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BIOTECHNOLOGICAL DESIGN TO OPTIMIZE THE PRODUCTION OF BIOACTIVE *LACTOBACILLUS PLANTARUM* BY-PRODUCTS: NOVEL CHRONIC WOUND TREATMENT

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ABSTRACT: *Lactobacillus plantarum* culture supernatants (LAPS) have antimicrobial, pro-healing and anesthetic properties, so our medical team applied whole cultures and LAPS in chronic wounds with encouraging results. Metabolites responsible for LAPS properties and its action mechanism were previously determined. **Objective:** to design methods and culture media to increase the production of LAPS metabolites and optimize it´s therapeutic effectiveness. Modifications in MRS broth composition were made and *L. plantarum* was cultivated in the resulting different media with different physicochemical conditions to obtain modified supernatants ($LAPS_m$). Antimicrobial and anti-biofilm activity (crystal violet) of $LAPS_m$ on strains typically isolated from chronic wounds (*Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis* and *Serratiamarcescens*), was tested. Concentration of barbiturates in $LAPS_m$ was quantified by GCMS and its relative anesthetic potency in healthy volunteers was evaluated. DNAase and auto inducers type 2biological activity in $LAPS_m$ were quantified. In addition, a safety assessment of $LAPS_m$ application on healthy skin of 200 volunteers was performed. $LAPS_m$ obtained from media with high concentrations of yeast extract possessed the greatest anesthetic power. Those containing greater amount of proteins, cations and surfactants had the highest capacity of biofilm disruption ($p<0.01$). When glucose and galactose concentration were increased in media, $LAPS_m$ had the greatest antimicrobial power ($p<0.05$). Finally, there is great safety in the application of $LAPS_m$ as no edema or erythema was observed. The results obtained will allow the manufacture of $LAPS_m$ with greater therapeutic effectiveness and even custom properties for each type of wound.

INTRODUCTION: 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). LAPS inhibit *in vitro* adhesion, growth, quorum sensing signals and virulence factors of bacteria typically isolated from chronic wounds [1-3].

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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* pre-clinical trials), compared to acetic acid or antiseptics typically used in the treatment of these infections [4]. Topical applications of LAPS on human infected chronic wounds (diabetic foot ulcers, burns and venous ulcers) reduces or eliminates the pathogenic bacteria load, the amount of necrotic tissue and the wound area. Also promotes angiogenesis, revascularization, debridement, the appearance of granulation tissue and wound healing with increased production of TGF-β, IL-8 and IL-8-R [5-7].

Chemical and enzymatic composition of LAPS was determined allowing us to propose a correlation between the chemical constituents and their biological activity [7, 8]. The metabolites responsible for the anti-pathogenic and pro-healing properties act synergistically, which positions the complex mixture of LAPS as a single active pharmaceutical ingredient (API) [9, 10]. However, the need for personalized treatments to suit different types of chronic wounds led to devise and develop this study. Besides, it is necessary to advance the field of alternative treatments that are financially accessible to the least economically developed countries [11]. Based on this, the aim of this work was to design methods and culture media to increase the production of LAPS metabolites and optimize it´s therapeutic effectiveness of the supernatants.

MATERIALS AND METHODS:

1. Bacterial strains: For antimicrobial and anti-biofilm activity tests (inhibition and disruption) four pathogenic strains were used: *Pseudomonas aeruginosa*, *Serratiamarcescens*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Clinical isolates from human chronic venous leg ulcers). Two strains were used for auto inducers type 2 (AI-2) bioassays: *Vibrio harveyi BB120* (wild type strain) as a source of external AI-2, *Vibrio harveyi*BB170as a reporter strain, which specifically responds to AI-2 by producing bioluminescence. A strain of *Lactobacillus plantarum* (ATCC 10241) was used to obtain all supernatants.

2. Modified culture media (M) design:
Thirty two culture media (M1 to M32) were prepared as shown in **Table 1**. Culture media design was based on changes in the deMan Rogosa and Sharpe (MRS) broth through modifications in the carbon, nitrogen and boron source and by increasing ionic strength and surfactant power.

3. *L. plantarum* growth in modified media: Modified media (M1 to M32) were inoculated with an overnight culture (8 h) of *L. plantarum* in MRS ($OD_{600}\approx 0.655$) (1 µL of inoculum per mL of fresh culture medium) and incubated for 12 h at 37°C (until stationary phase) under aerobic conditions (O_2 20% and CO_2 1%). In addition *L. plantarum* was grown in those media with changes in carbohydrate source (M10 to M16) under microaerophilic conditions (O_2 5% and $CO_2$10%). Finally, the OD_{600} (spectrophotometer Shimadzu UV-1800 UV-Visible) and pH (pH meter Broadley James Corporation-USA) was measured. Each medium was evaluated in octuplicate (n=8) and the result was expressed as mean ± SD.

4. *L. plantarum* supernatants:
Bacteria-free supernatants, were recovered after centrifugation (8000 rpm, 15 min) and subsequent filtration through Millipore filters (pore: 0.22 µm).When MRS broth was utilized, previously reported LAPS was obtained [1-10] and used as control of concentration and original activity. When M1 to M32 were used as culture media LAPS1 to LAPS32 were obtained **(Table 1**).

