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Interleukin-8 production by polymorphonuclear leukocytes from patients with chronic infected leg ulcers treated with *Lactobacillus plantarum*

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Abstract

Bacterial infection impairs the healing process, promoting the chronicity of inflammation and wounds. Because antibiotics fail to eradicate bacteria, especially in biofilm form, new therapeutic modalities may be required. In the present study, the effectiveness of bacteriotherapy with *Lactobacillus plantarum* on infected chronic venous ulcers was investigated and its effects on interleukin (IL)-8 production by cells from the ulcer bed and neutrophils isolated from peripheral blood that were previously challenged *in vitro* with *Pseudomonas aeruginosa* and *L. plantarum* were studied. Topical application of *L. plantarum* culture to lesions (25–60 cm^2) of 14 diabetic and 20 non-diabetic patients induced debridement, granulation tissue formation and total healing after 30 days in 43% diabetics and in 50% non-diabetics. No significant differences between the groups were observed. The cells from ulcer beds collected after treatment with *L. plantarum* for 10 days showed a decrease in the percentage of polymorphonuclear, apoptotic and necrotic cells and an enhancement of IL-8 production. IL-8 production by isolated neutrophils from these patients was compared with that in diabetics without ulcers, as well as normal subjects under basal conditions, and after infection of polymorphonuclear cells with *P. aeruginosa* preincubated either with or without *L. plantarum*. The basal values in diabetic and ulcer patients were higher than normal ($p < 0.001$) and were increased by *P. aeruginosa* infection in normal, diabetics ($p < 0.001$) and non-diabetics with ulcers ($p < 0.01$). Preincubation with *L. plantarum* decreased IL-8 production in patients with ulcers non-diabetic and diabetic ($p < 0.001$). *Lactobacillus plantarum* treatment reduced wound bacterial load, neutrophils, apoptotic and necrotic cells, modified IL-8 production and induced wound healing.

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Introduction

Chronic wounds are, by definition, wounds that remain in a chronic inflammatory state and therefore fail to follow the normal patterns of the healing process. Chronic wounds are rarely, if ever, sterile and achieving wound sterility is often an unrealistic and non-essential goal in wound care [1]. Bacteria infecting chronic wounds are producers of biofilm and so are extremely resistant both to antibiotics and to the host immune response [2].

Polymorphonuclear neutrophil (PMN) leukocytes are probably the most significant components of the host defence mounted against biofilm-forming bacteria, and their secretory and phagocytic arsenal often fails to eliminate bacteria in biofilm, especially in diabetics [1].

Microbial products induce the secretion of inflammatory mediators. One of the most important is interleukin (IL)-8, a potent chemoattractant of neutrophils. In this way, infection and inflammation contribute to the chronicity of the wound [3]. It has been estimated by the National Institute of Health of the USA that more than 80% of persistent bacterial infections are likely to involve biofilms [2]. Chronic wound infections often do not respond to traditional antimicrobial therapies [4], and new therapeutic modalities may be

required [5]. Financial and operative limitations in our hospitals have led us to investigate an alternative therapy, namely, bacteriotherapy with *Lactobacillus plantarum*, to debride chronic wounds and diminish infection. Bacteriotherapy consists of the use of harmless bacteria to displace pathogenic organisms and it is considered to be a promising alternative to fight infection [6–8].

We have previously demonstrated [9] the ability of the probiotic organism *L. plantarum* to inhibit the pathogenic activity of *Pseudomonas aeruginosa* both *in vivo*, using a burn wound mouse model, and *in vitro*, indicating that *L. plantarum* and/or its products are potential therapeutic agents for the local treatment of *P. aeruginosa* burn infections.

It has also been demonstrated that *L. plantarum* and *P. aeruginosa* induce antagonistic substances in the inflammatory response [10]. We used therapy with *L. plantarum* because Lactobacilli have an excellent overall safety record among probiotics and no spontaneous *L. plantarum* infections have been documented [11].

