Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, Early Online: 1–9
 DOI: 10.3109/13880209.2014.920037

informa
healthcare

ORIGINAL ARTICLE

Compounds from *Lactobacillus plantarum* culture supernatants with potential pro-healing and anti-pathogenic properties in skin chronic wounds

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Abstract

Context: It is necessary to advance the field of alternative treatments for chronic wounds that are financially accessible to the least economically developed countries. Previously we demonstrated that topical applications of *Lactobacillus plantarum* culture supernatants (LAPS) on human-infected chronic wounds reduce the pathogenic bioburden, the amount of necrotic tissue, and the wound area, as well as promote debridement, granulation tissue, and wound healing.

Objective: To study LAPS chemically and biologically and to find potential molecules responsible for its pro-healing and anti-pathogenic properties in chronic wounds.

Materials and methods: (1) *Chemical analysis*: extracts were subjected to a column chromatography and the fractions obtained were studied by GCMS. (2) *Quantification*: DL-lactic acid (commercial kit), phenolic compounds (Folin–Ciocalteu), H_2O_2 (micro-titration), and cations (flame photometry). (3) *Biological analysis*: autoinducers type 2 (AI-2) (*Vibrio harveyi* BB170 bioassay), DNAase activity (Agar DNAase), and *Pseudomonas aeruginosa* biofilm inhibition (crystal violet technique).

Results: According to its biological activity, the most significant molecules found by GCMS were the following: antimicrobials (mevalonolactone, 5-methyl-hydantoine, benzoic acid, etc.); surfactants (di-palmitin, distearin, and 1,5-monolinolein); anesthetics (barbituric acid derivatives), and AI-2 precursors (4,5-dihydroxy-2,3-pentanedione and 2-methyl-2,3,3,4-tetrahydroxytetrahydrofurane). *Concentrations measured* (μg/mL): DL-lactic acid (11.71 ± 1.53) and H_2O_2 (36 ± 2.0); phenolic compounds (485.2 ± 15.20); sodium (370 ± 17); potassium 920 ± 24); calcium (20 ± 4); and magnesium (15 ± 3). DNAase from LAPS had activity on genomic DNA from PMNs and *P. aeruginosa*.

Discussion and conclusion: The molecules and biological activities found in LAPS could explain the observed effects in human chronic wounds.

Keywords

Autoinducers type 2, bacteriotherapy, biofilm, *Pseudomonas aeruginosa*, skin chronic infections

History

Received 20 February 2014
Revised 11 April 2014
Accepted 28 April 2014
Published online 27 October 2014

Introduction

Chronic wounds are a worldwide problem for health systems because they produce large expenditures for hospitalization and treatments (Sen et al., 2009). Therefore, it is necessary to advance the field of alternative treatments that are financially accessible to the least economically developed countries (Greer et al., 2013). Given this, in recent years, our working group has developed a treatment for chronic wounds based on the application of *Lactobacillus plantarum* ATCC 10241 culture supernatants (LAPS).

Chronic wounds by definition are those that remain in a chronic inflammatory state and, therefore, fail to follow normal patterns of the healing process (Guo & DiPietro, 2010). Some factors that may contribute to this problem are diabetes, diseases of the veins or arteries, advanced age, and infections (Guo & DiPietro, 2010). The deleterious effect of microbial infection on wound healing has been recognized for decades and the control of bioburden is accepted as an important aspect of wound management (Wilkins & Unverdorben, 2013). Additionally, there is an increasing evidence that biofilm formation in wounds is the best unifying explanation for the failure of wound healing (Percival et al., 2012). Bjarnsholt et al. (2008) suggested that the lack of proper wound healing is

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in part caused by inefficient eradication of infecting opportunistic pathogens like *Pseudomonas aeruginosa*. They propose a model in which *P. aeruginosa* biofilm (microcolonies) amass at certain locations in every wound. Such microcolonies are capable of producing the polymorphonuclear neutrophil (PMN)-eliminating rhamnolipid, which would reduce the number of functional PMNs and this in turn may also play a beneficial role for additional colonizing bacteria (Bjarnsholt et al., 2008; Kirketerp-Møller et al., 2008). *Pseudomonas aeruginosa* cells within a biofilm are usually enmeshed in an extracellular matrix produced by the microorganism itself. This matrix is a complex mixture of exopolysaccharides (Ma et al., 2009, 2012), proteins (Toyofuku et al., 2012), and DNA derived from lysed cells (Webb et al., 2003). In addition, when the host fails to eradicate the infection, cellular components from necrotic neutrophils (DNA for example) can serve as a biological matrix to facilitate *P. aeruginosa* biofilm formation (Walker et al., 2005). It has been demonstrated that the expression of biofilm and virulence factors in *P. aeruginosa* are regulated by a cell-density-dependent signaling mechanism known as quorum sensing (Smith & Iglewski, 2003). This system has two components, las and rhl, and uses two autoinducers, *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone, respectively (Figure 1; Fuqua et al., 2001).

LAPS interferes with the pathogenic capacity of *P. aeruginosa* inhibiting *in vitro* adhesion, quorum sensing, biofilm, and virulence factors like elastase, pyocyanin, and rhamnolipids (Ramos et al., 2010a, 2012; Valdez et al., 2005).