5. Antimicrobial and anti-biofilmassays:
5.1Selected supernatants: For these assays the following supernatants obtained under aerobic conditions were selected: 1) Maximum concentration of sodium, potassium and magnesium salts (LAPS2, LAPS4 and LAPS6); 2) Maximum concentration of the surfactant Tween 80 (LAPS9); 3) Maximum concentration of glucose and galactose and mixed sugars (LAPS12, LAPS15 and LAPS16); 4) Maximum concentration of peptone, triptone, meat extract and mixed source of proteins (LAPS17, LAPS18, LAPS19 and LAPS22), 5) All concentrations of yeast extract (LAPS23 to LAPS26), 6) Supernatants with boric acid (LAPS28 and LAPS29). All selected supernatants complied with the requirement of having a pH greater than 4.2 which is the minimum allowed for application on skin [12]. For the same reason, supernatants obtained under microaerophilic conditions were discarded.

5.2 Antimicrobial activity:
The pathogenic strains were grown for 12 h at 37ºC in LB (*P. aeruginosa* and *S. marcesens*) or BHI broth (*S. aureus* and *S. epidermidis*) and suspensions of $OD_{600}\approx 0.150$ were prepared using fresh medium as diluent and blank.

In 96-well polystyrene microtiter plates (Costar Corning Inc. USA), 150 µL of each bacterial suspension were placed and respectively 50 µL

(25%) of LAPS (control for original activity), gentamicin (8µg/mL) (antimicrobial positive control), LB or BHI (growth control), MRS (control for original medium components activity),Saline (dilution control), human recombinant DNAase 1mg/mL (control for biofilm inhibition and biofilm disruption) and selected LAPS (see section 5.1), were added. The plates were incubated for 24 h at 37°Cand growth curves were obtained by measuring the OD_{600} once per hour in a microplate reader (BioTek FLx800TBID). Each curve was performed by octuplicate and every point was expressed as mean.

5.3Biofilm inhibition:

The content of the wells of the above assay (see section 5.2) was discarded and the wells were washed three times with saline. The remaining attached biomass (biofilm) was stained during 15 min with 200 µL of crystal violet (0.1%). Cell-attached dye was solubilized with 200 µL of ethanol and the OD_{540} of the resulting solution was measured in a microplate reader. The measured OD is directly proportional to the biomass (biofilm) formed. The percentages of stimulation or inhibition were calculated using the biomass formed in presence of LB or BHI as 100%. Results were expressed as percentage mean ± SD (n=8).

5.3.1Autoinducer type 2 (AI-2) activity in LAPS27 to LAPS32. There is AI-2 activity in LAPS [7, 8] and probably this is the main reason for which *L. plantarum* supernatants have biofilm inhibitory capacity.AI-2 has been proposed to serve as a 'universal' signal for interspecies communication [13, 14] and chemically generally are furanosyl borate diester[15, 16]. For this reason we measured AI-2 activity in LAPS27 to LAPS32 (which were obtained in presence of different sources of boron) by using the *V. harveyi* BB170 bioassay. Because of the acidic nature of supernatants could inhibit AI-2 detection[17], aliquots of them were neutralized with 8 M NaOH (NLAPS27 to NLAPS32).

***Vibrio harveyi* bioassay:** This bioassay was conducted according to Bassler *et al* [18]. *V. harveyi* BB170 was grown for 16 h in AB media and then diluted 5000 times in fresh AB media to obtain 10^5 CFU/mL. One mL of the mentioned *L. plantarum* supernatants tested for the presence of AI-2 (LAPS27 to LAPS32 and NLAPS27 to NLAPS32) 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) until4.5 h. A supernatant from a *V. harveyi* BB120 overnight culture was used as positive control and AB medium was used as negative control. Each experiment was performed in triplicates.

5.4 Biofilm disruption:

For each assay, five spherical glass beads (radius = 9 mm; weight = 5.4 g) were used. The total area of the beads was $[5.(4.\pi.r^2)] = 5086.8$ mm^2 and the total weight was approximately 27.0000 g. The beads and 20 mL of fresh media (LB or BHI, according to the strain) were placed respectively in separate glass flasks and autoclaved. Subsequently, each flask was inoculated with 200 µL of an overnight culture of each pathogenic strain and incubated at 37 °C for 16 h until mature biofilm formation on the bead´s surface. The supernatants were discarded and the beads washed thoroughly three times with sterile saline. The biofilm formed so far was considered the pre-disruption starting biomass (PDB).Then, 20 mL of a mixture (1:1) of fresh medium (LB or BHI) and selected supernatants (see section 5.1) were respectively added. Sterile saline was used as disruption negative control; MRS broth was used as control of the medium components effect and LAPS was used as original disrupting activity control.

The flasks were incubated at 37 °C for 6 h to allow disruption and the supernatants were discarded. Finally, the beads were washed, transferred to pre-weighed plastic containers, and dried at 60 °C to constant weight. Weighing was performed on analytical balance (sensitivity to 4 decimal points) and the weight of remaining biofilm was determined by the following weight difference: Remnant biomass = weight of beads with dry biomass – weight of beads.

Each experiment was performed in triplicates. The result was expressed as remnant biomass (µg) mean/ beads area (mm^2) to simulate what happens *in vivo* in a chronic wound.

5.4.1DNAase activity in LAPS17 to LAPS26:

The biofilm disrupting capacity of LAPS is partly due to the presence of extracellular DNAase [8, 19]. Because of this, we measure DNAase activity in those supernatants obtained in presence of various protein sources (LAPS17 to LAPS26) using LAPS as original DNAase activity control.

