ORIGINAL ARTICLE 10.1111/j.1469-0691.2005.01142.x

Interference of *Lactobacillus plantarum* with *Pseudomonas aeruginosa* *in vitro* and in infected burns: the potential use of probiotics in wound treatment

J. C. Valdéz[1], *M. C. Peral*[2], *M. Rachid*[1], *M. Santana*[3] *and G. Perdigón*[1,4]

[1]Cátedra de Inmunología, Facultad de Bioquímica, Química y Farmacia, [2]Departamento Biomédico and [3]Departamento de Bioestadística, Facultad de Medicina, Universidad Nacional de Tucumán and [4]Centro de Referencia para Lactobacilos, Tucumán, Argentina

ABSTRACT

This study evaluated the ability of the probiotic organism *Lactobacillus plantarum* to inhibit the pathogenic activity of *Pseudomonas aeruginosa*, both *in vitro and in vivo*, and investigated the mechanisms involved in such protection. *L. plantarum* whole cultures, culture filtrates (acid filtrate and neutralised acid filtrate) and isolated, washed cells were tested *in vitro* for their effects on the production of the *P. aeruginosa* quorum-sensing signal molecules, acyl-homoserine-lactones (AHLs), and two virulence factors controlled by these signal molecules, elastase and biofilm. All were inhibited by *L. plantarum* cultures and filtrates, but not by isolated, washed cells. The acid *L. plantarum* growth medium itself had some inhibitory activity, but the greatest activity was exerted by the whole culture. To test the in-vivo activity of *L. plantarum*, a burned-mouse model was used in which burns infected with *P. aeruginosa* were treated with *L. plantarum* at 3, 4, 5, 7 and 9 days post-infection. Samples from skin, liver and spleen taken after 5, 10 and 15 days demonstrated inhibition of *P. aeruginosa* colonisation by *L. plantarum*. There was also an improvement in tissue repair, enhanced phagocytosis of *P. aeruginosa* by tissue phagocytes, and a decrease in apoptosis at 10 days. These results indicate that *L. plantarum* and/or its by-products are potential therapeutic agents for the local treatment of *P. aeruginosa* burn infections.

Keywords Bacterial interference, burns, *Lactobacillus*, probiotic, quorum sensing, *Pseudomonas aeruginosa*

Original Submission: 11 March 2004; **Revised Submission:** 20 December 2004; **Accepted:** 24 December 2004

Clin Microbiol Infect 2005; 11: 472–479

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that infects primarily immunocompromised individuals, such as patients with cystic fibrosis, cancer or AIDS, or patients with indwelling medical devices or burns [1]. Resistance to antimicrobial agents and numerous virulence factors means that *P. aeruginosa* causes many recalcitrant infections [2]. *P. aeruginosa* virulence determinants include cell-associated (lipopolysaccharide endotoxin, flagellum, pili) and extracellular (alginate, exotoxin A, exoenzyme S, pyocyanin, elastase, etc.) factors. It has been demonstrated that expression of these virulence factors is regulated by a cell-density-dependent signalling mechanism known as quorum-sensing [3]. This system has two components, *las* and *rhl*, and uses two autoinducers, *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL), respectively, to control expression of the virulence factors [4]. The flagellum, pili and exopolysaccharide are important in pathogenicity because they allow the bacteria to form biofilms. Biofilms are extremely difficult to eradicate, since they are shielded from host defences such as phagocytes or antibodies, as well as antibiotics [5], and can be the source of chronic infections [2]. Also,

Corresponding author and reprint requests: J. C. Valdéz, Cátedra de Inmunología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, La Madrid, 4000, Tucumán, Argentina
E-mail: pumavaldez@yahoo.com.ar

polymorphonuclear neutrophils (PMNs), the dominant cells of the inflammatory response, and macrophages can be intoxicated through the activity of the type III secretion system of *P. aeruginosa* [6].

P. aeruginosa burn infection is a complicated process that involves inflammation, immune response and wound healing [7]. The interaction between a Gram-negative bacterium and its host is mediated by molecular structures produced by the pathogen, called pathogen-associated molecular patterns; these are recognised by pattern recognition receptors, associated with molecules called toll-like receptors (TLRs), on innate immune cells. For Gram-negative bacteria, a lipopolysaccharide receptor complex is associated with TLR-4, which regulates the inducible expression of various effector molecules, including different cytokines, chemokines and other molecules that are involved in the inflammatory and subsequent adaptive immune response [8]. The effects of *P. aeruginosa* infection of burns have been studied in mouse models, which have demonstrated excessive and uncontrolled production of different cytokines, such as inflammatory cytokines and chemokines, which appear to be very important in the infection mechanisms of *P. aeruginosa* [7,9].