The present study aimed to evaluate the efficacy of bacteriotherapy with *L. plantarum* culture on the chronic infected leg ulcers of diabetic and non-diabetic patients and to observe its effects on apoptosis, necrosis and IL-8 production by the cells from the ulcer bed (CUB). In addition, IL-8 production was analyzed in peripheral blood PMN (PBPMN) from patients with ulcers and compared with that in PBPMN from normal and diabetic subjects without ulcers under basal conditions, and after infection with *P. aeruginosa* either with or without preincubation with *L. plantarum*.

Material and Methods

Patients

The study comprised male and female individuals aged 40–70 years of age. Thirty-four patients from the plastic surgery and burns unit of the 'Zenon Santillan' Hospital with a chronic venous ulcer were included in a prospective uncontrolled study employing a local *L. plantarum* treatment. Fourteen patients suffered from moderately controlled type 2 diabetes mellitus (glycaemia level: 1.50 ± 0.30 mg/mL, HbA_{1C}: 7.8 ± 2.1%) and the 20 remaining patients were non-diabetic. Inclusion criteria included the presence of one venous ulcer confirmed by venous duplex ultrasound, with a surface of 25–60 cm^2, a bacterial load at a level $>10^5$ microorganisms per gram of tissue, which is generally accepted to justify a diagnosis of infection and is an important factor in delayed healing in chronic wounds [1], and no signs of healing in the past 3 months, despite conventional medical treatment.

Inclusion criteria comprised patients who had malignancy, autoimmune disease, an inclination to bleed or bleeding disease, and serious systemic infection.

Ten diabetic patients with similar glycaemia levels without lesions and 14 healthy subjects with normal glycaemia levels attending the 'Angel C. Padilla' Hospital were included as PBPMN donors. Both hospitals are located in the city of San Miguel de Tucumán, Argentina. All patients were informed about the aims of the study and provided their consent. The study was approved by the Hospital's Ethics Committee.

Treatment with *L. plantarum*

Wounds were cleaned, irrigated with saline and treated with topical applications of a whole culture of 10^5 *L. plantarum* ATCC 10241/mL in log phase, which was previously grown in De Man, Rogosa and Sharpe (MRS) broth for 5–6 h at 37°C. The culture was spread on a gauze pad and applied to the lesion, which was then covered with occlusive dressing. The culture was applied once-daily over a period of 10 days. Tolerable discomfort such as a burning sensation was observed after the first application of *L. plantarum*. The lesions were clinically monitored and evaluated weekly by the plastic surgeon.

Lesion biopsy samples

A 4-mm^3 sample of tissue was divided into two parts. One part of the biopsy was placed in RPMI (Roswell Park Memorial Institute) 1640-HEPES Medium (Sigma, St Louis MO, USA) with 100 mg/L of gentamicin, enzymatically digested to obtain CUB were stained with haematoxilin and eosin and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL). In addition, IL-8 was determined.

The other part of the biopsy was processed for microbial evaluation by routine techniques. Forty-eight hours after the 10-day treatment period, wound and blood samples were taken and incubated in MRS broth in an attempt to recover *L. plantarum*.

PBPMN

Heparinized venous blood samples were collected from all individuals. In the case of patients with ulcers, peripheral blood samples were obtained before performing bacteriotherapy. Neutrophils were isolated by dextran T-500 and Ficoll-Hypaque (Sigma) gradient centrifugation. The viability of neutrophils was >96%. Finally, the cells were suspended at 10^6 PBPMN/mL in RPMI 1640-HEPES Medium supplemented with foetal bovine serum 10% v/v (Gibco, Rockville, MD, USA).

Bacterial strains

The *P. aeruginosa* strains were a standard clinical isolate, PA100, which was grown in Luria–Bertani medium (Gibco) at 37°C. *Lactobacillus plantarum* ATCC 10241 was grown in MRS broth (Oxoid, Basingstoke, UK) at 37°C.

PBPMN infection

The PBPMN suspension (10^6/mL) was cultured on 25-mm diameter plates at 37°C for 1 h and infected for 1 h with 2×10^7 *P. aeruginosa* PA 100. Cells were fixed and IL-8 was determined.