For the manufacture of a calibration curve, standard solutions of human recombinant DNAase (Sigma) were used in different concentrations: 1000 µg/mL; 500 µg/mL; 250 µg/mL; 125 µg/mL; 62.5 µg/mL; 31.25 µg/mL; 15.63 µg/mL; 1.95 µg/mL; 0.98 µg/mL. In petri dishes (diameter: 15 cm), 20 mL of DNAase agar (Britania, Buenos Aires, Argentina) was placed. Twenty microliters of DNAase standard solutions, LAPS and LAPS17 to LAPS26 were respectively placed in wells (Diameter: 5 mm) made in the agar (each sample was assessed in triplicate). Then, petri dishes were incubated for 24 h at 37 °C and the enzymatic activity halos were revealed with HCl(1M)and measured (mm) with a Veniercaliper.

By plotting the halos of DNAase activity versus DNAase concentration in standard solutions, the calibration curve was found mathematically by the method of least squares. The equation which best represents the dataset was $y = 0.0027. e^{0.4629x}$ where "x" is the halo of enzyme activity (mm) and "y" is DNAase concentration(µg/mL).

TABLE 1: CULTURE MEDIA AND SUPERNATANTS OBTAINED AFTER THE GROWTH OF *L. PLANTARUM*. PEPTONE (P), TRYPTONE (T), MEAT EXTRACT (ME), YEAST EXTRACT (YE), GLUCOSE (G), GALACTOSE (GA), SURFACTANT SOURCE(SS), TWEEN 80 (T80), DIPOTASSIUM PHOSPHATE (DP), SODIUMACETATE (SA), AMMONIUMCITRATE (AC), BORICACID (BA), SODIUMBORATE (SB).

MEDIUM	COMPOSITION (w/v %)																	Supernatanto btained
	Nitrogen source				Carbon source		SS	Medium Salts					Ionic strength			Boron source		
	P	T	ME	YE	G	Ga	T80	DP	SA	AC	$MgSO_4$	$MnSO_4$	NaCl	KCl	$MgCl_2$	BA	SB	
MRS	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS
M1	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	0.1	-	-	-	-	LAPS1
M2	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	0.3	-	-	-	-	LAPS2
M3	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	0.1	-	-	-	LAPS3
M4	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	0.3	-	-	-	LAPS4
M5	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	0.1	-	-	LAPS5
M6	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	0.3	-	-	LAPS6
M7	1	-	1	0.5	2	-	0.2	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS7
M8	1	-	1	0.5	2	-	0.3	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS8
M9	1	-	1	0.5	2	-	0.5	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS9
M10	1	-	1	0.5	2.1	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS10
M11	1	-	1	0.5	2.2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS11
M12	1	-	1	0.5	2.3	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS12
M13	1	-	1	0.5	2	0.1	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS13
M14	1	-	1	0.5	2	0.2	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS14
M15	1	-	1	0.5	2	0.3	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS15
M16	1	-	1	0.5	2.1	0.2	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS16
M17	1.1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS17
M18	1	0.1	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS18
M19	1.1	0.1	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS19
M20	1	-	1.1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS20
M21	1	-	1.2	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS21
M22	1	-	1.3	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS22
M23	1	-	1	0.6	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS23
M24	1	-	1	0.7	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS24
M25	1	-	1	0.8	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS25
M26	1	-	1	0.9	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	-	LAPS26
M27	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	0.05	-	LAPS27
M28	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	0.1	-	LAPS28
M29	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	0.3	-	LAPS29
M30	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	0.05	LAPS30
M31	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	0.1	LAPS31
M32	1	-	1	0.5	2	-	0.1	0.2	0.5	0.2	0.02	0.005	-	-	-	-	0.3	LAPS32

6. Anesthetic properties of modified supernatants:

6.1 Quantification of 5,5-diethyl-barbituric acid (DEBA) in LAPS23 to LAPS26:

Previously it was discovered that the active principle potentially responsible for the anesthetic action of LAPS is DEBA and that yeast extract (YE) is a source of barbiturates precursors for *L. plantarum* [7, 8]. For this reason, this molecule was quantified in the supernatants obtained in presence of different concentrations of yeast extract (LAPS23 to LAPS26).

6.1.1 Extraction:

Five hundred milliliters of LAPS and LAPS23 to LAPS26 were extracted three times with ethyl acetate (PA Sintorgan. Ind. Arg) (70:30 v/v). The organic phases were collected, dried with anhydrous Na_2SO_4 and filtered. The sample was concentrated in rotary evaporator. The dried pellet was dissolved in ethyl acetate (5 mL) and stored at 4 °C until analysis by GCMS.

6.1.2 Calibration curve:

Standard solutions of DEBA (Anedra, Ind. Arg) were prepared in ethyl acetate (909.0µg/mL; 303.0µg/mL; 101.0µg/mL; 33.6µg/mL; 11.2 µg/mL 3.7µg/mL) and stored at 4 °C until analysis by GCMS.

6.1.3 GCMS:

Concentrated samples obtained from supernatant extraction and DEBA standard solutions were studied by gas chromatography (Thermo Electron Model trace GC ultra) in tandem with mass spectrometry (Thermo Electron Model Polaris Q). Each sample 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: 1.0mL/min; Column DB-5 30 m x 0.25 mm; Initial temperature: 60°C 4 min, Temperature ramp: 60-300°C at 10°C/min; Final temperature: 300°C 2 min). The compound was 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 reference spectrum. DEBA had a retention time of 15.30 minutes in all samples and standards. Absolute area of DEBA corresponding peaks in standard solutions and extracts were calculated by using the software Xcalibur Qual Browser 2.0. By plotting the absolute area versus DEBA concentration in standard solutions, the calibration curve was found mathematically by the method of least squares.

The equation which best represents the dataset was ($y= 6.10^{-5}x -3.01$) where "y" is DEBA concentration (µg/mL or mg/L) and "x" is absolute area from the corresponding peak of the gas chromatogram. Extrapolating the values of areas of the corresponding peaks in the unknown samples (extract from supernatants), DEBA concentrations were calculated. Finally, by using the values of incubation times (12 h) and the volume of culture medium used (500 mL = 0.5 L), the production rates of DEBA(µ= mg/L.h) were calculated.