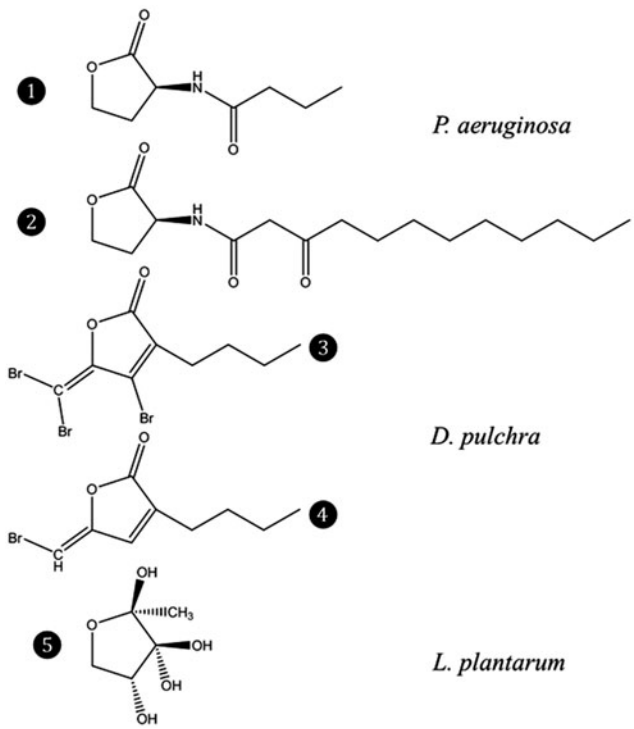

Figure 1. Structural similarity among the acyl homoserine lactones from *P. aeruginosa* (molecules 1 and 2), halogenated furanones from the alga *D. pulchra* (molecules 3 and 4), and the precursor of AI-2 found in the LAPS (molecule 5) is shown. There are several molecules chemically similar to AI-2 that produce quorum quenching in *P. aeruginosa* such as the mentioned halogenated furanones (Hentzer et al., 2003) and precursors of AI-2 (Ganin et al., 2009). Therefore, it is hypothesized that the molecule found in LAPS produces the same effect.

In addition LAPS showed bacteriostatic and bactericide properties and a great biofilm-disrupting capacity (Ramos et al., 2010a, 2012). LAPS are neither cytotoxic nor an inductor of necrosis-apoptosis in PMNs (*ex vivo*) (key cells in a chronic wound) or inflammatory response (*in vivo* in a mouse model), compared with acetic acid or antiseptics typically used in the treatment of these infections (Ramos et al., 2010b). According to the hypothesis of Bjarnsholt et al. (2008), *P. aeruginosa* would be responsible for the chronicity of wound infections and, in turn, it would be the predisposing factor for other infections. If so, treatment with LAPS would be extrapolated to any chronic wound. In fact, topical applications of *L. plantarum* cultures on infected chronic wounds (diabetic foot ulcers, burns, venous ulcers, and pressure ulcers) in humans are currently being carried out by our medical team with encouraging results, since *L. plantarum* reduce or eliminate the pathogenic bacterial load, the amount of necrotic tissue and the wound area, as well as they promote debridement, the appearance of granulation tissue, and wound healing with increased production of TGF-β, IL-8, and IL-8-R (Peral et al., 2009, 2010).

The objective of the present work was to perform a chemical and biological characterization of LAPS. This will provide the basis for the determination of the molecules responsible for the LAPS's anti-pathogenic and pro-healing properties. Besides, based on previously reported properties of the found metabolites, we will provide in the Discussion section, its potential biological targets within a chronic wound bed infected with *P. aeruginosa*.

Materials and methods

Bacterial strains

Lactobacillus plantarum ATCC 10241; *Pseudomonas aeruginosa* (mucoid clinical sample from a chronic wound); *Vibrio harveyi* BB120 (wild-type strain) used as a source of external autoinducers type-2 (AI-2); *Vibrio harveyi* BB170 is a reporter strain, which specifically responds to AI-2 by producing bioluminescence.

Lactobacillus plantarum supernatants (LAPS)

Lactobacillus plantarum was grown in MRS (Britania) broth 12 h at 37 °C. Supernatants were obtained by centrifugation (20 min, 10 000 rpm) and filtration (0.22 µm Millipore filter, Millipore Corporation, Billerica, MA). LAPS (pH 5.22 ± 0.43) was used to perform all assays mentioned below. Aliquots of LAPS were neutralized with 8 M NaOH (NLAPS).

Concentration of several components of LAPS

DL-Lactic acid

A commercial D- and L-lactic acid determination kit (Boehringer Mannheim GmbH, Mannheim, Germany) was used to determine the concentration of lactic acid in LAPS.

Phenolic compounds

The total phenolic content was determined using Folin–Ciocalteu's method (Nualkaekul & Charalampopoulos, 2011).

The total phenolic content of samples was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (Nualkaekul & Charalampopoulos, 2011) and expressed as μg gallic acid equivalent (GAE)/mL LAPS.

Hydrogen peroxide

The H_2O_2 concentration in LAPS (1 L) was measured by micro-titration with potassium permanganate (0.1 M) at 4 °C.

Cations

The concentration of sodium, potassium, calcium, and magnesium present in the LAPS was measured by flame photometry. The values obtained for DL-lactic acid, phenolic compounds, H_2O_2, and cations are the mean of the three samples.

Chemical analysis of LAPS

Extraction of supernatants

About 8 L of LAPS were extracted three times with ethyl acetate (70:30 v/v). The organic phases were collected, dried with anhydrous Na_2SO_4, and filtered. The sample was concentrated in a rotary evaporator.

Column chromatography

The extract was subjected to a column chromatography using silica gel CC (70–230 mesh) as a stationary phase. The dried sample weight was 27.8 g. The sample was separated into fractions, using as a mobile phase, by the following solvents of increasing polarity: (1) hexane; (2) hexane–chloroform (1:1); (3) chloroform; (4) chloroform–ethyl acetate (9:1), (7:3), (1:1), (3:7), (1:9); (5) ethyl acetate; (6) ethyl acetate–methanol (7:3), (1:1), (3:7); and (7) methanol. Fractions were analyzed by TLC (normal phase), and those which had the same distribution of spots were reunited and concentrated.