In pathogenic Gram-positive bacteria, peptidoglycan and lipoteichoic acid induce cell activation mediated by TLR-2, with the consequent production of pro-inflammatory cytokines [10]. Non-pathogenic Gram-positive bacteria, such as lactobacilli, induce a very different pattern of cytokines in peripheral blood cells [11]. It has been observed previously that lactobacilli are able to inhibit peritoneal macrophage apoptosis induced *in vitro* by infection with *Salmonella typhimurium* [12], and can also regulate the inflammatory infiltrate *in vivo* [13]. It has been shown that cells and/or the metabolic by-products of lactobacilli have antagonistic effects against pathogens *in vitro*, and also *in vivo* during trials with urinary and genital infections in humans and mice [14,15].

The present study investigated whether *L. plantarum* might interfere with the pathogenic properties of *P. aeruginosa*, both *in vitro* and *in vivo*, and aimed to define some of the mechanisms involved, with the possibility that this approach could be used for the local treatment of recalcitrant infections.

MATERIALS AND METHODS

Bacterial strains

The *P. aeruginosa* strains used in this study were a standard clinical isolate, PA100, which was grown in Luria–Bertani (LB) medium (Gibco, Rockville, MD, USA), and the *qsc* mutant 119 strain of *P. aeruginosa* (a generous gift from E. P. Greenberg and K. Lee, University of Iowa, USA). This latter strain has a random *lacZ* transcriptional fusion in the chromosome of a *lasI–rhlI* double mutant, and was used to detect the production of acyl-homoserine-lactones (AHLs) by PA100. The mutant strain produces neither 3O-C_{12}-HSL nor C_4-HSL, but responds to the presence of these compounds by increasing expression of β-galactosidase. The mutant strain was grown in LB medium containing gentamicin 100 mg/L [16].

L. plantarum ATCC 10241 was grown in MRS broth (Oxoid, Basingstoke, UK) at 37°C. The following preparations were used to study the in-vitro interference of *L. plantarum* with *P. aeruginosa*: (1) a whole-cell culture containing 10^6 *L. plantarum* CFU/mL (T preparation); (2) culture supernatants recovered following centrifugation and filtration through 0.22-μm filters (acid filtrate; AF preparation); (3) aliquots of AF neutralised with 8 M NaOH to pH 7 (neutralised filtrate; NF preparation); and (4) *L. plantarum* cells washed and resuspended in phosphate-buffered saline at a concentration of 10^6 CFU/mL (Lp preparation).

To measure growth of *P. aeruginosa* in the presence of *L. plantarum*, aliquots (1 mL) of an overnight culture of PA100, diluted 1:7 in LB medium, were grown in the presence of 1 mL of an *L. plantarum* preparation (T, AF, NF or Lp). After incubation for 16 h at 37°C, an aliquot of each mixture was spread on MacConkey agar plates for colony counting (*L. plantarum* does not grow on MacConkey agar).

In-vitro assays

Production of acyl-homoserine-lactones

Indirect assay

P. aeruginosa PA100 was grown to an OD_{600} of 0.7, centrifuged, washed in phosphate-buffered saline, and then resuspended in either T, AF, NF, MRS or LB medium. After incubation for 1 h at 37°C, the cultures were centrifuged and filtered. One part of this filtrate was added to one part of the *qsc* 119 mutant strain culture. Following incubation at 37°C for a further 1 h, β-galactosidase activity was measured by the Miller reaction [17].

Direct assay

One millilitre of *P. aeruginosa* culture supernatant filtrate was added to 1 mL of T, AF, NF, MRS broth or LB medium. β-Galactosidase activity was measured after incubation at 37°C for 1 h.

Biofilm assay

A static biofilm assay was performed as described previously [18], with measurements after 1 h (when bacterial growth was negligible) and 7 h. *P. aeruginosa* PA100 was cultured in 96-well polystyrene microtitre plates, and then stained with crystal violet. The cell-attached dye was solubilised with ethanol 95% v/v, and the absorbance was then measured at 540 nm.

Elastase assay

The elastolytic activity of the different samples was investigated after 1 h and 18 h, using elastin Congo red (Sigma, St Louis, MO, USA) as a substrate [19]. Insoluble elastin Congo red was removed by centrifugation, and the absorbance of the supernatants was measured at 495 nm. For the 1-h assay, *P. aeruginosa* at OD_{600} 0.7 was washed and resuspended in fresh LB medium, and then diluted 1:7 in T, AF, NF, Lp or MRS broth. After incubation for 1 h, the elastolytic activity of the samples was determined. For the 18-h assay, a 16-h *P. aeruginosa* culture was mixed with the *L. plantarum* samples and MRS broth, as described above, and elastolytic activity was determined after incubation for 18 h.

