Antipathogenic properties of *Lactobacillus plantarum* on *Pseudomonas aeruginosa*: The potential use of its supernatants in the treatment of infected chronic wounds

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Manuscript received: July 11, 2011
Accepted in final form: February 27, 2012

DOI:10.1111/j.1524-475X.2012.00798.x

ABSTRACT

Pathogenic bacteria delay wound healing through several different mechanisms such as persistent production of inflammatory mediators or maintenance of necrotic neutrophils, which release cytolytic enzymes and free oxygen radicals. One of the most frequent pathogens isolated from infections in chronic wounds is *Pseudomonas aeruginosa*. This bacterium is extremely refractory to therapy and to host immune attack when it forms biofilms. Therefore, antibiotics and antiseptics are becoming useless in the treatment of these infections. In previous works, we demonstrated that *Lactobacillus plantarum* has an important antipathogenic capacity on *P. aeruginosa*. The aim of the present work was to elucidate the mechanism involved in the control of growth of *P. aeruginosa* on different surfaces by *L. plantarum*. For this purpose, we investigated the effects of *L. plantarum* supernatants on pathogenic properties of *P. aeruginosa*, such as adhesion, viability, virulence factors, biofilm formation, and quorum sensing signal expression. *L. plantarum* supernatants were able to inhibit pathogenic properties of *P. aeruginosa* by a quorum quenching mechanism. The antipathogenic properties mentioned above, together with the immunomodulatory, tissue repair, and angiogenesis properties in the supernatants of *L. plantarum*, make them an attractive option in infected chronic wound treatment.

Chronic wounds are a significant cause of morbidity and mortality for many patients and therefore constitute a serious clinical concern. Multiple factors such as infection, ischemia, advanced age, malnutrition, and diabetes have been identified as contributors to impaired wound healing.[1] With regard to infection, pathogenic bacteria delay wound healing through several different mechanisms such as the induction of a persistent production of inflammatory mediators in the host, metabolic wastes and toxins, or inducing necrosis in neutrophils, which release cytolytic enzymes and free oxygen radicals. Therefore, this prolonged inflammatory response contributes to host injury and delays healing.[2]

One of the most frequent pathogens isolated from chronic infections is *Pseudomonas aeruginosa*, a gram-negative opportunist organism.[3] Chronic infections by *P. aeruginosa* cause alterations in the immune response, inflammation, and wound healing processes.[4] Biofilm and virulence factor formation are the main causes of *P. aeruginosa* participation in recalcitrant infections.[5] Cells within a biofilm are usually enmeshed in an extracellular matrix produced by the microorganism and the host. This matrix is a complex mixture of exopolysaccharides, proteins, and DNA.[6] *P. aeruginosa* virulence determinants include cell-associated (lipopolysaccharide endotoxin, flagellum, and pili) and extracellular (alginate, exotoxin A, exoenzyme S, rhamnolipids, pyocyanin, elastase, etc.) factors. It has been demonstrated that expression of these virulence factors and biofilm formation are regulated by a cell-density-dependent signaling mechanism known as quorum sensing.[7] This system has two components, las and rhl, and uses two autoinducers, N-(3-oxododecanoyl)-L-homoserine lactone (3-O-C12-AHL) and N-butyryl-L-homoserine lactone (C4-AHL), respectively.[8] *P. aeruginosa* cells in biofilm are shielded from host defenses such as phagocytes or antibodies, as well as from antibiotics and antiseptics.[9] Therefore, antibiotics and antiseptics are becoming useless in the treatment of these infections. Then, the need for novel antimicrobial agents with antipathogenic properties is clear and imminent.[2]

Extracts or supernatants from *Lactobacilli* cultures have been used for their medicinal effects, including wound healing, angiogenic, and immune system stimulating activity.[10] Besides, in previous works, we demonstrated that *Lactobacillus plantarum* has an important antipathogenic capacity on *P. aeruginosa*.[11,12] We also demonstrated that their supernatants are not cytotoxic and neither induce apoptosis (in vivo and ex vivo) in polymorphonuclears (PMNs) (key cells in a wound) compared with acetic acid or antiseptics typically used in the treatment of these infections.[13,14] Topical

treatments with *L. plantarum* whole cultures are currently being carried out by our medical team with infected burns and chronic venous ulcers in humans[14] with encouraging results.

The purpose of the present study was to investigate whether *L. plantarum* supernatants (Lps) might interfere with *P. aeruginosa* biofilm formation. We particularly focused our work on bacterial adhesion, cell viability, the expression of virulence factors and quorum sensing signals, and biofilm physicochemical characteristics. The final aim was to elucidate the mechanism involve in the interaction between Lps and the development of *P. aeruginosa* as sessile communities in a wound in order to establish the feasibility of using Lps in local treatment of infected chronic wounds.

MATERIALS AND METHODS

Bacterial strains, plasmid, and growth conditions

P. aeruginosa (standard clinical isolate) was grown for 12 hours at 37 °C in Luria–Bertani (LB) medium (Gibco, Rockville, MD). *L. plantarum* ATCC 10241 was grown for 12 hours in Man Rogosa Sharpe medium (MRS broth, Britania, Buenos Aires, Argentina) at 37 °C. *Agrobacterium tumefaciens KYC55* was used as biosensor of acyl-homoserine lactones (AHLs).[15] This strain carries the plasmids (pJZ372)(pJZ384)(pJZ410) and produces a blue color in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in response to the AHLs with N-acyl chain length from 4 to 12 carbons. *A. tumefaciens* biosensor strain was grown at 28 °C in AB solid medium[16] (1.2% agar) supplemented with gentamicin 20 μg/mL when required. *Bacillus subtilis* ATCC 6633 used in the rhamnolipid inhibition test was grown for 12 hours at 37 °C in LB medium.

Supernatant preparation

P. aeruginosa supernatants (Pas) obtained from 12-hour culture in LB broth (stationary phase culture with the highest concentration of AHLs) were recovered by centrifugation (10,000 × *g* for 15 minutes) and filtration through 0.22-μm pore size membrane filters. Lps were recovered from 12-hour culture in MRS broth, centrifuged, and filtered under the same conditions. pH values for Pas and Lps were 6.33 ± 0.27 and 5.22 ± 0.43, respectively. Aliquots of Lps were neutralized with 8 M NaOH to obtain neutralized Lps. A mixture with equal volumes of Pas and Lps was prepared and incubated for 48 hours at 37 °C (Pas-Lps) to evaluate the effect of Lps on *P. aeruginosa* signal molecules.

