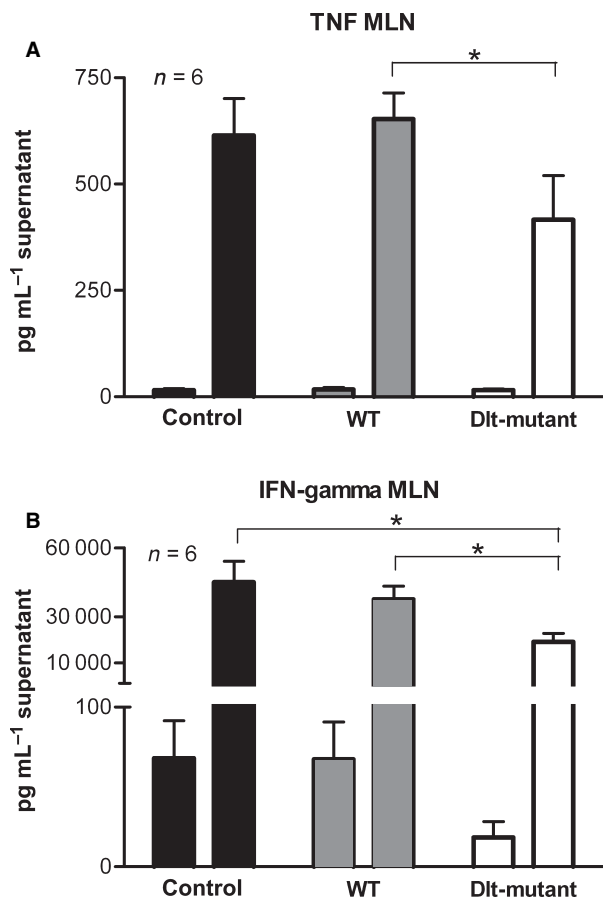


Figure 3 Effects of *L. plantarum* WT and *L. plantarum* Dlt⁻ mutant on concentration of TNF (A) and IFN-gamma (B) in supernatants of mesenteric lymphocytes. The left bar of a pair shows spontaneous release and the right bar release following 48 h of T-cell specific activation with anti-rat CD3/CD28. Data are presented as mean (SEM). Bars marked * are significantly different ($P < 0.05$) ($n = 6$).

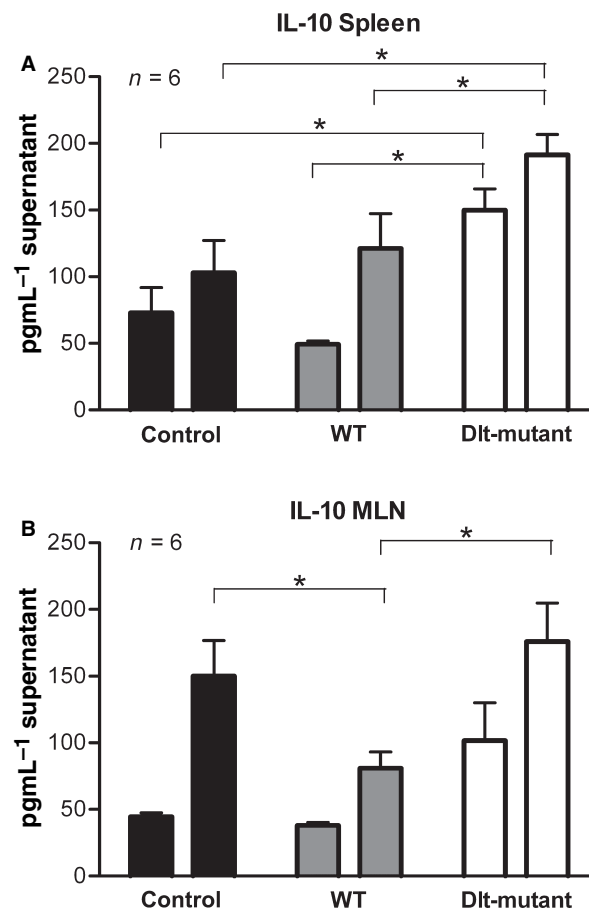


Figure 4 Effect of *L. plantarum* WT and *L. plantarum* Dlt⁻ mutant on concentration of IL-10 in supernatants of splenocytes (A) and mesenteric lymphocytes (B). The left bar of a pair shows spontaneous release and the right bar release following 48 h of T-cell specific activation with anti-rat CD3/CD28. Data are presented as mean (SEM). Bars marked * are significantly different ($P < 0.05$) ($n = 6$).