6.2 LAPS26 anesthesia evaluation on volunteer' shealthy skin: Anesthetic property of the supernatant obtained in the presence of the maximum concentration of yeast extract was evaluated (LAPS26).The supernatant was tested on healthy volunteers (n=40 which gave informed consent) using the following methodology: Two milliliters of LAPS26 or a control solution of lidocaine (2% + epinefrin 1:200000) was applied to the left or right forearm respectively. Then waited 10 minutes and the sensitivity of both forearms was assessed by applying various stimuli: 1) Distention, 2) Pressure, 3) Non-penetrative needling, 4) Temperature. To describe patient´s sensation intensity in response of each stimulus, a verbal scale was used [20]: 1) without sensation, 2) mild, 3) moderate, 4) intense.

7. Safety evaluation of LAPS12, LAPS19, LAPS26 and LAPS29. Response to application on human healthy skin:

Since there is no foreseeable risk from the use of modified supernatants is possible to evaluate the safety of their ingredients simply evaluating the appearance of irritation and/or allergy in clinical test. The use of the corrosion test in a model of healthy skin [21] is a methodology validated by the National Drug and Medical Technology

(Resolution Nº 288/90 ANMAT) in Argentina and in the Guide to Safety Assessment of the National Health Surveillance Agency in Brazil. This trial involved 200 healthy volunteers (with skin integrity in both forearms). The supernatant selection criterion was based on the concentrations of potentially irritating metabolites: LAPS, LAPS12 (maximum concentration of glucose), LAPS19 (mixture peptone-triptone), LAPS26 (maximum concentration of yeast extract), LAPS29 (maximum concentration of boric acid).To evaluate each selected supernatant, a panel of 40 volunteers (20 men and 20 women) was used. Each supernatant (2mL) was applied as a spray on the forearm of each volunteer (which gave informed consent)and the occurrence of edema and/or erythema at 20 minutes and 24 hours after application was evaluated. In addition, patients were surveyed about their skin sensations. The results were categorized as with or without sensation. In turn sensations were sub-categorized into: itching, burning, pain, numbness and redness.

8. Ethical considerations:

This study meets the ethical and scientists standards to design, conduct, recording and reporting studies that involve the participation of human beings (stipulated by the Ministry of Health of Argentina, Resolution Nº 1490/07). They are based in the International Declarations of Human Rights and Ethics Research (Nuremberg, 1948), Helsinki (1964 and updates), the Operational Guidelines for Ethics Committees (WHO 2000 - World Health Organization) and the International Ethical Guidelines for Health research Involving Human Subjects (CIOMS 2002 - Council for International Organizations of Medical Sciences).

9. Statistics:

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

RESULTS AND DISCUSSION:

1. *L. plantarum* growth in modified media:

In **Fig. 1**, the growth (OD_{600}) of *L. plantarum* (12 h) in the different media (MRS and M1 to M32) can be observed. *L. plantarum* possesses a facultative hetero fermentative metabolism [22] therefore may behave differently under aerobic and microaerophilic conditions. In aerobic conditions (O_2 20% and CO_2 1%), increasing the ionic strength in the culture media (M1 to M6) did not substantially alter the growth of *L. plantarum*. In contrast, in the culture media with increased surfactant strength (M7 to M9) reduced growth was observed. Naturally, the addition of proteins and carbohydrates to the culture media (M10 to M26), increased the growth of *L. plantarum*, except with the addition of yeast extract (M23 to M26) as it did not produce significant changes compared to MRS. Besides, the lower pH observed for these cases are logical as amino acids, glucose and galactose can enter to the aerobic glycolytic pathway with production of acetic and pyruvic acid [23]. Finally, the addition of boron as boric acid or its sodium salt to the media (M27 to M32) did not modify the growth of *L. plantarum*.