Lactic acid solutions

Taking into account the mean pH value and lactic acid (LA) concentration of *L. planturum* supernatants, a LA solution of 130 mM was prepared. The pH was regulated with NaOH 8 M to 5.22. The LA concentrations in Lps were determined from three *L. plantarun* independent cultures using a commercial kit (Test-Combination D-LA/L-LA UV-method, Boehringer Mannheim GmbH, Mannheim, Germany).

Extraction, analysis, and detection of AHLs

Extraction

Pas, Lps, and Pas-Lps were extracted two times, with dichloromethane (relation 7 : 3 vol:vol); each extraction was pooled and dried with anhydrous $MgSO_4$. Extracts were filtered with Whatman grade No. 1 filter paper and subjected to analytical thin-layer chromatography (TLC), gas chromatography and mass spectrometry (GCMS), and Fourier-transform infrared (FT-IR) spectroscopy.

AHL standards

AHLs were kindly supplied by M. Arena (University of Tucumán, Argentina). The standards used were C4-, C6-, C8-, and C12-AHL and their 3-oxo derivatives. The standards were stored dried at −20 °C. Standard stock solutions were prepared in ethyl acetate (high performance liquid chromatography [HPLC] grade).

TLC

Standards and supernatant extracts (1–4 μL) were applied to C18 reversed-phase TLC plates (200-mm layer; Baker, Buenos Aires, Argentina) and resolved using a solvent system methanol/water (60 : 40, vol/vol). Then, the solvent was evaporated, and the dried plates were overlaid with an overnight culture of *A. tumefaciens* KYC55 diluted 1/10 with fresh AB medium containing 1.12 g of melted agar and X-gal (100 μg/mL) in a Plexiglas jig designed to produce a uniform layer of agar (approximately 3 mm thick). After solidification, coated plates were incubated at 28 °C for 12–18 hours in a closed plastic container. AHLs were visualized by the appearance of blue spots. A tentative identification of AHLs was performed by comparing the *Rf* values of the samples and AHL standards.

GCMS

GCMS was used to detect the presence of AHLs according to Hong-Sink Moon procedure.[17]

FT-IR spectroscopy

Pas, Lps, and Pas-Lps were analyzed by FT-IR spectroscopy applying the film technique described by Helm et al.[18] Briefly, 100 μL of concentrated extracts (1 : 500) was transferred to ZnSe optical plates (13 mm diameters) and dried under moderate vacuum (0.1 bar) for 45 minutes to obtain transparent films. FT-IR spectra were acquired between 4,000 and 650/cm with 6/cm spectral resolution and 64 scans using a Spectrum One FT-IR spectrometer (PerkinElmer Instruments, Ettlingen, Germany). OPUS software (version 4.2; Bruker Optics GmbH, Ettlingen, Germany) and PerkinElmer software were used for data preprocessing. The band assignment of the vibrational modes corresponding to lactone carbonyl present in AHLs was obtained from Chhabra et al.,[19] who assigned the peaks at 1,785 and 1,774/cm to the carbonyl of the lactone group of 3-O-C12-AHL and C4-AHL, respectively.

Biofilm culture systems

P. aeruginosa biofilm formation was studied using continuous-flow chambers with either borosilicate slides or

ZnSe windows as substrates, which allowed monitoring of the biofilm structure and chemical composition in situ without disruption.[20] On the other hand, to evaluate the Lps disruption capacity on *P. aeruginosa* biofilm, a continuous-flow system using silicone hoses as substrate was used. To estimate the effect of Lps on formation, viability, and chemical composition of *P. aeruginosa* biofilm, a batch system in glass tubes filled with polypropylene beads was used.

For continuous-flow systems, either 0.08-mm-thick glass coverslips (20 mm × 20 mm in size) or ZnSe optical plates (13 mm × 2 mm, Korth Kristalle, Kiel, Germany) were used as growth surfaces. Both types of surfaces were introduced in respective 20-mL continuous-flow chamber systems. For each assay, biofilm culture chambers were inoculated with 2 mL of a *P. aeruginosa* planktonic cell culture (grown for 15 hours in LB at 37 °C). Bacteria were allowed to attach for 4 hours at 37 °C prior to the flow being initiated.[21] The sterile LB medium pumped through the flow chambers with a peristaltic pump (Apema Ind., Buenos Aires, Argentina) at a flow rate of 0.1 mL/min was continuously stirred and aerated at 37 °C. Surfaces with grown bacteria were withdrawn from the chambers at 12-, 24-, 36-, and 48-hour time points and softly washed with distillate water. Biofilms produced on coverslips were examined by optical microscopy after staining with crystal violet. The ones produced on ZnSe were analyzed by FT-IR spectroscopy (see FT-IR spectroscopy and data analysis).

In the case of biofilms produced on silicone hoses, every 12 hours a piece of 8 cm was cut and the biofilm adhered to it was removed and gently washed in a Petri dish with distillate water and the biomass produced was quantified by crystal violet staining procedure. The first 24 hours, sterile LB medium was pumped through the hoses with a peristaltic pump at a flow rate of 0.1 mL/min and then begun pumping a mixture LB-Lps (4 : 1) until 48 hours. A control curve was performed by pumping LB for 48 hours.

For batch systems, biofilms were grown on flat polypropylene beads (300 g; $f = 4.2$ mm, $h = 1.5$ mm, with an average density of 0.901 g/cm^3, Petroken, Buenos Aires, Argentina) in glass tubes ($f = 2$ cm, $h = 10$ cm, IVA [Industria Vidriera Argentina SA], Buenos Aires, Argentina). To elucidate the effect of Lps on biofilm development either from the beginning of the process (adhesion) or after 24 hours of biofilm growth, Lps, neutralized Lps, LA solution (130 mM, pH 5.22), and saline solution (NaCl, 0.85%) were added to LB medium (1 : 4). A planktonic culture of *P. aeruginosa* (100 µL, OD650 = 1.0) grown in LB medium was used as inoculums for each tube. Biofilm cultures were produced in three independent replicates. Tubes were incubated agitated (200 r.p.m.) at 37 °C for different time periods. At point intervals (every hour, the first 24 hours and then every 6 hours until 72), tubes were drained and washed, and the adhered biomass was removed by gently agitation as previously described by Bosch and colleagues.[21] Biofilm development was therefore monitored by viable-cell counting, crystal violet staining method, and FT-IR spectroscopy. Planktonic viability was assessed in the supernatant by counting colony-forming units per milliliter (CFU/mL).