Gas chromatography-mass spectrometry (GC-SM)

The concentrated fractions obtained from the column were studied by gas chromatography (ThermoElectron Model trace GC ultra, Thermo Electron Corp, Madison, WI) in tandem with mass spectrometry (ThermoElectron Model Polaris Q, Thermo Electron Corp, Madison, WI). Each fraction was injected (1 μL) and separated into their individual components by gas chromatography (injector 250 °C mode split 1/10; gas carrier: He, constant flow: 1.0 mL/min; column DB-5 30 m × 0.25 mm; initial temperature: 60 °C for 4 min, temperature ramp: 60–300 °C at 10 °C/min; final temperature: 300 °C for 2 min). When it was possible, components were identified by mass spectrometry (mass analyzer: ion trap; ionization type: electron impact at 70 eV; method of acquisition: full scan: 50–500 a.m.u.; ionization time: 0.25 min). For identification, the mass spectra library NIST MS Search 2.0 was used. The identification was based on a >90% similarity between the unknown and the reference spectrum. The identifiers used were the following: (1) CAS no. (identification number from the database of the American Chemical Society), (2) ChenSpider no. (identification number from the database of Royal Society of Chemistry), and (3) CheBI no. (identification number from the database and ontology of Chemical Entities of Biological Interest of the European Bioinformatics Institute).

Biological analysis of LAPS

AI-2 detection

AI-2 have been proposed to serve as a ''universal'' signal for interspecies communication (De Keersmaecker et al., 2006; Surette et al., 1999) and chemically and generally they are furanosyl borate diester (Figure 2; Chen et al., 2002; Schauder et al., 1999). *Lactobacillus plantarum* genome contains the *luxS* gene (GenBank accession no. NP_784522) which encodes for the enzyme Lux S (AI-2-synthase) (Figure 2; Winzer et al., 2002). For this reason, we measured AI-2 activity in different *L. plantarum* supernatants by using the *V. harveyi* BB170 bioassay.

AI-2 productions are dependent on the growth medium (De Keersmaecker & Vanderleyden, 2003). Consequently, we prepared supernatants of *L. plantarum* grown in three different media: (1) MRS, (2) MRS_{gal} (MRS in which we replaced glucose by galactose). This was done because glucose in cell-free culture fluids could hamper the detection of AI-2 by *V. harveyi* (De Keersmaecker & Vanderleyden, 2003). (3) MRS_{gal+BA} (MRS_{gal} supplemented with 10 mM of boric acid). This was done because boric acid in the growth medium

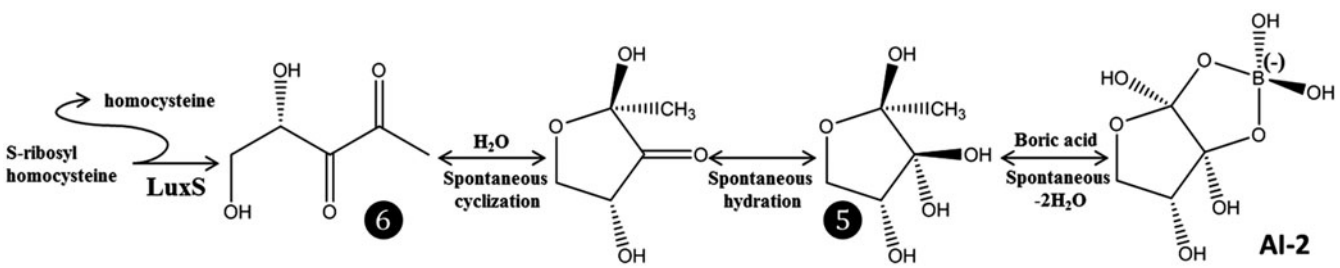

Figure 2. AI-2 synthesis in *V. harveyi* is shown. The activated methyl cycle is responsible for the generation of the major methyl donor in the cell, *S*-adenosyl-L-methionine, and the recycling of methionine by detoxification of *S*-adenosyl-L-homocysteine. The enzyme LuxS takes part in this cycle by salvaging the homocysteine moiety from the cycle intermediate *S*-ribosyl-homocysteine. As a by-product of this reaction, the direct AI-2 precursor 4,5-dihydroxy-2,3-pentadione (molecule 6) is formed. Molecule 6 undergoes further reactions to form distinct biologically active signal molecules generically termed AI-2. (2*S*,4*S*)-2-Methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate, the AI-2 signal of Vibrionales, is produced in the presence of boric acid without the help of any known enzyme. We assume that *L. plantarum* uses the same or a similar pathway for three reasons: (1) *L. plantarum* possesses in its genome the luxS gene encoding the enzyme LuxS. (2) We find in LAPS the molecules 5 and 6 (Table 1). (3) *Lactobacillus plantarum* supernatants obtained from the boric acid-added medium (LAPS gal + BA) induced a higher luminescence in the bioassay with *V. harveyi* BB170. This would indicate that the last step of the biosynthesis of AI-2 in *L. plantarum* requires boric acid, as it occurs in *V. harveyi*.

produces a significant induction of luminescence by supernatants of those cultures (Figure 2; De Keersmaecker & Vanderleyden, 2003).

Lactobacillus plantarum was grown separately in MRS, MRS_{gal}, and MRS_{gal+BA} for 12 h at 37 °C. Supernatants were obtained by centrifugation and filtration. These supernatants were called LAPS, $LAPS_{gal}$, and $LAPS_{gal+BA}$. Because of the acidic nature of supernatants which could inhibit AI-2 detection (De Keersmaecker & Vanderleyden, 2003), aliquots of them were neutralized with 8 M NaOH (NLAPS, $NLAPS_{gal}$, and $NLAPS_{gal+BA}$).