colorectal distension.¹⁶ This was accompanied by a decrease in afferent activity of dorsal root ganglion single units.¹⁶ These results were obtained in otherwise normal animals without prior infection or colonic inflammation. Other strains of probiotic lactic acid bacteria (LAB) have also been proven to be beneficial in visceral pain.²⁰ Although the mechanisms by which LAB protect from visceral pain perception are still unknown, we were able to show in previous experiments with *L. reuteri* that the protective effect was not only carried out by living bacteria but occurred also when the organisms were killed by heat treatment or gamma irradiation.¹⁶ Because of these findings, we questioned if cell wall components of LAB might be responsible for communicating the decrease in perception of visceral pain.

Several cell wall molecules of intestinal microbes have been established to be involved in bacteria-host communication in the gut.²¹ For intestinal inflammation, non-protein components in particular seem to play a significant role.²² Of those, LTA is a promising candidate. Grangette *et al.* have shown that the anti-inflammatory properties of *L. plantarum* in a mouse model of colitis were determined by the D-alanine content of its lipoteichoic acid (LTA).¹⁵ Depleting *L. plantarum* LTA of D-alanine to 1.1% compared with 41% of the parent strain did result in protection from inflammation. This was shown by the use of transgenic animals, not expressing Toll-like receptor-2, to be dependent on this Toll receptor. We assume from our previous results that the same mechanism of effect is likely in the rat although this cannot be determined because of the absence of a transgenic model.

Additionally, *L. plantarum* species have also been reported to be beneficial for relieving pain symptoms in patients with irritable bowel syndrome (IBS).^{23,24} We wondered therefore, if D-alanylation of *L. plantarum* LTA was also determining the inhibition of visceral pain perception in response to CRD. As immune-regulation of the *L. plantarum* Dlt⁻ mutant had only been shown in mice, on human peripheral blood mononuclear cells (PBMCs) and non-specific mouse bone marrow cells,¹⁵ but we were using rats for our pain experiments, we first examined if the immune regulatory effects of *L. plantarum* Dlt⁻ mutant and wild type could also be seen in normal *Sprague-Dawley* rats. Using rats as the mutant strain displayed similar immune regulatory properties *in vivo* described for human PBMCs *in vitro*.¹⁵ Feeding *L. plantarum* Dlt⁻ mutant resulted in a decrease of pro-inflammatory cytokine release (IFN-gamma and TNF) from mesenteric lymphocytes upon T-cell specific activation compared with cells of wild type fed rats. On the other hand, *L. plantarum* Dlt⁻ mutant increased the spontaneous secretion of IL-10 in splenocytes and was most effective in inducing IL-10 secretion upon T-cell specific activation in splenocytes and mesenteric lymphocytes. Plasma cytokine assessment was not possible because in our hands these are below the level of consistent detection. Feeding *L. plantarum* WT and Dlt⁻ mutant did not show changes in colonic tissue TNF or IFN-gamma, whereas IL-10 tissue levels were significantly reduced with feeding of *L. plantarum* Dlt⁻ mutant, and this possibly reflects local utilization. We also investigated if CRD by itself resulted in inflammatory signals and found that distension of the distal colon did not result in inflammatory changes as was reflected by low MPO levels and no detectable levels of IFN-gamma in the colon tissue after distension.