FT-IR spectroscopy and data analysis

ZnSe windows were removed from the continuous-flow chambers and deposit in Petri dishes with distillate water, gently washed three times to remove culture medium and nonadhered cells. Then, each window was dried at 40 °C for 45 minutes and transparent films for suitable FT-IR measurements[20,22] were obtained. FT-IR absorption/transmission spectra from 4,000 to 600/cm were acquired with a FT-IR spectrometer (Spectrum One, PerkinElmer) with 6/cm spectral resolution and 64 scan co-additions. To avoid interference with spectral water vapor bands, spectra were obtained under a continuous purge of dried air.[20,22] Three independent experiments were performed. In each experiment, one ZnSe window was removed from the chambers at 12-, 24-, 36- and 48-hour growth). Spectroscopic quantification of proteins and polysaccharides was determined by means of the corresponding band-area calculations: protein content was evaluated from the amide II band area (1,590–1,480/cm) and carbohydrate content from the carbohydrate band area (1,150–980/cm). Derivation and band area calculations were carried out by means of OPUS 4.0 (Bruker Optics GmbH) and PerkinElmer software (Spectrum 3.0).

Sessile cells from 8-cm hoses or from polypropylene beads were gently washed twice with distillate water, removed by vigorous stirring (vortex) for 5 minutes in 2.5 mL distilled water, and then were pelleted by centrifugation (8,000 *g* for 20 minutes at 15 °C). Both the washed biomass obtained from the hose peaces or from polypropylene beads were transferred to a ZnSe optical plate and dried in vacuum to obtain transparent films.[20] Absorption spectra were recorded as mentioned before.

Quantification of biofilm formation

Crystal violet staining

In the case of the biomass formed in the 8-cm hose, sessile cells were evaluated after 12- and 24-hour (biofilm growth) and after 36- and 48-hour (biofilm disruption). In the case of the biomass formed on polypropylene beads, sessile cells were evaluated in all time intervals (every hour, the first 24 hours and then every 6 hours until 72 hours). The adhered biomass was rinsed twice with phosphate-buffered saline (PBS) to remove unattached cells. Subsequently, sessile cells adhere to the hoses or the propylene beads were fixed for 20 minutes at 80 °C and stained by addition of 0.1% crystal violet (w/v) for 5 minutes. After exhaustive washing, 7 mL of decoloring solution consisting of ethanol/acetone (80 : 20) was added, and the mixture was incubated for 15 minutes to solubilize the stain. The absorbance of the eluted stain was measured at 590 nm (A590).

Molecular probe assay kits for cell viability

Live/dead bacteria in biofilms produced on polypropylene beads were detected using the Live Syto 9/Dead IP kit (BacLight Molecular Probes kit L-13152, Invitrogen, Buenos Aires, Argentina). Membrane-permeant Syto 9 dye labels live bacteria with green fluorescence, while the membrane-impermeant propidium iodide labels membrane-compromised bacteria with red fluorescence. In order to establish the percentage of living cells in a population by means of this technique, a calibration curve was constructed (see Kit L-13152 protocol). The percentage of live bacteria in a sessile cell suspension was calculated by extrapolation in the calibration curve.

CFU

Viable cell count determinations for planktonic cells in the supernatants were performed in the experiments carried out with polypropylene beads in glass tubes. Aliquots of the supernatants of each tube were taken and measured the pH and CFU/mL (successive dilution method).

Inhibition tests of Lps on *P. aeruginosa* virulence factors

Indirect inhibition tests

A mixture of LB-Lps (4 : 1) was inoculated with 100 µL of an overnight *P. aeruginosa* culture (LB) and incubated 12 hours at 37 °C. A control assay was carried out in parallel with a mixture LB-NaCl 0.85% (4 : 1).

Direct inhibition tests

A *P. aeruginosa* 12-hour supernatant was mixed with Lps and NaCl 0.85% (control) (4 : 1) and incubated for 1 hour at 37 °C.

Supernatants of both types of assays were used to measure elastase, pyocyanin, and rhamnolipids as described below. All tests were performed in triplicate.

Elastase

The elastolytic activity of supernatants was investigated using elastin Congo red (Sigma, St. Louis, MO) as substrate in elastin Congo red (ECR) buffer.[23] After 24-hour incubation of the supernatants with Congo red at 37 °C, the insoluble elastin Congo red was removed by centrifugation, and the supernatant absorbance was measured at 495 nm.

Pyocyanin

Supernatants were mixed with 1 : 1 with chloroform, agitated, and the lower organic layer was separated after. Two milliliters of HCl (0.2 M) was added to the organic phase and the pyocyanin-rich organic layer was separated, and the concentration was determined by measuring the absorbance at 520 nm.[24]

Rhamnolipids

For the detection of rhamnolipids, two independent tests were carried out. These included the hemolysis of erythrocytes by rhamnolipids[25] and the growth inhibition of *B. subtilis* exerted by rhamnolipids.[25] Supernatants were concentrated as follows: The culture supernatant pH was adjusted to 6.5 and $ZnCl_2$ was added to a final concentration of 75 mM.[25] The precipitated material was dissolved in 10 mL of 0.1 M sodium phosphate buffer (pH 6.5) and extracted twice with an equal volume of diethyl ether. The pooled organic phases were evaporated to dryness, and the pellets were dissolved in 100 µL of methanol. Concentrated culture supernatants were spotted onto paper filter discs (6.0-mm Whatman AA discs, Eurolab, Buenos Aires, Argentina), which were then put onto a layer of LB soft agar containing freshly grown *B. subtilis* cells (approximately 10^9/mL) or onto agar plates containing 5% sheep blood (Becton Dickinson, Buenos Aires, Argentina). Typically, 10 mL of a culture supernatant was concentrated down to 100 µL, and 10 µL of aliquots was applied to the paper discs. After incubation of the *B. subtilis* plates at 37 °C overnight and the blood agar plates at room temperature for 2 days, the corresponding inhibition halos and hemolysis were measured.