Vibrio harveyi *bioassay*

This bioassay was conducted according to Bassler et al. (1994). *Vibrio harveyi* BB170 was grown for 16 h in AB media (Bassler et al., 1994) and then diluted 5000 times in fresh AB media to obtain 10^5 CFU/mL. About 1 mL of the mentioned *L. plantarum* supernatants tested for the presence of AI-2 were added to 9 mL of these cells, mixed, and incubated at 30 °C with agitation (140 rpm). Bioluminescence measurements were taken every 30 min with a Microplate reader (BioTek FLx800TBID, BioTek, Anaheim, CA). Measurements taken after 5.5 h of incubation were normalized to the positive control (supernatant from a *V. harveyi* BB120 overnight culture) and then expressed as luminescence percentage. The AB medium was used as a negative control. Each experiment was performed in triplicates and the *t*-test was applied to determine the statistical significance of the results.

DNAase detection

Extraction of PMNs genomic DNA. Heparinized blood samples were collected by venipuncture from healthy individuals. MNs were isolated by dextran T-500 (Sigma, St. Louis, MO) sedimentation and Ficoll–Hypaque (Sigma, St. Louis, MO) gradient centrifugation. Cells were suspended in distilled water, frozen at −20 °C, and then thawed (thrice). DNA from lysed cells was purified by conventional techniques (Boom et al., 1990) and quantified at 260 nm.

Extraction of P. aeruginosa *genomic DNA*. An overnight *P. aeruginosa* culture in Luria Bertani broth was centrifuged (5 min, 8000*g*). The bacterial pellet was resuspended with PBS and 0.2 mL was transferred to a tube containing 0.5 mL of 0.1 mm diameter glass beads and 0.9 mL of lysis buffer (Boom et al., 1990). Bacteria were lysed by a 3 min pulse on a mini-bead-beater device, and RNA was digested by incubation of the lysate at 37 °C for 1 h. DNA was purified by conventional techniques (Boom et al., 1990) and quantified at 260 nm.

DNAse activity

Due to the presence of extracellular DNAse in *L. plantarum* supernatants (Caso & Suarez, 1997), we tested the enzymatic activity present in the LAPS on genomic DNA from *P. aeruginosa* and PMNs (components of *in vivo P. aeruginosa* biofilm matrix). For this assay, genomic DNA from *P. aeruginosa* or PMNs were used to prepare DNA agar (blue toluidine 100 µg/mL, tryptone 20 mg/mL, DNA 100 µg/mL, sodium chloride 5 mg/mL, and agar 15 mg/mL). The molten agar was placed in Petri dishes to form a uniform layer (thickness: 5 mm). When the agar solidified, five wells (diameter: 5 mm) were made in which the following samples were placed (50 µL): (1) PBS (negative control), (2) pancreatic DNAase 0.01 mg/mL (low-concentration positive control), (3) pancreatic DNAase (Sigma, St. Louis, MO) 1 mg/mL (high-concentration positive control), (4) LAPS, and (5) NLAPS. The dishes were incubated for 24 h at 37 °C and the enzymatic activity was observed as a light violet halo.

Effect of heat and proteases

Aliquots of LAPS were treated with (1) heat (2 h, 90 °C) ($LAPS_{heat}$), (2) papain (0.15 mg/mL, 4 h, 37 °C) ($LAPS_{papain}$), (3) trypsin (0.10 mg/mL, 72 h, 37 °C) ($LAPS_{tripsin}$), (4) pepsin (0.10 mg/mL, 18 h, 37 °C) ($LAPS_{pepsin}$), (5) proteinase K (0.20 mg/mL, 24 h, 37 °C) ($LAPS_{proteninase}$), (6) collagenase (0.50 mg/mL, 24 h, 37 °C) ($LAPS_{collagenase}$), and (7) pronase E (0.50 mg/mL, 24 h, 37 °C) ($LAPS_{pronase}$). The times of incubation and concentrations utilized were selected to obtain a maximum enzymatic activity and to guarantee the total destruction of the samples proteins.

Biofilm inhibiting capacity

In this assay, we evaluate whether treated supernatants obtained in the previous step conserve the inhibitory capacity on *P. aeruginosa* biofilm formation. For this, a static biofilm assay using *P. aeruginosa* was performed as described previously (O'Toole & Kolter, 1998). An overnight *P. aeruginosa* culture in the LB medium was diluted 1:7 in LB and placed (150 µL) in 96-well polystyrene microtiter plates (BD Bioscience, Franklin Lakes, NJ). Respectively, 50 µL of the treated supernatants were added and incubated for 6 h at 37 °C. The biomass formed was stained with 20 µL of crystal violet 0.1% (15 min) and then washed thrice with PBS. The cell-attached dye was solubilized with 200 µL of ethanol 95% (v/v), and the absorbance of the resulting solution was measured at 540 nm in a microplate reader (BioTek FLx800TBID, BioTek, Anaheim, CA). The measured absorbance is directly proportional to the biomass (biofilm) formed. With these data, the percentage of inhibition relative to control (MRS) was calculated. The values obtained are the mean of three samples. To estimate the direct effect of proteases on *P. aeruginosa* biofilm, the same assay was performed with solutions of the proteases in PBS at the same concentrations which were used to treat LAPS.

Results

Concentration of different components of LAPS

The measured concentrations of the different components in LAPS were the following: (1) D-lactic acid: 9.01 ± 1.02 mg/mL (100 mM); (2) L-lactic acid: 2.7 ± 0.51 mg/mL (30 mM); (3) hydrogen peroxide: 36 ± 2.0 µg/mL (approx. 2 vol); (4) phenolic compounds: 485.2 ± 15.2 GAE µg/mL; (5) sodium: 370 ± 17 µg/mL; (6) potassium: 920 ± 24 µg/mL; (7) calcium: 20 ± 4 µg/mL; and (8) magnesium: 15 ± 3 µg/mL.