Cytotoxicity and apoptosis assays

Adult inbred BALB/c mice, weighing 20–25 g, from the laboratory stock were used in all experiments. Cells were cultured in RPMI-1640 medium–HEPES modified (Sigma) supplemented with fetal bovine serum 10% v/v (Natocord, Cordoba, Argentina). Peritoneal polymorphonuclear neutrophils (PPMNs) were obtained with a slight modification of the method of Nind [20]. PPMN-enriched peritoneal exudates were collected by lavage with Hanks' solution and resuspended in cell culture medium to a concentration of 1×10^6 cells/mL. Peritoneal macrophages were obtained from normal mice by peritoneal lavage and resuspended in cell culture medium to a concentration of 1×10^6 cells/mL.

A tetrazolium reduction assay, CellTiter 96 AQ_{ueous} One Solution (Promega, Madison, WI, USA), was used to measure cytotoxicity activity. PPMNs were cultured in 96-well tissue culture plates at 5×10^4 cells/100 μL/well and were infected with three different PA100 inocula (10^2, 10^4 or 10^8 CFU/100 μL). The absorbance of reduced tetrazolium was determined at 490 nm.

To assay apoptosis, 10^6 adherent cells (peritoneal macrophages or PPMNs) were infected with PA100 at 10^7 CFU/mL. After 2 h, the presence of DNA fragmentation was determined by the terminal deoxytransferase-mediated dUTP nick end-labelling (TUNEL) test using the Fluorescein Apoptosis Detection System (Promega). Fluorescent cells were counted, with apoptosis expressed as the percentage of fluorescent cells/200 cells.

In-vivo experiments

The burned-mouse model of Stieritz and Holder, modified by Rumbaugh *et al*. [21], was used. The protocol was approved by The Animal Care and Use Committee at Texas Tech University Health Science Centre. Briefly, the mice were anaesthetised, their backs were shaved, and they were placed in water at a temperature of 90°C for 10 s to burn 15% of the body surface. Immediately following the burn, the mice were randomised into three groups: (1) group BPs, injected subcutaneously directly under the burn with 200–300 CFU of *P. aeruginosa* PA100 (100 μL); (2) group BPs + Lp, comprising some of the mice from group BPs, injected in the burned and infected area, on days 3, 4, 5, 7 and 9 after the initial infection, with 100 μL of a suspension of *L. plantarum* (10^5 CFU/mL) grown in MRS broth; and (3) group B, comprising mice injected in the wound with 100 μL of phosphate-buffered saline.

On days 5, 10 and 15 after the initial infection, mice were anaesthetised with ether, blood was collected, and skin, connective tissue and panniculus carnous muscle from the burned area were processed. Paraffin sections were stained with haematoxylin and eosin. Skin and connective tissue sections from the burned area, spleen and liver of each animal were also obtained and the CFU/g of tissue were determined by plating on MacConkey agar.

Tissue phagocytes (TPs) were obtained from the connective and muscular tissue sections underneath the burned skin; these were washed twice to eliminate the necrotised PMNs and then digested with Hanks' solution containing pronase E (Sigma) 0.05% w/v, collagenase type VIII (Sigma) 0.05% w/v and deoxyribonuclease type I (Sigma) 0.001% w/v. The adherent cells from this suspension (mostly PMNs) were isolated by allowing them to adhere to the plastic surfaces of Petri dishes. TP phagocytosis of *P. aeruginosa* and *L. plantarum* was detected by indirect immunofluorescence, using specific polyclonal antibodies against each microorganism. The cells were analysed by double or triple fluorescence under an epifluorescence microscope. *P. aeruginosa* cells inside phagocytes were detected by cytoplasmic red fluorescence (Rodamine TRICT-conjugated; Jackson Immuno Research Laboratory, West Grove, PA, USA), while *L. plantarum* cells were detected by cytoplasmic green fluorescence (FITC-conjugated; Sigma), and the morphology of nuclei was visualised by light blue fluorescence (DAPI; Molecular Probes, Eugene, OR, USA). Results were expressed as the percentage of phagocytes containing microorganisms/200 cells. TP apoptosis was analysed for the presence of DNA fragmentation by the TUNEL assay.

The *P. aeruginosa*-specific IgG antibody response of mice in groups BPs and BPs + Lp was measured by ELISA at day 15 after infection. Briefly, ELISA plates (NUNC Maxisorb; Nalgene, Roskilde, Denmark) were coated by adding 100 μL of heat-killed *P. aeruginosa* suspension. Diluted (1:100) sera from animals in each group, a positive control from heat-killed *P. aeruginosa*-immunised mice and sera from normal mice were then added (100 μL). IgG was detected using peroxidase-conjugated goat anti-mouse IgG (Sigma). The plates were developed with *o*-phenylene diamine (Sigma) and read at OD_{490}.

Statistical analysis

Analysis of data was performed by using random effect in an ANOVA model with 95% confidence intervals (CIs).