Statistics

The *t*-test was used for statistical analysis. $p < 0.05$ was considered statistically significant.

RESULTS

Lactobacillus supernatant (Lps) effect on quorum sensing signals

If AHL-destroying enzymes (lactonase and acylases) are present in Lps, incubation of the mixture (Pas-Lps) for an extended period of time (48 hours at 37 °C) would ensure the total destruction of AHLs present in Pas. However, by using TLC, GCMS, and FT-IR spectroscopy, C4-AHL and 3-O-C12-AHL molecules in Pas and Pas-Lps mixture solution were detected (Figure 1 and Table 1). This would suggest that Lps has no destructive effect on AHLs.

Lactobacillus supernatant effect on *P. aeruginosa* biofilms

Biofilm formation

P. aeruginosa biofilm formation was studied using continuous-flow chambers with either borosilicate slides or ZnSe windows as substrates, which allowed monitoring of the biofilm structure and chemical composition in situ without disruption. When FT-IR samples of the biofilm formed on ZnSe optical plates were analyzed, peaks corresponding to those identified as characteristic of a *P. aeruginosa* mucoid strain (Figure 2) were found in all time point spectra (12, 24, 36, and 48 hours). This strain produced biofilm with characteristic bands near 1,650 (amide I), 1,550 (amide II), 1,240 (P=O stretching, C–O–C stretching, and/or vibration of amide III), 1,100–1,000 (C–OH and P–O stretching), 1,450, and 1,400/cm. Besides the *P. aeruginosa*, clinical isolates used spectra showed a relatively higher absorbance at 1,060 (C–OH stretching of alginate) and 1,250/cm (C–O stretching of O-acetil group in alginate) as described by Nivens et al.[26]

Biofilms produced on coverslips were examined by optical microscopy after staining with crystal violet. The structures found at different culture times on the coverslips corresponded to the different stages of biofilm formation: adhesion, microcolonies, and mature biofilm (Figure 3).

Biofilm disruption

Figure 4 shows the biomass curve measured with crystal violet on 8-cm hose. The control curve shows a normal

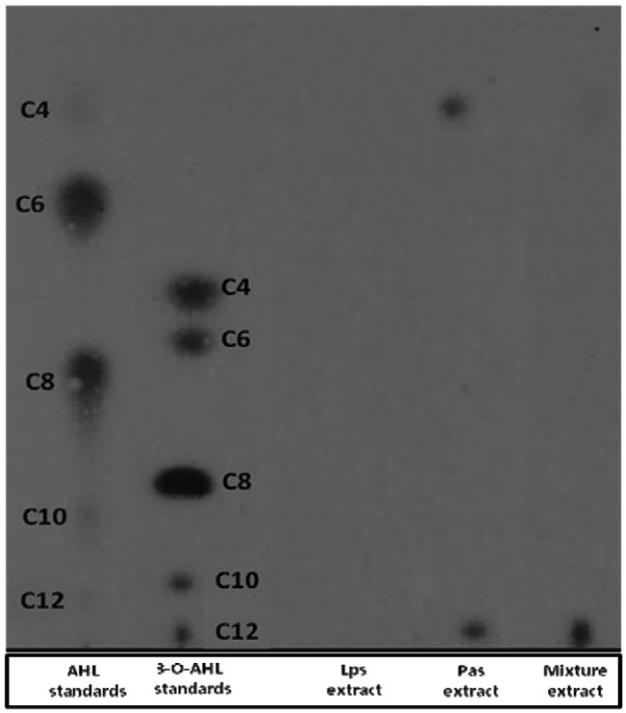

Figure 1. C18 reversed-phase TLC plates (200-mm layer; Baker), the chromatograms were developed with methanol/water (60 : 40, vol/vol) and revealed with *Agrobacterium tumefaciens* KYC55 bioassay. C4-AHL (*Rf* = 0.76) and 3-O-C12-AHL (*Rf* = 0.08) were present in *Pseudomonas aeruginosa* supernatant extracts and mixture extracts. AHL, acyl-homoserin lactone; Lps, *Lactobacillus plantarum* supernatant; Pas, *Pseudomonas aeruginosa* supernatant; TLC, thin-layer chromatography.

evolution of biomass formation in the hose while the curve with Lps represents a kinetics of biofilm disruption. As it is shown in this curve, there is a steep slope of biofilm formation in the first 12 hours. In the second period (12–24 hours), the slope was decreased because of hose saturation. Thereafter (when start pumping LB/Lps 4 : 1), in the third period (24–48 hours), a significant decrease in biomass in the hose was observed (this not occur in the control curve). This could be due to the presence of Lps in the mix pump and indicate destabilization and subsequent disruption of the biofilm matrix.

When we analyzed by FT-IR samples of biofilm formed on the hoses (before and after the addition of Lps), we found in all cases peaks corresponding to those identified as characteristic of a *P. aeruginosa* mucoid strain as mentioned before (data not shown). However, despite biomass decreased, chemical composition of the biofilm remained intact. This was demonstrated by means of the corresponding peak ratios especially those associated with the matrix components such as DNA or alginate. This would indicate that biofilm disruption is not based in the destruction of a particular component of the matrix. It is hypothesized that the Lps destabilizes the entire biofilm matrix, producing a subsequent lack of adhesion and biofilm detachment.

Biofilm inhibition

Figure 5 shows the kinetics of biomass formation measured with crystal violet on polypropylene beads in batch cultures. In this figure, the normal kinetics (with 0.85% NaCl) with those produced with the addition of LA solution (130 mM pH 5.22), Lps, and neutralized Lps was compared.