Table 1. Esters, fatty acids, alcohols, antimicrobial compounds, surfactants, barbiturates, phenolic compounds, AI-2, and derivatives detected in LAPS by GC-MS.

Group	Compound	Fraction	t_R	Identifiers
Esters	Decanedioic acid dibutyl ester	C	22.60	CAS no. 98781-27-2
	Hexanedioic acid mono(2-ethylhexyl) ester	C-EA (9:1)	24.47	CAS no. 4337-65-9
	Octadecanoic acid 9,10-dihydroxy-methyl ester	C-EA (9:1)	25.30	CAS no. 1115-01-1
	Lactic acid, 3-phenyl-methyl ester	C-EA (9:1)	13.92	CAS no. 97508-25-3
	Benzenepropanoic acid, α-hydroxy-methyl ester	C-EA (9:1)	13.85	CAS no. 13673-95-5
	Decanoic acid, 3-hydroxy-methyl ester	C-EA (9:1)	14.93	CAS no. 56618-58-7
	Butanedioic acid, monomethyl ester	C-EA (7:3)	9.93	CAS no. 3878-55-5
	Butanoic acid, 2,3-dimethyl-2-(1-methylethyl)-methyl ester	C-EA (7:3)	14.21	CAS no. 112474-09-6
	Pentanoic acid, 2-hydroxy-4-methyl-methyl ester	C-EA (7:3)	7.28	CAS no. 40348-72-9
	L-Valine, *N*-(*N*-acetyl-L-alanyl)-, butyl ester	C-EA (1:1)	9.98	CAS no. 55712-41-9
	3-Hydroxy-hexanoic acid, ethyl ester	EA	3.09	CAS no. 2305-25-1
	Acetic acid, heptyl ester	EA	3.32	CAS no. 112-06-1
	2-Butenoic acid, ethyl ester	EA	4.04	CAS no. 623-70-1
	4,4-Dimethyl-3-oxo-pentanoic acid, ethyl ester	EA	4.18	CAS no. 17094-34-7
	Acetic acid, 3-methylbutyl ester	EA	4.70	CAS no. 29732-50-1
	N-Acetyl-L-phenylalanine, methyl ester	EA	18.01	CAS no. 3618-96-0
Fatty acids	Oleic acid	C-EA (9:1)	21.81	ChEBI no. 16196
	9-Hexadecenoic acid	C-EA (9:1)	22.45	ChEBI no. 72004
	n-Decanoic acid	C-EA (9:1)	13.73	ChEBI no. 30813
	Dodecanoic acid	C-EA (9:1)	16.25	ChEBI no. 30805
	2-Methyl-3-[4-*t*-butyl]phenyl propanoic acid	C-EA (9:1)	17.77	CAS no. 66735-04-4
	E-9-Tetradecenoic acid	C-EA (9:1)	18.33	CAS no. 544-64-9
	Tetradecanoic acid	C-EA (9:1)	18.54	ChEBI no. 28875
	Pentadecanoic acid	C-EA (9:1)	19.28	ChEBI no. 42504
	Octadecanoic acid	C-EA (9:1)	22.60	ChEBI no. 28842
	Phenyl propanedioic acid	C-EA (9:1)	12.33	CAS no. 2613-89-0
	2-Hydroxy-3-methyl butanoic acid	C-EA (7:3)	8.64	CAS no. 4026-18-0
	2-Hydroxy-4-methyl pentanoic acid	C-EA (7:3)	10.50	CAS no. 20312-37-2
	4-Butoxy-butanoic acid	C-EA (1:1)	9.29	CAS no. 55724-73-7
	Palmitic acid	C-EA (1:1)	20.62	ChEBI no. 15756
	2-Hydroxy-2,3-dimethylsuccinic acid	C-EA (1:1)	16.97	ChemSpider no.: 347725
Alcohols	1-Phenoxypropane-2-ol	C-EA (9:1)	12.02	CAS no. 770-35-4
	2,3-Dimethyl-2,3-butanediol	C-EA (7:3)	4.53	CAS no. 76-09-5
	2-(Dodecyloxy)-ethanol	EA	13.57	CAS no. 4536-30-5
Antimicrobial compounds	Benzoic acid	C-EA (9:1)	10.85	ChEBI no. 30746
	5-Methyl hydantoin	C-EA (7:3)	22.18	ChEBI no. 27612
	3-Isobutyl 2,5 piperazinedione	C-EA (7:3)	22.89	CAS no. 845-67-0
	Mevalonolactone	C-EA (1:1)	7.34	ChEBI no. 67849
	Lactic acid	C-EA (1:1)	12.74	ChEBI no. 28358
	Succinic acid	C-EA (1:1)	13.36	ChEBI no. 15741
	Acetic acid	C-EA (1:1)	14.17	ChEBI no. 15366
	Butyric acid	C-EA (1:1)	12.76	ChEBI no. 30772
	Ethanol	M	8.98	ChEBI no. 16236
Surfactants	1-Mono-linolein	C-EA (3:7)	21.88	CAS no. 26545-74-4
	1,2-Di-Palmitin	EA	25.61	CAS no. 761-35-3
	Distearin	EA	27.19	CAS no. 6904-15-6
Barbiturates	2,5-Diethy barbituric acid (barbital)	C-EA (7:3)	15.94	ChEBI no. 31252
	5-Butyl-5-ethyl-1,3-diazinane-2,4,6-trione (Buthetal)	C-EA (7:3)	17.93	CAS no. 77-28-1
	5-Ethyl-5-isopropylpyrimidine-2,4,6(1H,3H,5H)-trione (probarbital)	C-EA (7:3)	19.12	CAS no. 76-76-6
	5-Ethenyl-5-pentan-2-yl-1,3-diazinane-2,4,6-trione (vinilbital)	C-EA (7:3)	27.69	CAS no. 2430-49-1
Phenolic compounds	2,4-Di-*tert*-buthyl-phenol	H-C (1:1)	15.55	CAS no. 96-76-4
	Glycyl-L-phenil alanine	C-EA (1:1)	18.03	CAS no. 3321-03-7
	N-Formyl-D-phenylalanine	C-EA (1:1)	25.38	CAS no. 4289-95-6
	5-oxo-DL-proline	C-EA (3:7)	14.48	ChEBI no. 16010
	2,4-Bis(1,1-dimethylethyl)-phenol	C-EA (3:7)	15.55	CAS no. 96-76-4
AI-2 and derivatives	4,5-Dihydroxy-2,3-pentanedione (Figure 2, molecule 6)	EA-M (7:3)	7.85	CAS no. 142937-55-1
	2-Methyl-2,3,3,4-tetrahydroxytetrahydrofurane (Figure 2, molecule 5)	C-EA (9:1)	14.80	ChemSpider no.: 395434