We then tested both *L. plantarum* strains in the CRD model and found that feeding of *L. plantarum* Dlt⁻ mutant is superior to wild type in controlling the cardio-autonomic response to noxious CRD. The experiments were conducted in a constitutive visceral pain model in the absence of inflammation to exclude parameters that alter visceral homeostasis. Neither MPO nor neutrophil infiltrates changed following CRD. Therefore, the anti-nociceptive effect can be solely attributed to a specific agent – in this case a *L. plantarum* with a modified cell wall LTA. The cardio-autonomic response to CRD in healthy rats is the result of a sympathico-afferent, vago-efferent pathway originating from sensory neurons in the intestinal wall. The neuronal circuit includes neurons from the inferior mesenteric ganglion and the enteric nervous system.²⁵ This brain-gut connection is part of

a network informing the brain of conditions in the intestine and ensuring that potentially noxious stimuli are recognized. It is widely accepted that IBS may occur subsequent to infectious diarrhea and that the syndrome may often occur as result of low grade intestinal inflammation, as exemplified by elevated pro-inflammatory cytokines in the plasma.¹³ The anti-inflammatory cytokine IL-10 has been proven to reverse this effect²⁶ and promote anti-nociception. Experiments have also shown that administration of exogenous IL-10 protects mice from dynorphin-induced neuropathic pain in a dose-dependant manner²⁷ possibly, by blocking NF-kappa B induced pro-inflammatory cytokine production. The Dlt⁻ mutant of *L. plantarum* did increase the amount of IL-10 and might thereby impair pain perception. The Dlt⁻ mutant also lowered pro-inflammatory cytokine secretion (TNF-alpha and IFN-gamma). Decreased secretion of TNF may also be relevant to the decreased visceral pain perception observed because inhibition of TNF binding to its receptor has been shown to be associated with decreased spinal nociception.²⁸ As cytokines have been proven previously to be involved in inflammation-induced visceral pain, we would like to stress the point that we have observed a decrease in intestinal pain perception in parallel with an anti-inflammatory cytokine profile in a non-inflammatory model. This observation suggests a constitutive association between specific cytokine levels and nociceptive capacity of the nervous system, not only under pathological but also under physiological conditions.

The results that we have obtained are to some extent supported by previous work on IBS patients which showed that baseline IL-10 release is decreased in PBMCs.¹⁴ Inflammatory changes are arguably controversial in tissues from these patients. However, the baseline pro-inflammatory plasma cytokine increase in IBS¹³ does support the suggestion that these symptoms may somehow be related to intrinsic cytokine synthesis by immune cells and a subsequent imbalance in pro-inflammatory to anti-inflammatory signals. IBS patients show significantly more heterozygote TNF high genotypes than healthy controls.²⁹ Furthermore, treatment with *Bifidobacterium infantis*, another Gram-positive probiotic, not only improved IBS symptoms when given to patients but also normalized increased baseline IL-10/IL-12 ratios.¹⁴ Our study showed that the cell wall component LTA of *L. plantarum* plays a crucial role in mediating protection from intestinal pain, due to CRD in healthy rats and does increase the T-cell activation specific release of IL-10 from splenocytes and the spontaneous IL-10

release in these animals. We do not know if LTA structure is also involved in the anti-nociceptive effect of other Gram-positive probiotics, such as *L. reuteri*. This needs further investigation.

In conclusion, we have shown for the first time that the extent of LTA D-alanylation might be a determinant in explaining the regulatory effect of *L. plantarum* and possibly *L. reuteri* in visceral pain perception. The fact that this same feature characterizes the anti-inflammatory properties of *L. plantarum*¹⁵ provides supportive evidence that the same mechanisms by which LAB provoke their beneficial effects upon inflammation may also be effective in protection from intestinal pain. It further suggests the possible involvement of anti-inflammatory cytokines, such as IL-10, in down-regulating not only immune events but also the visceral perception of pain even in healthy rats.

ACKNOWLEDGMENTS

The authors would like to thank Ursula Kadela, Jolanta Stanizs and Xianjun Yang for their invaluable technical support and Paul Forsythe and Hanneke van der Kleij for helpful discussions. The help of Annick Mercenier is also gratefully acknowledged, as is the grant support of the Crohn's and Colitis Foundation of Canada. Competing interest: the authors have no competing interests.

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