In all cases, four well-defined periods were observed. In the first period, which lasts approximately 5 hours, the cells are adhering to the polypropylene beads. During this time, the lowest percentage of adherence compared with normal kinetics was obtained with Lps (40–60%), followed by neutralized Lps (65–75%), and finally with LA solution that begins and ends this period with the same percentage (80%). In the second period, which was called latency (Figure 5), an increase in biomass with time was not observed. This could be due to phenotypic transformation experienced by bacteria to switch to a biofilm mode of growth. This phase lasted 3–4 hours in normal kinetics and was extended to 5–6 hours in the presence of neutralized Lps or Lps, respectively. This would indicate that in the supernatants of *L. plantarum*, some factor that interferes with normal phenotypic transformation might be present. The third period corresponds to biofilm formation represented as biomass increase on the beads, reaching bead saturation in the fourthperiod. The growth rate in the third period was measured by the curve slopes. The steeper slope

Table 1. Detection of *Pseudomonas aeruginosa* quorum sensing signals in Lps, Pas, and Pas-Lps extracts. Results obtained with different methodologies. TLCs were revealed with *Agrobacterium tumefaciens*KYC55 bioassay. Positive (+) means that the molecule was found by the method. Negative (–) means that the molecule was not found. Positive/negative (+/–) means that the molecule was found only in some samples

	TLC		GCMS		FT-IR	
Sample	C4-AHL	3-O-C12-AHL	C4-AHL	3-O-C12-AHL	C4-AHL	3-O-C12-AHL
Lps extract	–	–	–	–	–	–
Pas extract	+	+	+	+	+	+
Pas-Lps extract	+	+/–	+	+/–	+	+

AHL, acyl-homoserin lactone; FT-IR, Fourier-transform infrared; GCMS, gas chromatography and mass spectrometry; Lps, *Lactobacillus plantarum* supernatant; Pas, *Pseudomonas aeruginosa* supernatant; TLC, thin-layer chromatography.

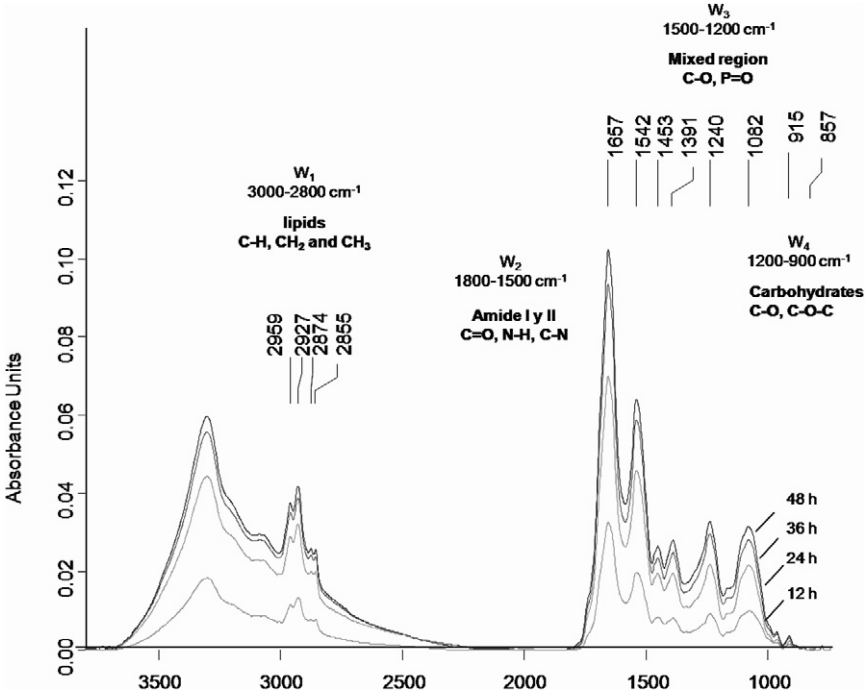

Figure 2. Fourier-transform IR absorption/transmission spectra of *Pseudomonas aeruginosa* sessile cells recovered from polypropylene beads at different time points during biofilm growth (12, 24, 36, and 48 hours). The main important windows of the whole IR spectra and a tentative assignment of the bands corresponding to the main functional groups in biomolecules are indicated.[23] IR, infrared.

was obtained with normal kinetics (0.296) (Figure 5) and reached saturation within 24 hours. LA solution had a lower slope (0.260) and saturation was delayed until 72 hours. The slopes with the supernatants of *L. plantarum* (Lps and neutralized Lps) were markedly lower (0.124 and 0.145, respectively) and never reached the saturation of the beads (Figure 5). This could be seen more clearly when the percentage of biomass with respect to normal kinetics in each time was calculated. In the presence of Lps at 12 hours, only 51% of the normal biomass formed, reaching 56% at 72 hours. With neutralized Lps, the values range from 60% at 12 hours to 63% at 72 hours. Instead, with LA solution, the curve was more similar to normal with values ranging from 75% at 12 hours to 95% at 72 hours. Apparently, this partially could be related to the supernatant pH because there was lower adhesion and biofilm formation, while the supernatant was more acidic. The pH values of supernatants can be seen on the curves in Figure 5. In addition, it was found that cells exposed to the LA solution produced pyoverdin after 48 hours of incubation (appearance of green fluorescence pigment in the supernatant). Because of this, it was deduced that the LA solution is more stressful for *P. aeruginosa* than Lps.

When analyzing the FT-IR spectra of biofilm removed from polypropylene beads at various times, it was noted that the characteristic peaks of biofilm of *P. aeruginosa* mucoid strain are still present and the ratio between peaks remained unchanged in the presence of Lps (data not shown). When performing the computational analysis of the spectra, it was

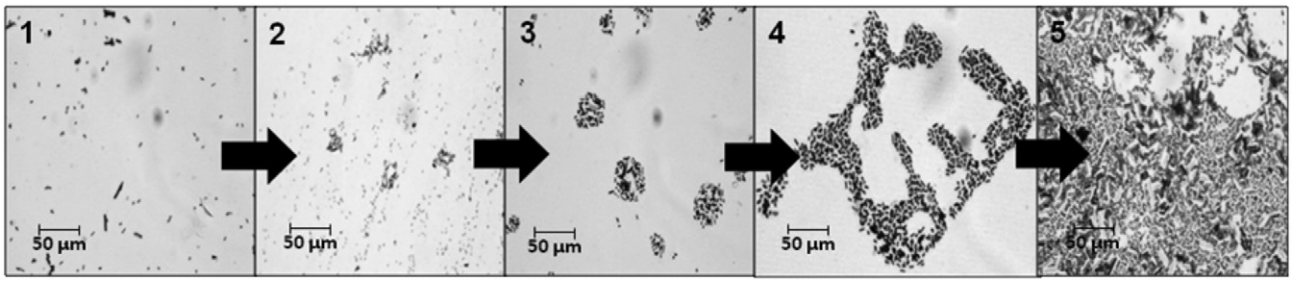

Figure 3. Biofilm development stages on coverslips. The different stages of biofilm formation are indicated: (1) adhesion; (2) microcolony formation; (3) microcolonies; (4) biofilm structure formation; and (5) mature biofilm.