RESULTS

In-vitro effects of *L. plantarum* on *P. aeruginosa*

No viable *P. aeruginosa* PA100 cells were detected after culture in the T and AF *L. plantarum* preparations. Although NF showed a reduced growth-inhibiting capacity, it produced a significant reduction in the *P. aeruginosa* viable count, compared with PA100 cultured in LB medium ($p < 0.001$). The Lp preparation had no inhibitory effect.

The *L. plantarum* preparations also inhibited production of AHL by PA100 in both indirect and

direct assays. Using the indirect assay, significant inhibition by the *L. plantarum* AF ($p < 0.001$), T ($p < 0.001$) and NF ($p < 0.01$) preparations was observed, compared with PA100 cultured in LB medium. The MRS control also showed a lesser inhibitory effect ($p < 0.05$) (Fig. 1). In the direct assay, significant inhibition was seen with the AF ($p < 0.01$) and T ($p < 0.001$) preparations, as well as a lesser effect with the MRS control ($p < 0.05$), but no significant inhibition was observed with the NF preparation (Fig. 2).

Fig. 3 shows the effect of the various preparations on the formation of biofilm by PA100. Compared to *P. aeruginosa* grown in LB medium, the T and AF preparations, and the MRS control, had a significant inhibitory effect ($p < 0.001$) on biofilm production after incubation for 7 h.

Fig. 4 shows that elastolytic activity was inhibited significantly by the T ($p < 0.001$), AF ($p < 0.01$) and NF ($p < 0.05$) preparations compared with *P. aeruginosa* PA100 cultured in LB medium.

The in-vitro cytotoxicity, compared with non-infected control cells, exerted by 10^2, 10^4 or 10^8 CFU of *P. aeruginosa* PA100 on PPMNs was

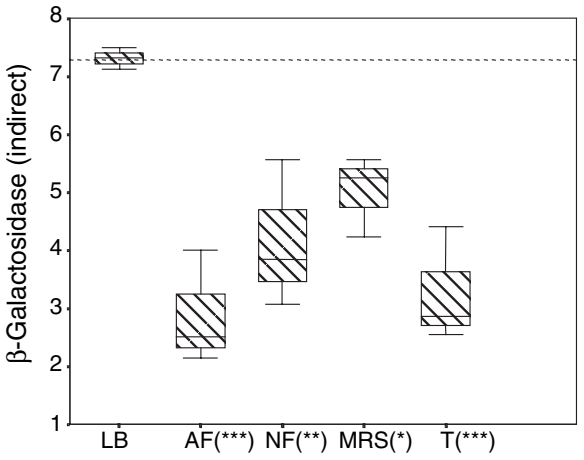

Fig. 1. Inhibitory effect of different preparations of *Lactobacillus plantarum* on acyl-homoserine-lactones (AHLs) produced by *Pseudomonas aeruginosa* as measured by β-galactosidase activity (indirect assay). AHL production by *P. aeruginosa* grown in Luria–Bertani (LB) medium is indicated by the dotted line. The solid line inside each box is the mean of three samples. LB, *P. aeruginosa* in LB medium; AF, *P. aeruginosa* in the presence of *L. plantarum* acid filtrate; NF, *P. aeruginosa* in the presence of *L. plantarum* neutralised filtrate; MRS, *P. aeruginosa* in MRS broth; T, *P. aeruginosa* in the presence of 10^6 CFU *L. plantarum*/mL. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

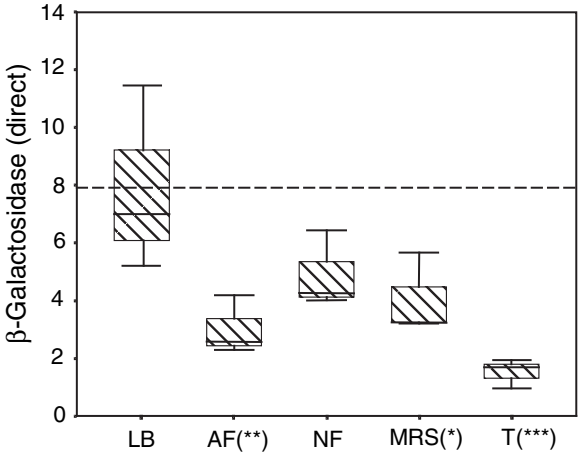

Fig. 2. Inhibitory effect of different preparations of *Lactobacillus plantarum* on the production of acyl-homoserine-lactones (AHLs) by filtrates of *Pseudomonas aeruginosa* culture supernatant (direct assay). AHL production by filtrate of *P. aeruginosa* culture supernatant is indicated by the dotted line. The solid line inside each box is the mean of triplicate samples. LB, *P. aeruginosa* in Luria–Bertani medium; AF, *P. aeruginosa* in the presence of *L. plantarum* acid filtrate; NF, *P. aeruginosa* in the presence of *L. plantarum* neutralised filtrate; MRS, *P. aeruginosa* in MRS broth; T, *P. aeruginosa* in the presence of 10^6 CFU *L. plantarum*/mL. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

49%, 68%, and 73%, respectively. PPMNs and peritoneal macrophages also showed a high susceptibility to in-vitro apoptosis induced by *P. aeruginosa* PA100 (98% and 97%, respectively, vs. 2–3% for non-infected control cells). PA100 also induced apoptosis *in vivo* (see below).