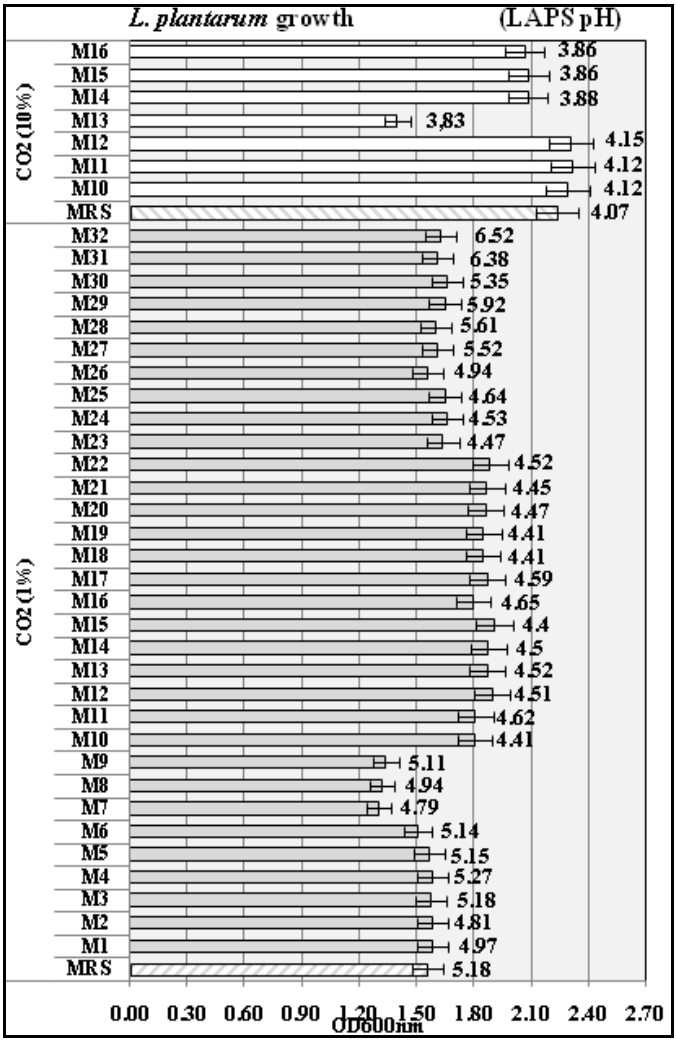

FIG. 1: GROWTH OF 12 HOURS OF *L. PLANTARUM* (OD_{600}) IN MODIFIED CULTURE MEDIA UNDER AEROBIC AND MICROAEROPHILIC CONDITIONS. THE NUMERICAL VALUE BESIDE EACH BAR CORRESPONDS TO THE pH REACHED.

On the other hand, the growth in microaerophilic conditions (O_2 5% and CO_2 10%) were significantly higher ($p<0.05$) than those obtained under aerobic conditions while the pH of the respective supernatants were markedly lower. This is due to the large production of lactic acid from anaerobic glycolysis. The minimum allowable pH for pharmaceutical topical products is 4.2 [12]. For this reason all supernatants obtained in microaerophilic conditions were discarded for the remaining assays.

Chronic wounds [25, 26]. The modified media were designed to maximize production of antimicrobial molecules present in the original LAPS as organic acids (acetic, pyruvic, lactic, etc.), hydrogen peroxide, ethanol, benzoic acid, 5-methyl hydantoin, 2,5-mevalonolactone, isobutyl piperazinedione and others [8]. Therefore, this assay allowed us to identify the supernatants that retain or increase the bacteriostatic and bactericidal activity present in the original LAPS [2, 3, 7].

Fig. 2 and **3** shows respectively the supernatants antimicrobial activity on gram negative and gram positive bacteria. MRS stimulated the growth of *S. marcescens*, *S. aureus* and *S. epidermidis* compared to the control LB or BHI (**Fig. 2B**, 3A and **3B**), whereas inhibit the growth of *P. aeruginosa*(**Fig. 2A).** Gentamicin had bacteriostatic activity on *S. marcescens* and *S. epidermidis* and bactericidal against *P. aeruginosa* and *S. aureus* (**Fig. 2** and **3**). Saline and DNAase produced growth curves similar to the control (LB or BHI), which shows that dilution of the medium does not significantly affect the growth of the tested bacteria (**Fig. 2** and **3**). LAPS and the majority of modified supernatants showed at least a bacteriostatic effect on all tested strains.

However, all strains were adapted to the hostile environment generated by LAPS19, LAPS28and LAPS29 and so resumed their growth between 10 and 20 hours of incubation (**Fig.2** and **3**). In addition, the gram positive bacteria were adapted to LAPS16 (**Fig.3A** and **3B)** and *S. marcescens*, *S aureus* and *S. epidermidis* were adapted to LAPS26. Apparently an overload of nutrients (such as the dual source of carbohydrates and proteins in LAPS16 and LAPS19 or the maximum concentration of yeast extract in LAPS26) can positively affect the bacteria, enabling to overcome the antimicrobial effect of the supernatants.

Furthermore, the presence of boric acid (as is the case of LAPS28 and LAPS29) somehow prevents the antimicrobial effect of these supernatants. One possible explanation is that LAPS28 and LAPS29 were significantly more alkaline than the rest of the supernatants (**Fig.2**).