Lactobacillus plantarum interference

For the study of *L. plantarum* interference with *P. aeruginosa*, PBPMN cultures (10^6/mL) were preincubated for 1 h at 37°C with 2×10^5 *L. plantarum.* The viability of PBMN from normal subjects, measured with trypan blue, was 76 ± 7%. Cells were subsequently infected with 2×10^7 *P. aeruginosa* for 1 h at 37°C Thereafter, cells were fixed and IL-8 was determined. Apoptosis was determined by TUNEL. DNA breaks were detected *in situ* by using a fluorescein apoptosis detection system kit Promega (Madison, WI, USA). Fluorescent cells were counted and expressed as percentage of fluorescent cells in a total count of 200 cells.

Intracellular IL-8 was determined by an immunoperoxidase assay using anti human IL-8 goat polyclonal IgG antibodies and an ABC staining system from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stained cells were counted and expressed as a percentage of positive cells in a count of 200 cells.

Statistical Analysis

Analysis of data was performed by using random effect in an analysis of variance model with 95% confidence intervals. To compare the reduction in the wound area as a function of time a multivariate analysis of variance (MANOVA) for repeated measurements was used. $p < 0.05$ was considered statistically significant.

Results

Wound healing

As shown in Table 1 the application of *L. plantarum* cultures to wounds induced, in approximately 8 days (range, 6–10 days), a coverage of granulation tissue of more than 90% in 50% and 55% of the diabetic and non-diabetic patients, respectively. After 30 days of treatment, a reduction of more than 90% of the lesion area was observed in 43% and 50% of the diabetics and non-diabetic patients, respectively. As shown in Fig. 1a, the difference between the values at day 0 and at day 30 of wound closure area in both groups was statistically significant ($p < 0.001$) using the MANOVA test.

TABLE 1. Effect of *Lactobacillus plantarum* treatment on granulation tissue and area of ulcers of diabetic and non-diabetic patients

		Diabetics (*n* = 14)	Non-diabetics (*n* = 20)
Area coverage with granulation tissue	>90%	*n* = 7; *P* = 0.50 day 0: 8% (0–12%) day 8: 94% (90–100%)	*n* = 11; *P* = 0.55 day 0: 6% (0–10%) day 8: 96% (92–100%)
Percentage (mean/range) before (days 0) and after 8 days (range, 6–10) of treatment	<90%	*n* = 7; *P* = 0.50 day 0: 7% (0–10%) day 8: 39% (30–60%)	*n* = 9; *P* = 0.45 day 0: 8% (0–10%) day 8: 43% (34–58%)
Ulcer area reduction	>80%	*n* = 6; *P* = 0.43 93% (80–100%)	*n* = 10; *P* = 0.50 92% (80–100)
Percentage (mean/range) and size (cm^2) (median/ mean/(range) before/ after 30 days of treatment	<80%	Before: 26/31.7/(25–50) After: 1.2/2.2/(0–6.2) *n* = 8; *P* = 0.57 52% (range, 35–70%) Before: 46/45,8/(26–60) After: 26/24/(15–30)	Before: 38/39.5/(25–54) After: 4/4.7/(0–10) *n* = 10; *P* = 0.50 51% (range, 37–75) Before: 48/47.6/(27–60) After: 25/25/(11–37)

n, no of patients; *P*, proportion of patients.

This indicates a continuous healing process. No significant differences were observed between the diabetic and non-diabetic groups. The degrees of the reduction of the wound areas 30 days after the start of treatment in the diabetic and non-diabetic patients is shown in Fig. 1b.