In-vivo effect of *L. plantarum* on *P. aeruginosa* infection

The burned skin of mice in the different groups differed in macroscopic appearance. Group B showed fewer purulent exudates than group BPs + Lp, and group BPs + Lp showed fewer purulent exudates than group BPs. There was a delay in wound healing in groups BPs and BPs + Lp (30 days) compared with group B (25 days).

Histological studies on day 5 showed oedema, vascular congestion and necrotic areas containing inflammatory infiltrates, while neutrophils were distributed throughout the entire thickness of the dermis and muscular surrounding tissue in all groups. These infiltrates were larger in groups BPs and BPs + Lp than in group B. On day 10, the wound repair process was significantly advanced

in the group B mice compared to the other groups, but there were some differences between groups BPs and Bps + Lp. In group BPs + Lp, the areas of necrosis were smaller and the inflammatory infiltrates were more diffuse than for the mice in group BPs. In addition, dermal regeneration from wound margins, characterised by granulation tissue rich in fibroblasts, collagen matrix, and capillary vessels formed by endothelial cells with prominent nuclei and a centripetal re-epithelialisation process, was improved by *L. plantarum* treatment. However, the healing time did not differ between the two groups, and skin repair occurred after *c*. 30 days.

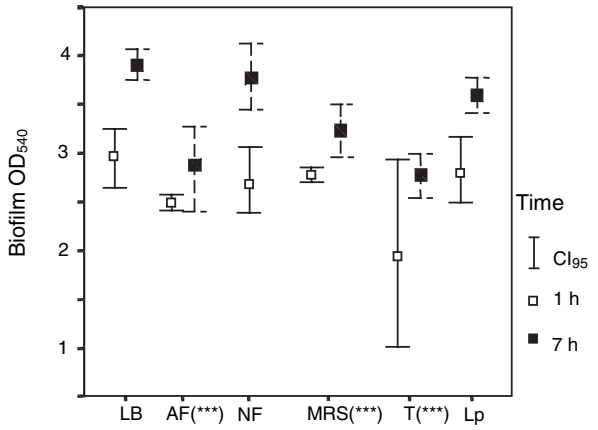

Fig. 3. Effect of different preparations of *Lactobacillus plantarum* on *Pseudomonas aeruginosa* biofilm production in static culture. Biofilm formation was monitored after incubation for 1 h and 7 h. Each point is the average of three measurements. LB, *P. aeruginosa* in Luria–Bertani medium; AF, *P. aeruginosa* in the presence of *L. plantarum* acid filtrate; NF, *P. aeruginosa* in the presence of *L. plantarum* neutralised filtrate; MRS, *P. aeruginosa* in MRS broth; T, *P. aeruginosa* in the presence of 10^6 CFU *L. plantarum*/mL; Lp, *P. aeruginosa* in the presence of *L. plantarum* cells washed and resuspended in phosphate-buffered saline at a concentration of 10^6 CFU/mL. ***p < 0.001; CI_{95}, 95% CI for the mean.

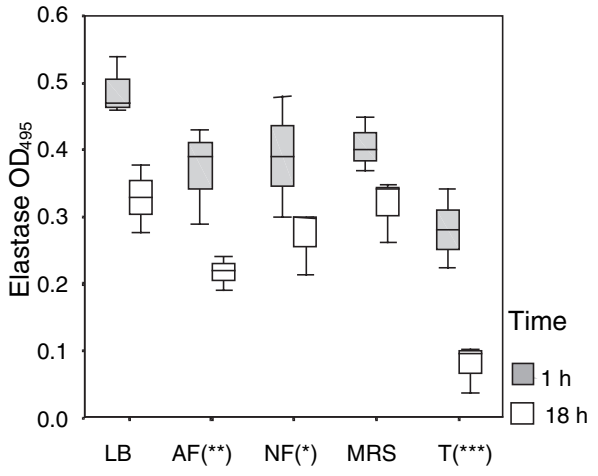

Fig. 4. Inhibitory effect of different preparations of *Lactobacillus plantarum* on elastase produced by *Pseudomonas aeruginosa*. The solid line inside each box is a mean of triplicate samples. LB, *P. aeruginosa* in Luria–Bertani medium; AF, *P. aeruginosa* in the presence of *L. plantarum* acid filtrate; NF, *P. aeruginosa* in the presence of *L. plantarum* neutralised filtrate; MRS, *P. aeruginosa* in MRS broth; T, *P. aeruginosa* in the presence of 10^6 CFU *L. plantarum*/mL. *p < 0.05; **p < 0.01; ***p < 0.001.