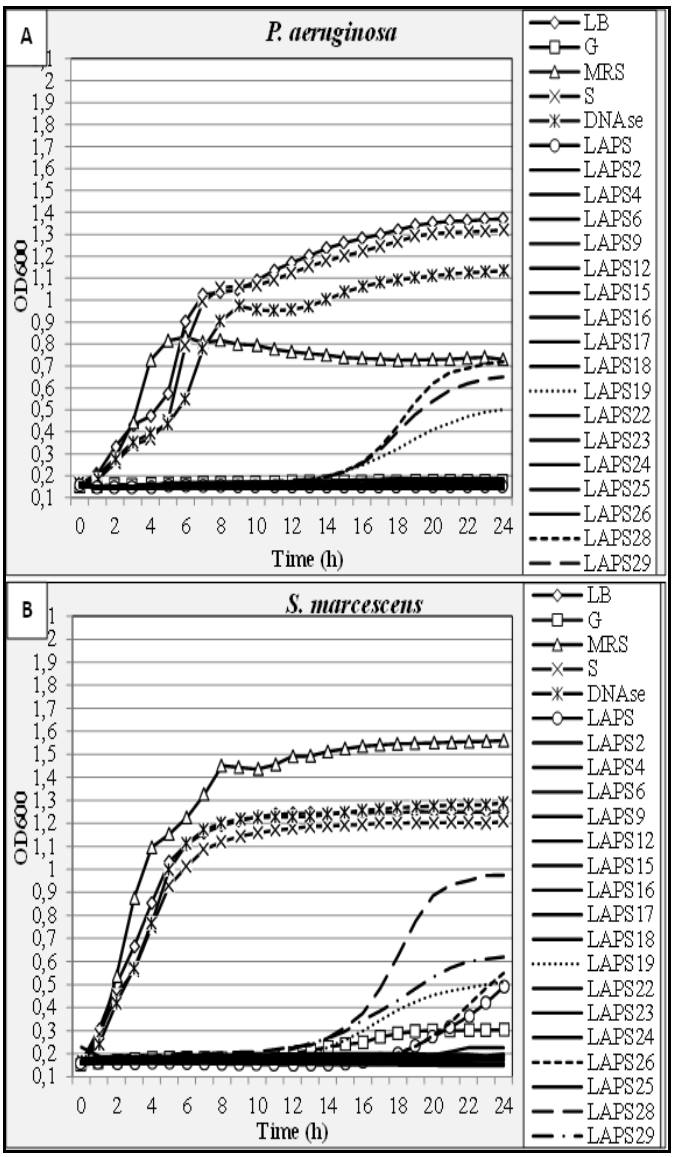

FIG.2: GROWTH CURVES OF THE PATHOGENIC GRAM NEGATIVES BACTERIA *P. AERUGINOSA* (A) AND *S. MARCESCENS* (B) OBTAINED IN PRESENCE OF SELECTED MODIFIED SUPERNATANTS (SEE SECTION 5.1 MATERIALS AND METHODS). LB (GROWTH CONTROL), G (GENTAMICIN 8 µg/mL) (ANTIMICROBIAL POSITIVE CONTROL), MRS (CONTROL FOR ORIGINAL MEDIUM COMPONENTS ACTIVITY), LAPS (CONTROL FOR ORIGINAL ANTIMICROBIAL ACTIVITY), S (SALINE) (DILUTION CONTROL).

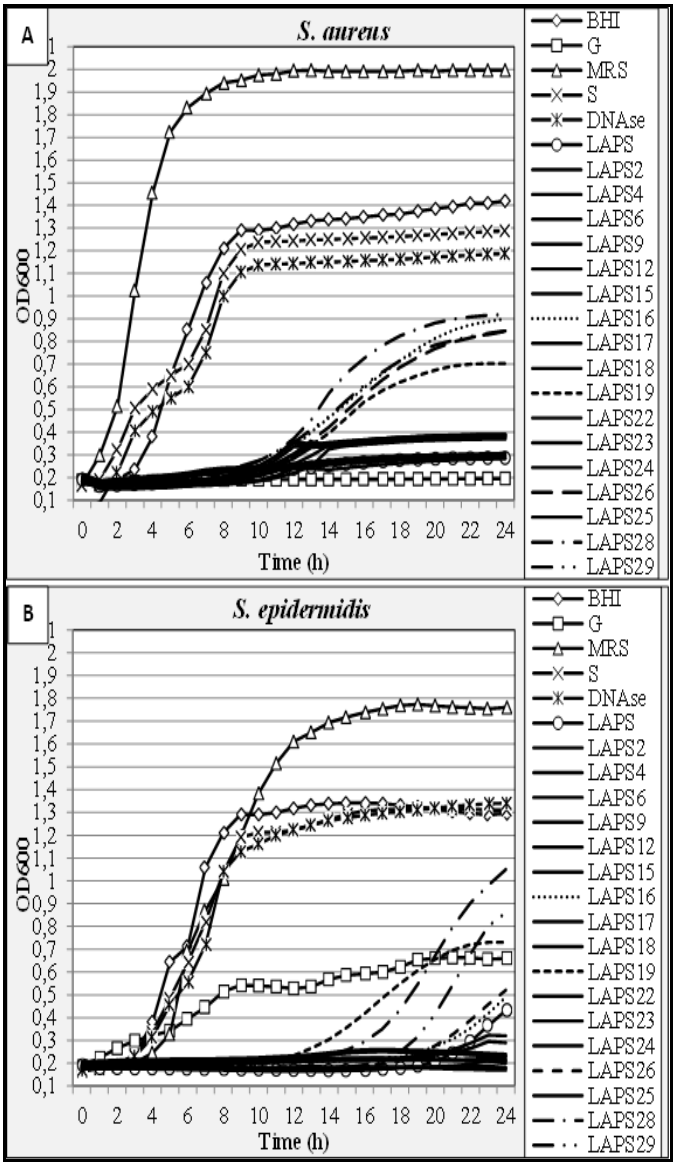

FIG.3: GROWTH CURVES OF THE PATHOGENIC GRAM POSITIVES BACTERIA *S. AUREUS* (A) AND *S. EPIDERMIDIS* (B) OBTAINED IN PRESENCE OF SELECTED MODIFIED SUPERNATANTS (SEE SECTION 5.1 MATERIALS AND METHODS). BHI (GROWTH CONTROL), G (GENTAMICIN 8 µg/mL) (ANTIMICROBIAL POSITIVE CONTROL), MRS (CONTROL FOR ORIGINAL MEDIUM COMPONENTS ACTIVITY), LAPS (CONTROL FOR ORIGINAL ANTIMICROBIAL ACTIVITY), S (SALINE) (DILUTION CONTROL).

3. Supernatants biofilm inhibition activity:

Besides the presence of plank tonic bacteria, there is an increasing evidence to believe that biofilm formation in wounds is the best unifying explanation for the failure of wound healing [27]. That is why one of the main properties sought in modified supernatants is the increase or preservation of biofilm inhibitory activity shown by the original LAPS [2, 3, 7]. The percentages of inhibition and stimulation of biofilm formation produced by controls and modified supernatants are shown in **Fig.4**. Control with gentamicin showed biofilm inhibitory capacity secondary to its antimicrobial activity. MRS behaved antagonistically on gram positive and gram negative bacteria. Inhibited biofilm formation of *P. aeruginosa* and *S. marcescens* possibly by its content in the surfactant Tween 80 (**Table 1**) and stimulated biofilm formation of both *staphylococci* perhaps encouraged by the extra supply of nutrients (**Fig.4**). Saline solution stimulated significantly the biofilm formation of gram positive bacteria. This would be due the contribution of NaCl to the system since staphylococcal biofilm formation is stimulated under high osmolarity conditions [28].