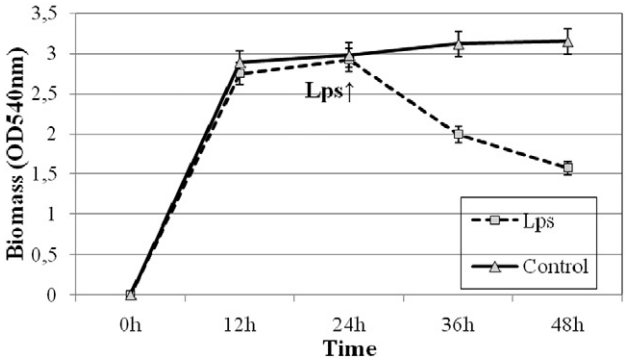

Figure 4. Biofilm growth curve of *Pseudomonas aeruginosa* on silicone hoses cultured under continuous flow rate. Lps:LB (4 : 1) was pumped after 24 hours of growth. LB, Luria–Bertani; Lps, *Lactobacillus plantarum* supernatant.

concluded that bacterial growth (represented by the peaks of amide I and II) and biofilm (represented by the peaks of alginate) were expected. This means that mature biofilm was obtained in each sample and time. However, in Lps spectra, the absorbance units of each peak were smaller than the corresponding normal kinetic spectra (data not shown). This would indicate that mature biofilm was formed in the presence of Lps but in smaller amounts. That is, Lps only inhibits biofilm formation without affecting the chemical composition of its matrix. The spectra obtained in the presence of neutralized Lps and LA solution showed less inhibition (data not shown).

Lps effects on cell viability

Biofilm cell viability

Figure 6 shows the kinetics of biofilm live bacteria (%) measured with Syto 9-PI on polypropylene beads in batch culture. The higher bacterial mortality was produced by the LA solution, reaching 81.4% of live bacteria at 48 hours. The solution of LA decreased the viability of *P. aeruginosa* regardless of its acidity. On the other hand, in the presence of Lps, the percentage of live bacteria was lower when the pH decreased. Besides, the kinetics of biofilm live bacteria produced with neutralized Lps was very similar to normal kinetics (0.85% NaCl), which could indicate that the death caused by Lps was due solely to its acidity.

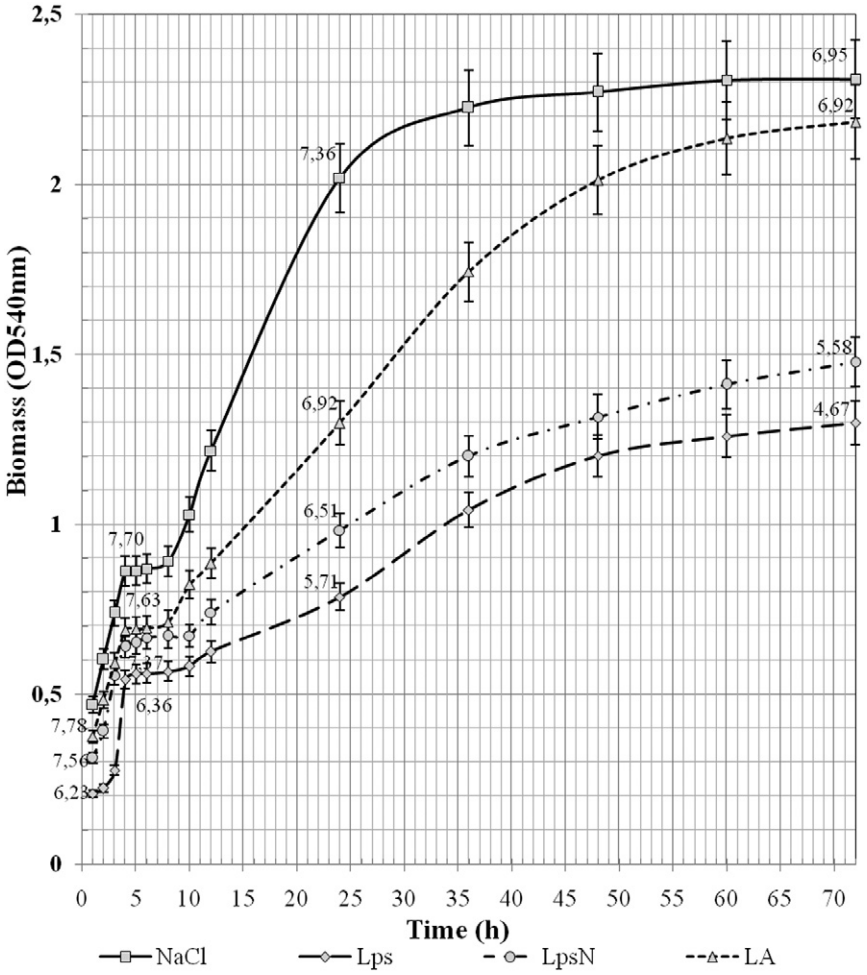

Figure 5. Kinetics of adhesion and biofilm formation on polypropylene beads in a batch culture in the presence of NaCl 0.85% (normal kinetics), lactic acid (LA) solution, Lps, and Lps neutralized (LpsN). The supernatant pH is indicated above each curve. Lps, *Lactobacillus plantarum* supernatant.