Significantly lower numbers of PA100 were detected in all tissues analysed from mice in group BPs + Lp than in those from mice in group BPs ($p < 0.001$). Fig. 5 shows the skin bacterial counts at days 5 and 10 (other tissues had a similar profile). At day 15, 60% of the mice in group BPs + Lp showed clearance of bacteria, compared to 40% in group BPs.

At day 5, the percentage of phagocytes containing PA100 was similar for groups BPs and BPs + Lp, and was reduced for both groups by day 10 ($p < 0.001$). However, by day 10, the number of cells exhibiting phagocytosis of PA100 in group BPs + Lp was significantly greater than in group BPs ($p < 0.001$) (Fig. 6). The percentage of TPs in apoptosis at day 5 was similar in groups BPs and BPs + Lp, i.e., approaching 100%. This percentage was lower in all groups at day 10, but there were significant differences between the groups: group B (5 ± 2%) vs. group BPs (41 ± 7%)

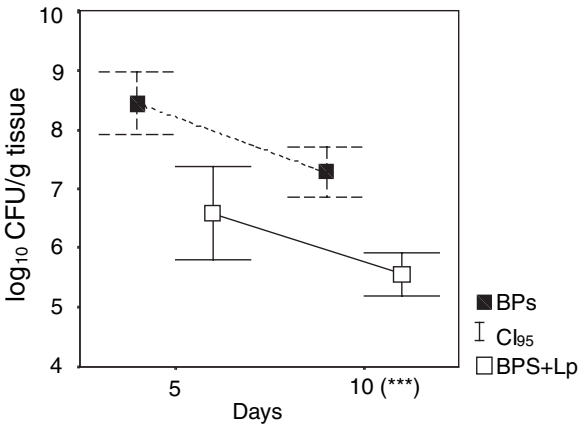

Fig. 5. Comparison of *Pseudomonas aeruginosa* viable counts in tissue obtained from burn wounds at days 5 and 10 post-infection. Values represent the average of five independent experiments. BPs, burn wounds infected with *P. aeruginosa*; BPs + Lp, burn wounds infected with *P. aeruginosa* and treated with *Lactobacillus plantarum*. ***p < 0.001; CI_{95}, 95% CI for the mean.

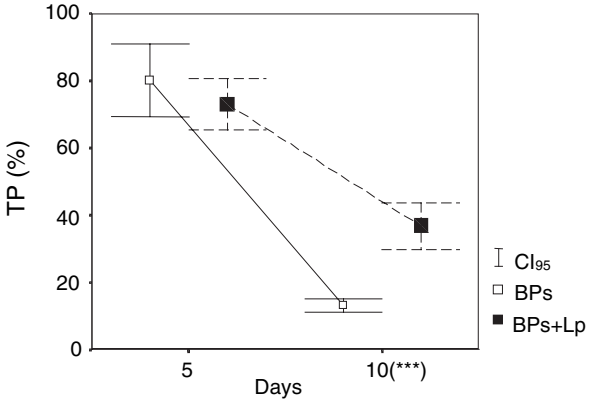

Fig. 6. Tissue phagocytosis (TP) of *Pseudomonas aeruginosa* at days 5 and 10 in the burned-mouse model. Each point is the average of five experiments and each experiment was performed with cells from one mouse. BPs, burn wounds infected with *P. aeruginosa*; BPs + Lp, burn wounds infected with *P. aeruginosa* and treated with *L. plantarum*. ***$p < 0.001$; CI_{95}, 95% CI for the mean.

($p < 0.001$); group BPs (41 ± 7%) vs. group BPs + Lp (25 ± 4%) ($p < 0.001$).

Infection elicited high levels of antigen-specific IgG antibodies, but no significant difference was observed between groups BPs + Lp and BPs.

DISCUSSION

P. aeruginosa infection is often difficult to eradicate because of resistance to many antibiotics and disinfectants [22,23]. Consequently, this organism is an emerging therapeutic problem [24] and is one of the most serious complications seen in burn wounds [7]. The probiotic lactobacilli have been used mainly to treat gastrointestinal disorders [25]. However, their ability to secrete acids, bacteriocins and other by-products that may neutralise infection caused by pathogens, and the fact that they are considered to be harmless microorganisms that regulate the host's inflammatory and immune responses, mean that lactobacilli may be useful for the treatment of clinical infections in other parts of the body, such as recurrent bladder infections caused by *Escherichia coli*, vaginosis caused by anaerobic microbes, wound infections caused by *Staphylococcus aureus*, and others [11–14,26,27].