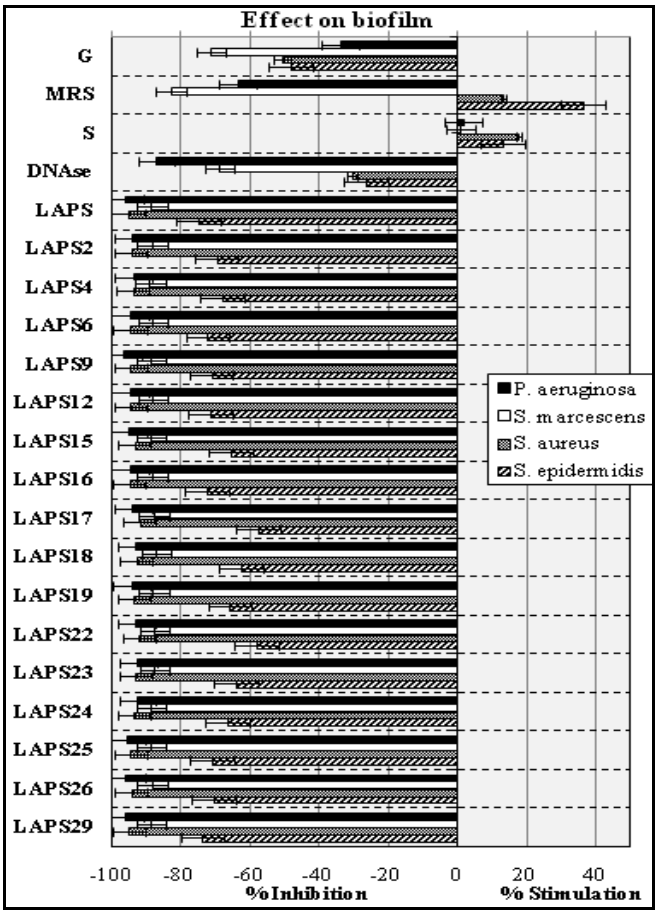

FIG.4: EFFECT ON BIOFILM FORMATION OF PATHOGENIC BACTERIA IN PRESENCE OF SELECTED MODIFIED SUPERNATANTS (SEE SECTION 5.1 MATERIALS AND METHODS). G (GENTAMICIN 8 µg/mL) (ANTIMICROBIAL CONTROL), MRS (CONTROL FOR ORIGINAL MEDIUM COMPONENTS ACTIVITY), S (SALINE) (DILUTION CONTROL), DNASE (BIOFILM INHIBITION CONTROL), LAPS (CONTROL FOR ORIGINAL ANTI-BIOFILM ACTIVITY).

Biofilm matrix of the tested strains contains DNA from bacterial secretion (*in vitro*) and/or from lysed host cells (*in vivo*) [29-31]. Because of this, it is logical observe biofilm inhibition in presence of DNAase

solution. Is worth noting that gram positive were less affected by DNAase, then it follows that gram positive biofilm matrix contain less DNA than gram negatives (**Fig. 4**). All modified supernatants showed significant inhibitory activity on all strains biofilm. However, this inhibition was similar to that shown by the original supernatant (LAPS).

Fig. 5 shows the results of *V. harveyi* BB170 bioassay. When supernatants were used without neutralizing (LAPS, and LAPS27 to LAPS32), no significant difference was observed between samples and negative controls (AB or MRS). In contrast, neutralized supernatants (NLAPS, and NLAPS27 to NLAPS32) induced a significant production of luminescence ($p<0.01$ for NLAPS, NLAPS27, NLAPS28, NLAPS30, NLAPS31 and NLAPS32 and $p<0.001$ for NLAPS29) (**Fig.5**).

Therefore *L. plantarum* produces AI-2 and these molecules are present in their supernatants. Greater induction of luminescence produced in presence of NLAPS29 (maximum concentration of boric acid), indicate that the chemical structure of the AI-2 present in the supernatants is possibly the aforementioned furanosil borate diester [16]. This is consistent with the fact that *L. plantarum* genome has the *lux S* gene (encoding the AI-2 synthase) (Gen Bank accession Nº NP_784522). Previously it was hypothesized that the molecule responsible for the inhibition of biofilm is AI-2 [8]. However, the supernatants obtained in the presence of boric acid (LAPS27 to LAPS29) do not show greater inhibitory activity than the original supernatant (LAPS) (**Fig.4**).

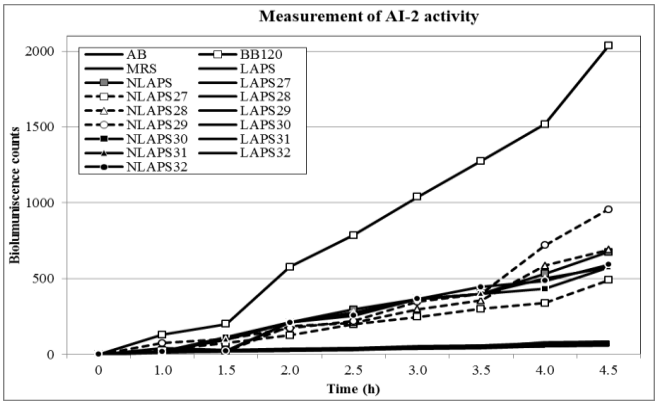

FIG.5: INDUCTION OF BIOLUMINESCENCE IN *V. HARVEYI* BB170 PRODUCED BY SUPERNATANTS OBTAINED IN PRESENCE OF DIFFERENT SOURCES OF BORON (LAPS27 TO LAPS32). AB (NEGATIVE CONTROL), BB120 (SUPERNATANT OF *V. HARVEYI* BB120 AS 100 % CONTROL), MRS (CONTROL OF CULTURE MEDIUM EFFECT).