Decrease in colony forming units (CFU) with treatment

Before treatment with *L. plantarum*, wound microbial counts were 5×10^5 to 10^8 bacteria/g tissue. The bacteria isolated included *Staphylococcus aureus* (45%), *P. aeruginosa* (35%) and *Staphylococcus epidermidis* (15%), as well as others such as *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Enterococcus faecalis* (5%). All these bacteria were biofilm producers when assayed for biofilm formation in 96-well polyvinyl chloride microtitre dishes stained with 0.1% crystal violet. The number of CFU was lower than 10^5 after 5 days of treatment and dropped further (to $<10^3$) after 10 days ($p < 0.001$). No significant differences were observed between diabetic and non-diabetic patients (p 0.97). Forty-eight hours after the end of the treatment with *L. plantarum*, this bacterial species was not recovered from either peripheral blood or wound samples.

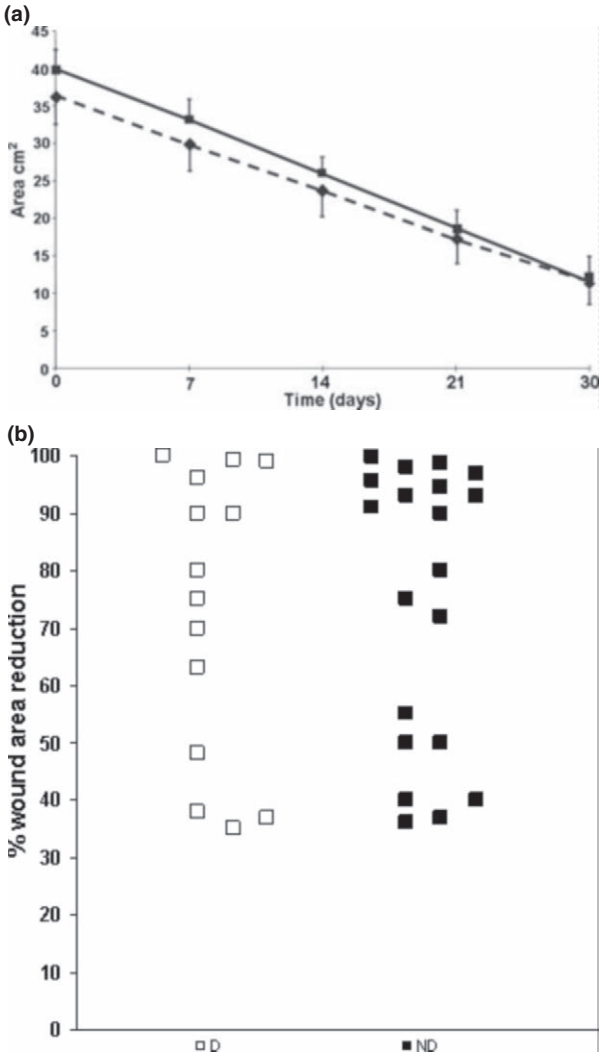

FIG. 1. (a) Ulcer area size and time of treatment in diabetic (filled line) and non-diabetic (dotted line) patients. Each point represents the mean ± SD wound area (diabetics n = 14; non-diabetics n = 20). (b) Each symbol represents the percentage of wound reduction area in each individual patient on day 30 after the start of the treatment. Open squares, diabetics (D); filled squares, non-diabetics (ND).

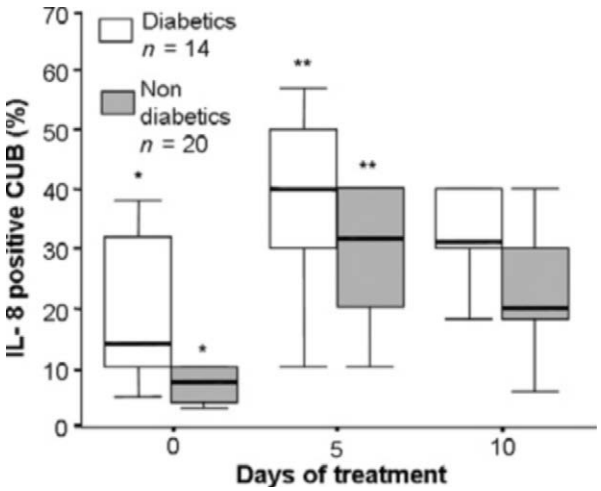

FIG. 2. Percentage of interleukin (IL)-8-positive cells from the ulcer bed from non-diabetics (filled squares) and diabetics (open squares) with ulcers as a function of time. Boxes refer to the 25th (bottom) and 75th (top) percentiles, and the median is the horizontal line inside; fences refer to maximum and minimum values and open circles represent outliers. *Significantly lower compared to days 5 and 10 ($p < 0.001$) within the same group. **Significantly higher compared to day 10 ($p < 0.05$) within the same group.