The present study investigated the in-vitro and in-vivo inhibition exerted by *L. plantarum* on the virulence of *P. aeruginosa*. Growth of *P. aeruginosa* was inhibited fully by the *L. plantarum* T and AF preparations, while the NF preparation resulted in 97% inhibition. The *P. aeruginosa* quorum-sensing molecules *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone are important for the control of elastase rhamnolipid and the formation of differentiated biofilm [5,7,18,28]. The findings of the present study agreed with previous data, in that direct inhibition of AHL activity and/or blockage of their synthesis (indirect assay) decreased virulence factor production and resistance to antimicrobial agents. The same inhibition profile of the T, AF and NF preparations was obtained for elastase and biofilm production with different concentrations of *P. aeruginosa*. This inhibition was not dependent on the growth of *P. aeruginosa*, as this was negligible at 1 h. Recent investigations have demonstrated that quorum-sensing molecules play no role in early biofilm formation, but are important in the later stages [28]. The inhibitory effect shown in the present study may be associated with the inhibition of quorum-sensing molecules, but a direct inhibitory action of *L. plantarum* secondary metabolites on elastase and biofilm formation cannot be ruled out. The acid *L. plantarum* growth medium itself had some inhibitory activity, but the greatest inhibitory effect was observed with the T and AF preparations.

In order to investigate the in-vivo *L. plantarum* inhibitory effect, a burned-mouse model of *P. aeruginosa* infection was treated with whole cultures of *L. plantarum*. At day 10, treatment with *L. plantarum* (group BPs + Lp) enhanced *P. aeruginosa* phagocytosis by tissue phagocytes significantly, and led to a decrease in apoptosis. Concomitantly, there was a decrease in the bacterial counts in skin, liver and spleen. The effect of the treatment was more pronounced on day 15, with macroscopically diminished purulent exudates, a more diffuse inflammatory cell infiltrate, and fast regeneration and tissue repair.

It has been shown in animal models of infection that the ability of *P. aeruginosa* to colonise the host, as well as its capacity to induce inflammation and cause death, is attenuated in the absence of the complete *las* and/or *rhl* quorum-sensing systems [21]. The direct inhibition of AHL activity and/or blockage of their synthesis by *L. plantarum* and its by-products could decrease significantly the production of virulence factors and resistance to

antimicrobial agents. Previous studies have demonstrated that 3O-C_{12}-HSL regulates the immune response and stimulates the inflammatory response directly, since it induces the expression of COX2, prostaglandin E_2 and other cytokines, as well as chemokines such as interleukin-1a, interleukin-6, interferon-γ, tumour necrosis factor-α, MIP-2, MIP-1B and IP-10 (MIP-2 is mainly a chemotactic factor for PMNs) [29]. Production of AHL molecules *in vivo* by *P. aeruginosa* has been demonstrated in infected mice [30], and the production of pro-inflammatory cytokines is exacerbated by *P. aeruginosa* infection of burned wounds [9]. In immunocompetent individuals, the induction of inflammation is beneficial, but in immunodeficient subjects, who mount a potent inflammatory response, such induction would produce significant tissue destruction and the ability to combat the infection would be reduced severely [30]. It has been demonstrated that interleukin-18, another inflammatory cytokine, inhibits *P. aeruginosa* infection [31]. However, the interaction of lactobacilli with fibroblasts, epithelial cells and inflammatory cells produces a very different pattern of cytokines and chemokines in comparison with pathogenic bacteria [11,32]. Perhaps these antagonisms confer on lactobacilli the anti-inflammatory activity reported previously [13,15,32]. The cells contained in the whole-cell culture preparation of *L. plantarum* (T) would have an ability to interfere with the infective capacity of *P. aeruginosa* cells by inhibiting the excessive PMN influx induced by the pathogen and allowing effective bacterial clearance. Moreover, *P. aeruginosa* has a toxic effect on phagocytes [6], and *Lactobacillus* protects them from the apoptosis caused by pathogens [12]. While this therapy is not as effective as some novel antibiotics, such as novospirin-10 or protegrin-1, in the *P. aeruginosa* burned-wound infection model [33], the apparent activity of *L. plantarum* and its by-products deserves further investigation for possible use in topical wound treatments.

ACKNOWLEDGEMENTS

The authors thank P. Greemberg and K. Lee, Department of Microbiology, University of Iowa, for the *qsc P. aeruginosa* mutant. This research was supported by grants from Consejo de Investigación de la Universidad Nacional de Tucumán, Argentina No. D/231.