C, chloroform; EA, ethyl acetate; M, methanol; t_R, retention time.

Other molecules present in LAPS

Table 1 shows the molecules found by GCMS in the fractions obtained from column chromatography. The molecules were divided into eight groups according to their chemical structure or biological activity: esters, fatty acids, alcohols, antimicrobial compounds, surfactants, barbiturates, phenolic compounds, AI-2, and derivatives.

Presence of AI-2 in LAPS

Figure 3 shows the results of the *V. harveyi* BB170 bioassay. When supernatants were used without neutralizing

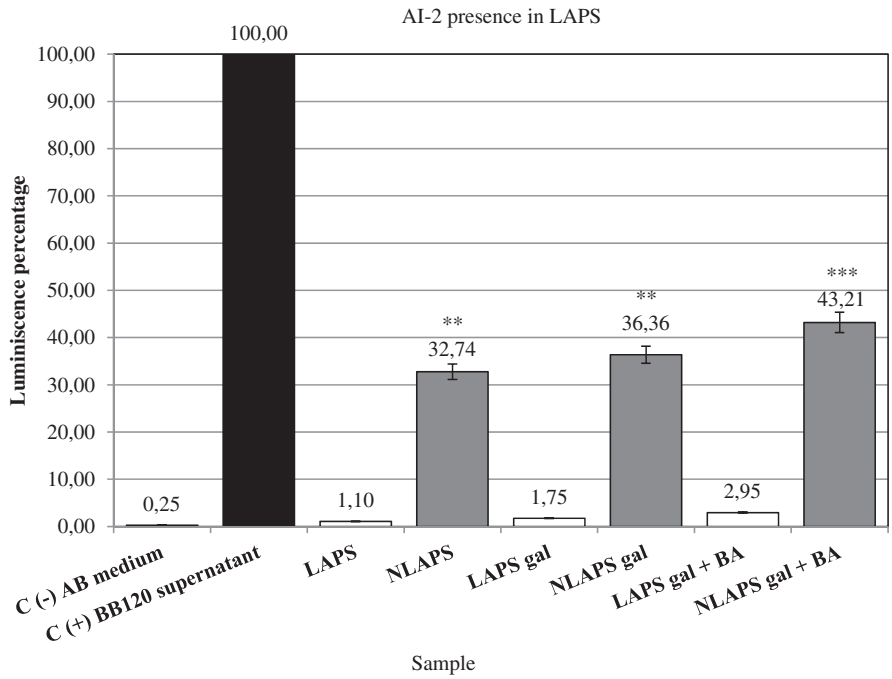

Figure 3. Induction of bioluminescence in *V. harveyi* BB170 bioassay produced by *L. plantarum* supernatants grown in different media: MRS, MRS replacing glucose by galactose (MRS_{gal}), and MRS_{gal} + boric acid (MRS_{gal+BA}). These supernatants were called LAPS, $LAPS_{gal}$, and $LAPS_{gal+BA}$. The same neutralized supertantants were used to perform the *V. harveyi* bioassay (NLAPS, $NLAPS_{gal}$, and $NLAPS_{gal+BA}$). Measurements taken after 5.5 h of incubation were normalized to the positive control (supernatant from the overnight culture of BB120) and then expressed as luminescence percentage. Only significant differences were found with respect to the negative control (AB medium) when neutralized supernatants were used: $^{**}p<0.01$; $^{***}p<0.001$.

(LAPS, $LAPS_{gal}$, and $LAPS_{gal+BA}$), no significant difference was observed between samples and negative control. In contrast, neutralized supernatants (NLAPS, $NLAPS_{gal}$, $NLAPS_{gal+BA}$) induced a significant production of luminescence ($p<0.01$ for NLAPS and $NLAPS_{gal}$ and $p<0.001$ for $NLAPS_{gal+BA}$) (Figure 3). Therefore, *L. plantarum* produces AI-2 and these molecules are present in their supernatants. Greater induction of luminescence produced in the presence of boric acid would indicate that the chemical structure of the AI-2 present in the supernatants is possibly the furanosil borate diester (Chen et al., 2002; Figure 2, molecule 8). This is consistent with our previous findings (De Keersmaecker & Vanderleyden, 2003) that *L. plantarum* genome has the *luxS* gene (AI-2 synthase; Figure 2) (GenBank accession no. NP_784522). Furthermore, to substantiate this, the molecules 4,5-dihydroxy-2,3-pentanedione (Figure 2, molecule 5) and 2-methyl-2,3,3,4-tetrahydroxy-tetrahydrofurane (Figure 2, molecule 7) were found in LAPS (GCMS) (Table 1).

