ORIGINAL ARTICLE 10.1111/j.1469-0691.2009.02793.x

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.

Keywords: Bacteriotherapy, interleukin-8, lactobacillus, polymorphonuclear, ulcers
Original Submission: 13 July 2008; **Revised Submission:** 15 September 2008; **Accepted:** 21 November 2008
Editor: G. Greub
Article published online: 9 June 2009
Clin Microbiol Infect 2010; **16**: 281–286

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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).