REFERENCES

1. Kievit TR, Iglewski BH. Bacterial quorum sensing in pathogenesis relationships. *Infect Immun* 2000; **68**: 4839–4849.
2. Lewis K. Riddle of biofilm resistance. *Antimicrob Agents Chemother* 2001; **45**: 999–1007.
3. Van Delden C, Iglewski BH. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* 1998; **4**: 551–560.
4. Fuqua C, Parsek MR, Greenberg EP. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Ann Rev Genet* 2001; **35**: 439–468.
5. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; **284**: 1318–1322.
6. Dechaeux D, Toussaint B, Richard M, Brochier G, Croize J, Attree I. *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. *Infect Immun* 2000; **68**: 2916–2924.
7. Rumbaugh K, Griswold JA, Hamood AN. The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microb Infect* 2000; **2**: 1721–1731.
8. Zhang G, Ghosh S. Toll-like receptor-mediated NFkB activation: a phylogenetically conserved paradigm in the innate immunity. *J Clin Invest* 2001; **107**: 13–19.
9. Rumbaugh KP, Colmer JA, Griswold JA, Hamood AN. The effects of infection of thermal injury by *Pseudomonas aeruginosa* PAO1 on the murine cytokine response. *Cytokine* 2001; **16**: 160–168.
10. Shwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 1999; **274**: 17406–17409.
11. Hessle C, Andersson B, Wold A. Gram-positive bacteria are potent inducers of monocytic IL-12 while gram-negative bacteria preferentially stimulate IL-10 production. *Infect Immun* 2000; **68**: 3581–3586.
12. Valdéz JC, Rachid M, Gobbato N, Perdigón G. Lactic acid bacteria induce apoptosis inhibition in *Salmonella typhimurium* infected macrophages. *Food Agric Immunol* 2001; **13**: 189–197.
13. Perdigón G, Valdéz J, Rachid M. Antitumor activity of yogurt: study of possible immune mechanism. *J Dairy Res* 1998; **65**: 120–138.
14. Reid G. Probiotic agents to protect the urogenital tract against infection. *Am J Clin Nutr* 2001; **73**: 437S–443S.
15. Ashahara T, Nomoto K, Watanuki M, Yokokura T. Antimicrobial activity of intraurethrally administered probiotic *Lactobacillus casei* in a murine model of *Escherichia coli* urinary tract infection. *Antimicrob Agents Chemother* 2001; **45**: 1751–1760.
16. Whiteley M, Lee KM, Greenberg EP. Identification of genes controlled by quorum sensing on *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 1999; **96**: 13904–13909.
17. O'Toole GA, Kolter R. The initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Mol Microbiol* 1998; **28**: 449–461.
18. Gambello M, Iglewski B. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional

activator of elastase expression. *J Bacteriol* 1991; **173**: 3000–3009.
19. Miller JH. *A short course in bacterial genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1992.
20. Nind AF. Neutrophil chemotaxis: technical problems with nitrocellulose filters in Boyden-type chambers. *J Immunol Meth* 1981; **40**: 39–43.
21. Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN. Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. *Infect Immun* 1999; **67**: 5854–5862.
22. Hancock RE. Resistance mechanisms in *Pseudomonas aeruginosa* and other non-fermentative gram-negative bacteria. *Clin Infect Dis* 1998; **27**(suppl): 93–99.
23. McDonell G, Russell D. Antiseptic and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* 1999; **12**: 147–179.
24. Luzzaro F, Mantengoli E, Perilli M *et al.* Dynamics of a nosocomial outbreak of multidrug-resistant *Pseudomonas aeruginosa* producing the PER-1 extended-spectrum β-lactamase. *J Clin Microbiol* 1999; **39**: 1865–1870.
25. Marteau PR. Probiotics in clinical conditions. *Clin Rev Allergy Immunol* 2002; **22**: 255–273.
26. Harder B. Germs that do a body good. *Sci News* 2002; **161**: 72–77.
27. Gan BS, Kim J, Reid G, Cadieux P, Howard JC. *Lactobacillus fermentum* RC-14 inhibits *Staphylococcus aureus* infection of surgical implants in rats. *J Infect Dis* 2002; **185**: 1369–1372.
28. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* display multiple phenotypes during development as biofilm. *J Bacteriol* 2002; **184**: 1140–1154.
29. Smith RS, Harris SG, Phipps R, Iglewski BH. The *Pseudomonas aeruginosa* quorum sensing molecules N-(3-Oxododecanoyl) homoserine lactone contributes to virulence and induces inflammation in vivo. *J Bacteriol* 2002; **184**: 1132–1139.
30. Wu H, Song Z, Hentzer M *et al.* Detection of N-acyl-homoserine lactone in lung tissue of mice infected with *Pseudomonas aeruginosa*. *Microbiology* 2000; **146**: 2481–2493.
31. Schultz MJ, Knapp S, Florquin S *et al.* Interleukin-18 impairs the pulmonary host response to *Pseudomonas aeruginosa*. *Infect Immun* 2003; **71**: 1603–1634.
32. Blum S, Haller D, Pfeifer A, Shiffrin EJ. Probiotics and immune response. *Clin Rev Allergy Immunol* 2002; **22**: 287–309.
33. Steinstraesser L, Tack BF, Waring AJ *et al.* Activity of novospirin G10 against *Pseudomonas aeruginosa* in vitro and infected burns. *Antimicrob Agent Chemother* 2002; **46**: 1837–1844.