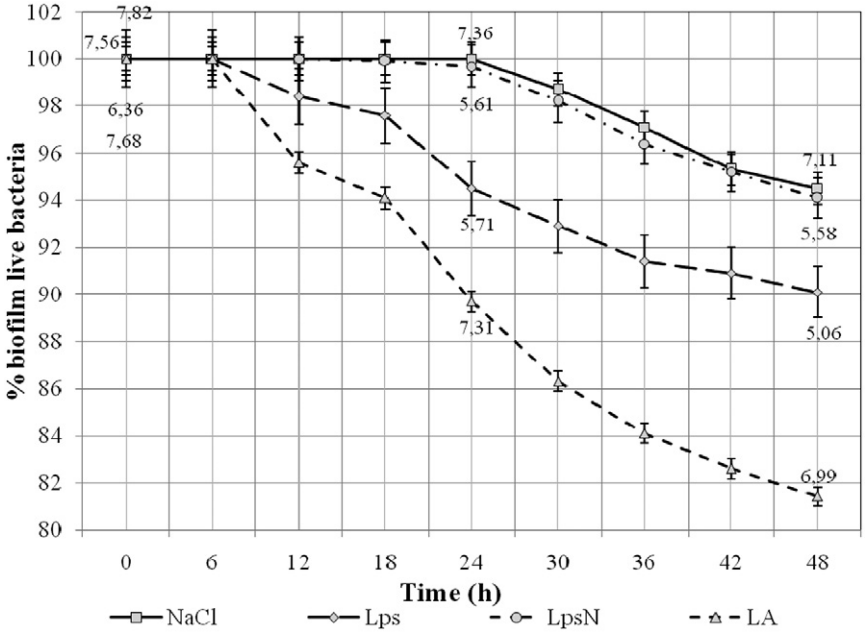

Figure 6. Kinetic of bacterial viability in biofilm. The viability of biofilm bacteria on polypropylene beads in a batch culture in the presence of NaCl 0.85% (normal kinetics), lactic acid (LA) solution, Lps, and Lps neutralized was measured by fluorescent staining with Syto 9-PI. The numerical values observed on each curve correspond to supernatant pH. Lps, *Lactobacillus plantarum* supernatant; LpsN, Lps neutralized.

In all cases, the loss of viability was more pronounced during the so-called third period of the biofilm growth. This would indicate that inhibition occurs when *P. aeruginosa* is metabolically active.

Planktonic cell viability

The curves CFU/mL vs. time (Figure 7) show the effect of the LA solution, Lps, and neutralized Lps on the viability of *P. aeruginosa* planktonic cells. It was observed that LA solution and Lps have a great growth inhibitory capacity (Figure 7). This means that the low pH of the sample is closely related to the observed inhibitory capacity. However, the low pH does not fully explain this observation as the neutralized Lps retains some of its inhibitory activity. Supernatant pH values were indicated on the curves in Figure 7.

Lps effect on P. aeruginosa virulence factors

Effect on elastase

Table 2 shows that Lps inhibited the production of elastase by 37.5% ($p < 0.01$) (indirect inhibition) and inhibited the elastolytic activity of Pas by 58.9% ($p < 0.01$) (direct inhibition).

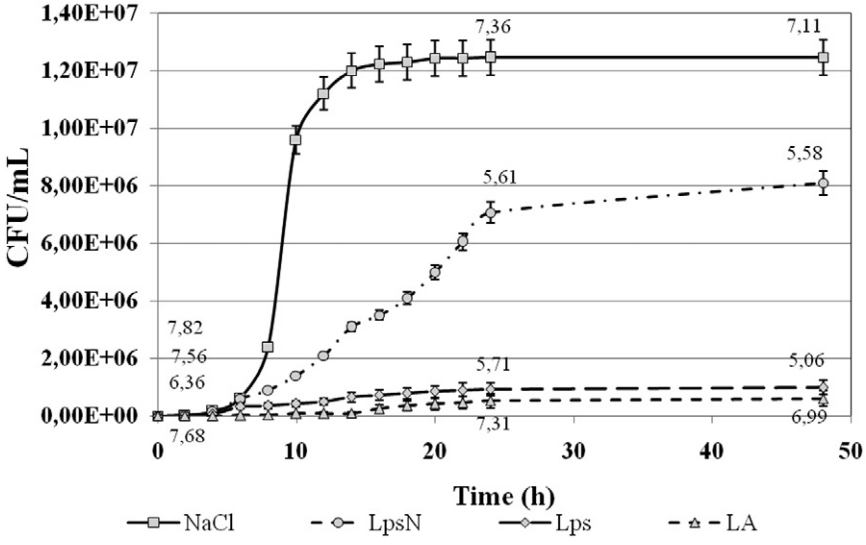

Figure 7. Growth bacterial curves represented by CFU/mL supernatant of batch cultures in the presence of NaCl 0.85% (normal kinetics), lactic acid (LA) solution, Lps, and neutralized Lps. The numerical values observed on each curve correspond to supernatant pH. CFU, colony-forming units; Lps, *Lactobacillus plantarum* supernatant; LpsN, Lps neutralized.

Effect on pyocyanin

Table 2 shows that Lps inhibited the production of pyocyanin by 49.7% ($p < 0.001$) (indirect inhibition) and showed no direct inhibition on it (no significant difference between control and Lps).

Effect on rhamnolipids

Table 2 shows that Lps inhibited significantly ($p < 0.01$) the production of rhamnolipids (indirect inhibition) represented as hemolysis inhibition (sheep red blood cells) of 41.2% and besides as a decrease of *B. subtilis* growth inhibition of 44.0%. Furthermore, no significant direct inhibition was observed.

DISCUSSION

In this work, it was established that Lps does not destroy *P. aeruginosa* signal molecules (C4-AHL and 3-O-C12-AHL). This was demonstrated by biological (TLC bioassays) and chemical (GCMS and FT-IR) assays (Figure 1, Table 1). Therefore, destroying enzymes such as acylases, lactonases might not be present in Lps. However, with this methodology, the lactones were not quantified. Because of this, in previous works, another bioassay with the reporter strain *P. aeruginosa* 129b was carried out.[11,13] This strain has a random lacZ transcriptional fusion in the chromosome of a lasI–rhlI double mutant and was used to quantify the biological activity of AHLs. The mutant strain produces neither 3-O-C12-AHL nor C4-AHL but responds to the presence of these compounds by increasing expression of β-galactosidase.[27] In the direct assay, the reporter strain was stimulated with Pas alone and mixed (vol:vol) with LB, Lps, neutralized Lps, and MRS. In the indirect assay, a culture of *P. aeruginosa* was centrifuged and washed with PBS, and bacteria were resuspended in the different samples above. β-galactosidase activity was quantified by Miller reaction.[28] β-galactosidase expression was decreased in the presence of Lps to a lesser extent in the presence of neutralized Lps and MRS.[11] This implies that the presence of Lps decreases the biological response of *P. aeruginosa* to its AHLs. Therefore, Lps interferes with the AHL biological activity and prevents the normal functioning of *P. aeruginosa* quorum sensing. This process, called quorum quenching, is essential in the antipathogenic activity exerted on *P. aeruginosa*.