IL-8 changes in CUB with *L. plantarum* treatment

For the collection of CUB, samples were taken on days 0, 5 and 10 from the deep bed tissue. Most cells on days 0, 5 and 10 were PMN. The percentage of CUB positive for IL-8 in diabetics and non-diabetics was similar before treatment (day 0) (Fig. 2), which is in agreement with a previous study (12), although there is a tendency indicating lower levels in CUB from the non-diabetic group (p 0.058).

IL-8 values increased after 5 days of treatment ($p < 0.001$) followed by a decrease after 10 days compared to 5 days of treatment ($p < 0.05$). These variations were independent of the diabetic or non-diabetic condition of the patients. IL-8 was expressed mainly by PMN.

Cell population changes in CUB with *L. plantarum* treatment.

Because the percentage values of different cellular populations of CUB from diabetic and non-diabetic patients showed no significant differences, both groups were combined. Before *L. plantarum* treatment (day 0), 77 ± 13% of CUB were PMN, which showed 25% necrosis, 7% apoptosis and 46% prolongation of life span morphology. After 10 days of treatment, the percentage of PMN decreased (55 ± 19%) and the number of macrophages (25 ± 12%) and fibroblasts/endothelial cells (19 ± 9%) increased. None of these cells showed signs of necrosis or apoptosis.

Determination of IL-8 in PBPMN

Healthy individuals (N) had basal values of IL-8 positive PBPMN that were significantly lower than basal values of all other groups, diabetic patients without ulcers (D), diabetic patients with ulcers (DU) and non-diabetic patients with ulcers (NDU) ($p < 0.001$) (Fig. 3).

After *P. aeruginosa* infection, the percentage of IL-8 positive PBPMN was increased significantly in groups N, D and DU compared to basal values ($p < 0.001$) and, to a lesser extent, in the NDU group ($p < 0.01$). There were

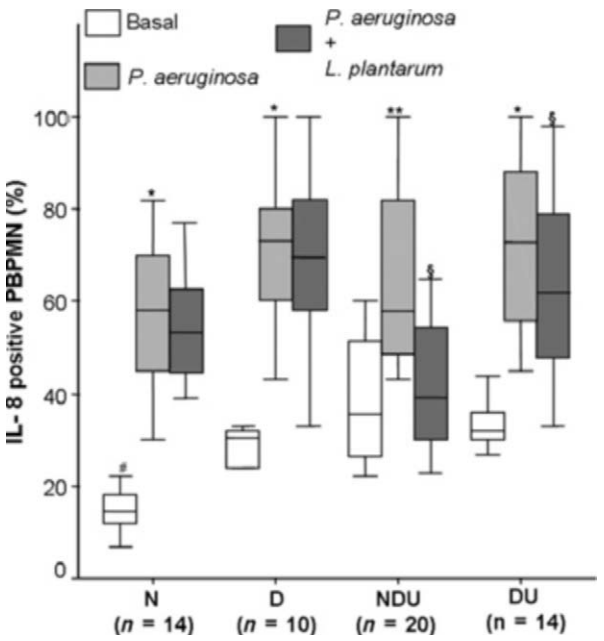

FIG. 3. Percentage of positive peripheral blood polymorphonuclear neutrophils (PBPMN) for interleukin (IL)-8 production from different groups of individuals: normal (N), diabetics without ulcers (D), non-diabetics with ulcers (NDU), diabetics with ulcers (DU). The assay was carried out with PBPMN under basal conditions (open squares) and with PBPMN infected with *Pseudomonas aeruginosa* preincubated either with (dark grey squares) or without *Lactobacillus plantarum* (light grey squares). Boxes refer to the 25th (bottom) and 75th (top) percentiles, and the median is the horizontal line inside; fences refer to maximum and minimum values and open circles represent outliers. Significantly lower than the other basal values: $^{\#}p < 0.001$. Significantly higher than the basal values within the same group: $^{*}p < 0.001$; $^{**}p < 0.01$. Significantly lower than *P. aeruginosa* infected alone within the same group: $^{\S}p < 0.001$.