DNAase activity in LAPS

The extracellular DNAase of *L. plantarum* (Caso & Suarez, 1997) showed activity on both genomic DNAs (PMNs and *P. aeruginosa*) (Figure 4). The halos obtained with NLAPS (pH 7.0) were significantly lower than those obtained with LAPS (pH 5.22 ± 0.43) which means that *L. plantarum* DNAase has an optimum activity at acidic pH.

Effect of heat and proteases on LAPS

LAPS was treated with heat and proteases to verify if its ability to inhibit biofilm formation is caused by an enzyme or another protein. Consequently, treated supernatants were used in biofilm inhibition assays (Figure 5). As demonstrated previously, LAPS inhibited approximately 45% *P. aeruginosa* biofilm formation. This ability is partially lost when the supernatant is neutralized (NLAPS) (Ramos et al., 2010a; Valdez et al., 2005). The heat-treated supernatant (LAPS heat)

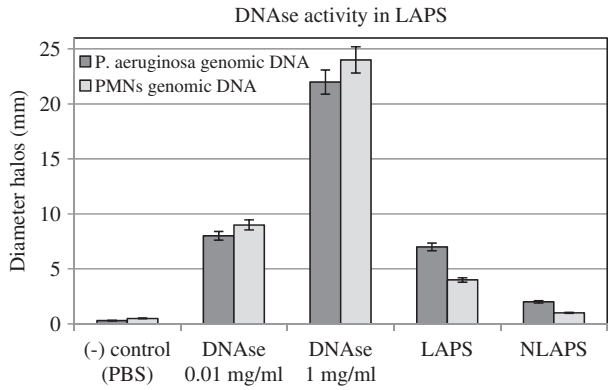

Figure 4. LAPS DNAse activity measured as halos of enzymatic activity in DNA agar prepared with *P. aeruginosa* and PMNs genomic DNA. The DNAase present in LAPS may use genomic DNA from *P. aeruginosa* and genomic DNA from PMNs (two essential components of *in vivo P. aeruginosa* biofilm matrix) as a substrate. This partially explains the biofilm disrupting ability of LAPS.

almost completely lost its inhibitory capacity (Figure 5). Thus the inhibitory factor is heat sensitive. In contrast, the protease-treated supernatants showed an exacerbation in the original inhibitory capacity (black bars). Therefore, we conclude that the inhibitory factor is not a protein. To assess the contribution of proteases in biofilm inhibition, we performed the same experiment with protease solutions in the same concentrations used to treat LAPS. Figure 5 shows that proteases are capable of inhibiting biofilm formation by themselves (white bars), and the exacerbation observed with protease-treated supernatants is possibly the sum of the inhibitory contributions of each one (LAPS + protease).

Discussion

In our previous work, we have shown that LAPS interferes with the pathogenic capacity of *P. aeruginosa* inhibiting *in vitro* adhesion, quorum sensing, biofilm, and virulence

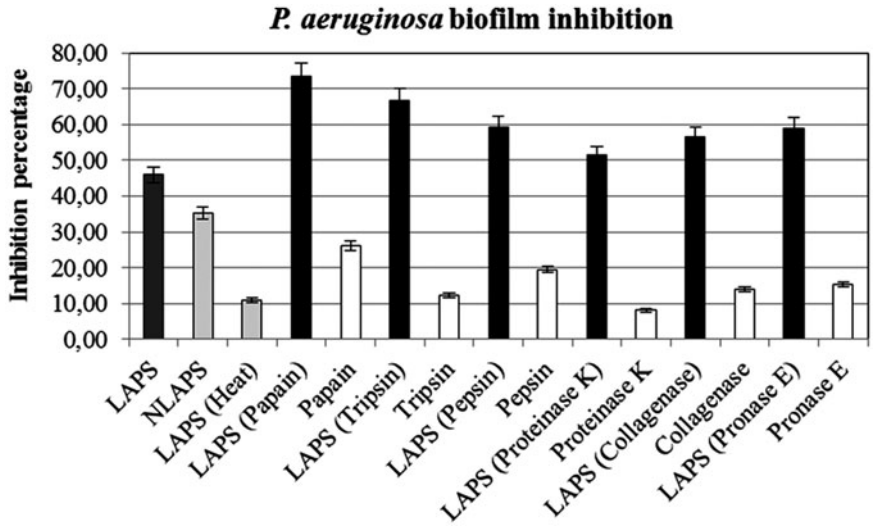

Figure 5. *Pseudomonas aeruginosa* biofilm inhibition produced by LAPS treated with heat and different proteases. The same assay was carried out with proteases solutions with the same concentration utilized to treat LAPS. The heat-treated supernatant (LAPS heat) almost completely lost its inhibitory capacity so it follows that the inhibiting factor present in LAPS is heat-sensitive. Proteases (white bars) are capable of inhibiting biofilm formation by themselves, and the exacerbation observed with protease-treated supernatants (black bars) is possibly the sum of the inhibitory contributions of each one (LAPS + protease). This would indicate that the inhibitory factor of biofilm formation present in LAPS is not a protein.

factors (Ramos et al., 2010a, 2012; Valdez et al., 2005). In addition, LAPS showed antimicrobial properties and a great biofilm disrupting capacity (Ramos et al., 2010a, 2012). Based on this, we seek in this work the molecules present in LAPS potentially responsible for these properties:

(a) We found low molecular weight molecules of organic acids (lactic, butyric, acetic, and succinic acids), H_2O_2, alcohols, benzoic acid, 5-methyl hydantoin, 2,5-mevalonolactone, and isobutyl piperazinedione in LAPS (Table 1). All these molecules were previously reported as antimicrobials by other authors (Ricke, 2003; Servin, 2004). Many of them proved to have growth inhibitory activity on Gram-negatives and to synergize each other's antimicrobial action, especially in the presence of lactic acid (Paavola et al., 1999).