By using disruption tests in continuous culture, it was demonstrated that Lps is able to produce disruption of a previously formed *P. aeruginosa* biofilm (Figure 4). However, when comparing biofilm matrix composition with the resulting postdisruption matrix, chemical changes were not detected. Possibly, Lps might destabilize the biofilm substrate adhesion, with the consequent detachment of it. On the other hand, through inhibition assays in batch cultures, it was elucidated the stage at which supernatants of *L. plantarum* act on *P. aeruginosa* biofilm formation. These supernatants inhibited bacterial adhesion and biofilm growth, and even they were able to prolong the latency period (Figure 5). However, the microscopic study of *P. aeruginosa* biofilm in the presence of Lps and neutralized Lps showed structures consistent with the known stages of biofilm formation (bacterial adhesion, microcolonies formation, and mature biofilm). Besides, as in disruption tests, no changes in the chemical composition of the matrix in the presence of Lps were found. Therefore, the supernatants of *L. plantarum* are able to inhibit biofilm formation and even produce the disruption of it, without affecting the matrix chemical composition. The main components of *P. aeruginosa* biofilm matrix are alginate, proteins, and DNA.[6] This allowed discarding the presence of disrupting enzymes as alginases and proteases in the Lps. However, it was recently shown the presence of DNAses in Lps[29] so that this could partially explain the Lps disruption capacity.

In the viability assays, the higher bacteriostatic and bactericidal capacity of the LA solution on *P. aeruginosa* (planktonic and biofilm forms) was demonstrated (Figures 6 and 7). LA solution had the same pH and the same LA concentration than Lps; however, Lps did not showed the bactericidal power and growth inhibitory activity was shown by LA solution. In solution, LA is in its undissociated form and therefore readily crosses the *P. aeruginosa* membrane. In contrast, Lps LA is found partially as lactate and therefore does not pass through the membrane. LA could be considered to be a key antimicrobial compound produced by lactobacilli.[30] The exact mode of action underlying this observed antimicrobial effects of LA has not yet been completely clarified, although there have been cases where virulence factor expressions were affected by LA.[31] Besides exerting its activity through lowering the pH and through its

Table 2. Inhibition assays on the production of virulence factors (indirect inhibition) and inhibition assays of virulence factors once they are produced (direct inhibition)

		Indirect inhibition		Direct inhibition	
Virulence factor		Control	Lps	Control	Lps
Elastase ($OD_{495\,nm}$)		(0.512 ± 0.091)	(0.320 ± 0.075)**	(0.377 ± 0.052)	(0.222 ± 0.070)**
Pyocyanin ($OD_{520\,nm}$)		(0.646 ± 0.076)	(0.325 ± 0.027)***	(0.551 ± 0.099)	(0.543 ± 0.062)
Rhamnolipids	Halo of hemolysis (mm)	(17 ± 2)	(10 ± 1)**	(22 ± 3)	(22 ± 2)
	Halo of *Bacillus subtilis* inhibition (mm)	(25 ± 3)	(14 ± 3)**	(27 ± 2)	(26 ± 1)

Significant difference compared with respective control: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Lps, *Lactobacillus plantarum* supernatant.

undissociated form, LA is also known to function as a permeator of the gram-negative bacteria outer membrane,[32] allowing other compounds to act synergistically with LA. In addition, organic acids such as LA can capture elements essential for growth, such as iron, by their chelating properties.[33]

In this work, an indirect inhibition of virulence factors (elastase, pyocyanin, and rhamnolipids ($p < 0.01$) was observed (Table 2). *P. aeruginosa* regulates the production of these virulence factors by a quorum sensing mechanism and this in turn depends on bacterial growth.[7,34] The supernatants of *L. plantarum* showed the capacity to inhibit both the bacterial growth and the biological activity of *P. aeruginosa* AHLs[11,13] so that it is logical to observe an inhibition in the production of these factors.

In previous works, we showed that Lps is able to decrease ex vivo necrosis and apoptosis produced by supernatants of *P. aeruginosa* on human PMNs.[13] Besides, Lps are capable of inhibiting in vivo inflammatory infiltration in Balb/c mice produced by supernatants of *P. aeruginosa*.[13] Furthermore, Lps proved to be less harmful on PMNs than acetic acid, an antiseptic widely used in our hospitals in the infected chronic wound treatment.[13]

Antipathogenic properties mentioned above, together with the immunomodulatory,[35] tissue repair, and angiogenic properties[14,36] in the supernatants of *L. plantarum*, make it an attractive option in infected chronic wound treatment. In fact, topical treatment with *L. plantarum* whole cultures is currently being carried out by our medical team with infected burns and chronic venous ulcers in humans with encouraging results.[14]

Lps acidity and the presence therein of organic acids such as LA,[36] antimicrobial factors such as hydrogen peroxide (H_2O_2),[36] ethanol,[36] and other molecules,[37] bacteriocins such as plantaricin I and II,[38] and enzymes such as DNAse[27] explain almost all the properties found in the supernatant. However, the Lps factors capable of producing *P. aeruginosa* quorum quenching remain to be elucidated. One possible explanation might be autoinducer type 2 (AI-2) (Interspecies communication) in the presence in the Lps,[39] which could modify the *P. aeruginosa* phenotypic expression or may interfere with the normal functioning of quorum sensing system.

In conclusion, in this work, we demonstrate that Lps are able to reduce the viability of *P. aeruginosa* in both planktonic and biofilm forms. Also, these interfere with normal functioning of *P. aeruginosa* quorum sensing system that inhibits both biofilm and virulence factor (elastase, pyocyanin, and rhamnolipids) formation. In addition, Lps disrupts the *P. aeruginosa* biofilm without affecting its matrix chemical composition and decreases its adhesion to surfaces. Antipathogenic properties mentioned above, together with the immunomodulatory, tissue repair, and angiogenic properties of Lps, show that the use of Lps in the treatment of infected chronic wounds is highly feasible.

ACKNOWLEDGMENTS

This work was financially supported by grant BID OC-AR PICT 1728 2006 No 1458 from the National Agency for Promotion of Science and Technology, Argentina, and Research Council National University of Tucuman CIUNT Program No. 26/D453.

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