no significant differences among D, DU and NDU groups. Preincubation of PBPMN with *L. plantarum* prior to infection with *P. aeruginosa* inhibited the number of IL-8 positive cells compared to cells that were not preincubated only in the DU and NDU groups ($p < 0.001$). No significant differences were observed between D and DU patients.

Discussion

In the present study, we observed the effects of bacteriotherapy on the modification of the inflammatory response and on infection in chronic venous ulcer leg wounds in diabetic and non-diabetic patients. The wounds showed polymicrobial infections with biofilm-producing bacteria.

The application of *L. plantarum* caused a debridement and granulation of wounds, a decrease both in the bacterial load and in the area of the lesion in a significant percentage of diabetic and non-diabetic patients, with no significant differences between the two groups. Previously, we demonstrated that *L. plantarum* antagonized *P. aeruginosa in vitro* by inhibiting the synthesis of quorum-sensing signals, biofilm formation and virulence factors. In an *in vivo* mouse experimental model, *L. plantarum* decreased the infective capacity of *P. aeruginosa*, enhancing the phagocytic activity and inhibiting the apoptosis of PMN [9]. Other studies have shown the same activity of *Lactobacillus fermentum RC-14* on *S. aureus* infection [8].

Lactobacillus plantarum treatment decreased apoptosis and necrosis of neutrophils and promoted the appearance of fresh PMN and other cells (i.e. fibroblasts and endothelial) in the ulcer bed. These cells were viable and exhibited changes in IL-8 production. A decrease in *P. aeruginosa*-induced IL-8 production in PBPMN was only seen in patients with ulcers.

At present, we have no explanation for the differences observed between individuals with and without ulcers. The greater percentage of basal IL-8 in PBPMN found in diabetics and patients with ulcers with respect to normal subjects would predispose the former to inflammation.

Although it has been well established that, in diabetics, basal inflammation states are increased, data on the production of IL-8 and wound healing are contradictory [12]. We found no significant differences in the behaviour of the CUB and PBPMN in diabetics and non-diabetics, most likely because the former did not have advanced macro- and/or microvascular complications.

The *L. plantarum* inhibition of IL-8 induced by *P. aeruginosa* in PBPMN is in accordance with the effects observed in human leukocytes when stimulated *in vitro* with *P. aeruginosa* or *L. plantarum*. *Lactobacillus plantarum* preferentially induces IL-12 and tumour necrosis factor-α, whereas *P. aeruginosa* preferentially induces IL-10 and IL-6 [10]. Bacteria may have intra- and interspecies communication for synergistic or actions [13], as has been demonstrated in habitats such as the gut and vagina [14] and the lung in cystic fibrosis [15].

On the basis of these and previous findings obtained in experimental models, we hypothesize that the antipathogenic and biofilm-disrupting activity of *L. plantarum* treatment regulates IL-8 levels and modulates the entry and activity of the newly-arrived PBPMN to the ulcer bed. A decrease in apoptosis and necrosis and a more effective phagocytosis would decrease the number of bacteria, the debris and the inflammatory response. Consequently, the number of PMN would decrease and would promote an increase in macrophages and fibroblasts, as well as tissue repair processes.

The importance of this treatment lies in the easy access to and application of *L. plantarum*, its efficacy, innocuousness and low cost. In Argentinian hospitals, treatment is free and low budgets make costly treatments impossible. Because the present study comprises a first approach to the subject, with the limitation of the absence of comparator groups and the small sample size, further studies investigating the treatment of chronic infected wounds with *L. plantarum* are warranted.

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Transparency Declaration

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