(b) The presence of AI-2 in LAPS was determined chemically (GCMS, Table 1) and biologically (*V. harveyi* bioassay, Figure 3). *Pseudomonas aeruginosa* is able to detect and modify their virulence in response to AI-2 (Duan et al., 2003). Similarly, in the presence of LAPS, *P. aeruginosa* modifies its phenotypic expression to a planktonic mode of growth and decreased production of virulence factors (Ramos et al., 2012; Valdez et al., 2005). The inhibitory factor of biofilm formation present in LAPS is heat-sensitive but resistant to proteases (Figure 5). The thermal sensitivity of AI-2 (Chen et al., 2002) and its presence in LAPS (Figure 3 and Table 1) led us to the hypothesis that these molecules are responsible for the inhibition of biofilm and virulence factors which were demonstrated in the previous work (Ramos et al., 2012). There are no receptors for AI-2 in *P. aeruginosa* (Rezzonico & Duffy, 2008), thus our hypothesis is that these molecules could act as structural analogues of acyl homoserine lactones of *P. aeruginosa* (Figure 1) and, therefore, hinder the normal functioning of its quorum-sensing system (quorum quenching) as previously observed (Ramos et al. 2010a; Valdez et al. 2005). There are other molecules chemically similar to AI-2 that produce quorum quenching in *P. aeruginosa* such as halogenated furanones isolated from the alga *Delisea pulcra* (Hentzer et al., 2003; Figure 1) and precursors of AI-2 (Ganin et al., 2009). In this work, the precursor molecules 5 and 6 were found in LAPS (Figures 1 and 2 and Table 1).

(c) We found different agents with a great biofilm disrupting capacity on *P. aeruginosa* as DNAase (Figure 4), cations (sodium: 370 ± 17 µg/mL; calcium: 20 ± 4 µg/mL; and magnesium: 15 ± 3 µg/mL), surfactants (Table 1), and chelating agents (Table 1) in LAPS. Interestingly, all these agents have been previously reported in the literature. DNAase affects the capability of *P. aeruginosa* to form biofilms when it is present in the initial developmental stages (Whitchurch et al., 2002; Xavier et al., 2005). In contrast, alginate gel strength and the total biofilm protein of *P. aeruginosa* are reduced by the salts of sodium, magnesium, and calcium (Chen & Stewart, 2002; Gordon et al., 1991). Also, it was shown that the presence of surfactants and/or chelating agents in the medium reduces the total biofilm protein and produces major reductions in alginate gel strength of a *P. aeruginosa* biofilm (Chen & Stewart, 2000, 2002).

In the previous work, we have shown that topical applications of LAPS on human-infected chronic wounds reduce or eliminate the necrotic tissue and the wound area, as well as they promote debridement, the appearance of granulation tissue, and wound healing (Peral et al., 2009, 2010). Based on this, we seek in this work the molecules present in LAPS potentially responsible for these properties:

(a) We found phenolic compounds (GCMS, Table 1) in a high concentration (485.2 ± 15.20 GAE µg/mL) in the LAPS. During the inflammatory phase of wound healing, there is a continuous influx of PMNs, attracted by bacterial presence. Necrotic PMNs release free oxygen radicals generating a highly oxidizing environment that only contributes to tissue injury and the maintenance of the inflammatory phase of healing (Guo & DiPietro, 2010). We postulate that the elevated concentration of phenolic compounds present in LAPS through their antioxidant properties may decrease the concentration of free oxygen radicals in the ulcer bed indirectly attenuating the deleterious effect. Indeed, the phenolic

compounds were already reported as pro-healing agents (Sen et al., 2002).

(b) We found lactic acid (GCMS, Table 1) in a high concentration (D-lactic acid: 9.01 ± 1.02 mg/mL; L-lactic acid: 2.7 ± 0.51 mg/mL) in LAPS. Angiogenesis is imperative for later stages in wound healing (Tonnesen et al., 2000). Endothelial growth and proliferation are directly stimulated by lactic acid in the wound (Rendl et al., 2001). When growth factor-producing cells are no longer in a lactic acid-filled environment, they stop producing angiogenic factors (Rendl et al., 2001; Tonnesen et al., 2000). We postulate that the lactic acid found in LAPS would be important in stimulating macrophages and platelets to produce endothelial growth factors with subsequent neovascularization and increased wound tissue perfusion.

(c) Finally, we found a variety of molecules derived from barbituric acid in LAPS. The most abundant was 5,5-diethyl barbituric acid (Table 1). Yeast extract (a main component of MRS broth) is a water-soluble extract from an autolysate of *Saccharomyces cerevisiae* cells. The 5-methyl barbituric acid is the final metabolite in *S. cerevisiae* pyrimidines metabolism (Sievers & Wolfenden, 2005). We propose that barbituric acid derivatives found in LAPS come from changes made by *L. plantarum* to 5-methyl barbituric acid. It was previously demonstrated that some barbiturates can promote wound healing via enhancing collagenization (Ajwee et al., 2012). Besides, these molecules would have a slight local anesthetic action (Henn & De Eugenio, 2009).

Conclusion

Molecules and enzymes found in this work explain the properties of LAPS and scientifically support its therapeutic effectiveness in chronic wound treatment.

Acknowledgements

We thank Dr. Fabio Rezzonico (Agroscope Changins-Wädenswil ACW, Division of Plant Protection, Wädenswil, Switzerland) for the generous donation of *V. harveyi* strains used in this work.

Declaration of interest

The authors declare no competing financial interest. This work was supported by grant BID 1728 OC-AR PICT 2006 no. 1458 from National Agency for Scientific and Technological Promotion, Argentina and the Research Council of the National University of Tucumán CIUNT Program No. 26/D453.

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