4. Supernatants biofilm disruption activity:

Chronic wounds infecting bacteria are generally present in biofilm form [32].Therefore pharmaceutical products with biofilm disrupting activity are extremely necessary for chronic wound treatment. It was demonstrated that the biofilm disruption showed by LAPS [7, 8] is due to synergism between surfactants (Tween 80, 1-mono-linolein, 1,2-di-palmitin and distearin) [8, 33], an extracellular DNAase [8, 19] and variations in the ionic strength (Na, K and Mg salts) [8, 34]. This assay was designed to identify the supernatants that retain or increase the biofilm disrupting activity present in the original LAPS. The disrupting activity of controls and supernatants can be seen in **Fig. 6**.

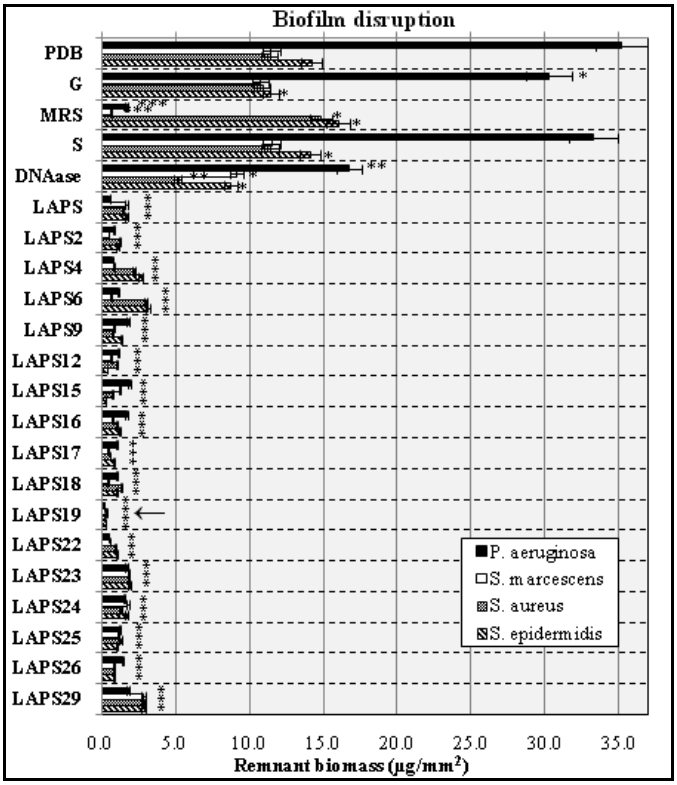

FIG. 6: BIOFILM DISRUPTION OF PATHOGENIC BACTERIA IN PRESENCE OF SELECTED MODIFIED SUPERNATANTS (SEE SECTION 5.1 MATERIALS AND METHODS). PDB (PRE-DISRUPTION BIOMASS), G (GENTAMICIN 8 µg/mL) (ANTIMICROBIAL CONTROL), MRS (CONTROL FOR ORIGINAL MEDIUM COMPONENTS ACTIVITY), S (SALINE) (DILUTION CONTROL), DNAASE (CONTROL FOR BIOFILM DISRUPTION), LAPS (CONTROL FOR ORIGINAL BIOFILM DISRUPTING ACTIVITY). STATISTICAL SIGNIFICANCE WAS CALCULATED COMPARED TO THE RESPECTIVE PRE-DISRUPTION BIOMASS (PDB) (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

PDB represent the pre-disruption biomass before treatment with controls and supernatants. Gentamicin and saline did not affect the biomass formed. Here it can be seen the well-known high resistance to antibiotics that have the biofilm

bacteria. DNAase had some disruptive activity. MRS also had a significant biofilm disruption activity on gram negatives probably due to its content in Tween 80 although stimulated biofilm formation in gram positives. LAPS and all modified supernatants showed intense disrupting activity. As expected, the most active supernatant were those with modifications in ionic strength (LAPS2, LAPS4 and LAPS6) and surfactant power (LAPS9). Besides the maximum activity was observed withLAPS19 (double source of proteins peptone-triptone) (black arrow) followed byLAPS26 (maximum concentration of yeast extract).

On the other hand, the highest concentrations of DNAase were obtained in LAPS19 and LAPS26 (**Table 2**) which would explain the greater biofilm disrupting activity observed for these supernatants (**Fig.6**).

TABLE 2: DNASE CONCENTRATION IN SUPERNATANTS OBTAINED FROM CULTURE MEDIA WITH MODIFICATIONS IN THE PROTEIN SOURCE. S (SUPERNATANT), X (AVERAGE HALOS OF ENZYMATIC ACTIVITY), Y= DNASE CONCENTRATION (µg/mL) CALCULATED THROUGH CALIBRATION CURVE.

S	X (mm)	$Y=0.0027.e^{0.4629X}$
LAPS	9,67	0,24
LAPS17	11,67	0,60
LAPS18	12,83	1,03
LAPS19	14,33	2,01
LAPS20	10,67	0,38
LAPS21	11,67	0,60
LAPS22	13,33	1,29
LAPS23	10,00	0,28
LAPS24	11,33	0,51
LAPS25	12,83	1,03
LAPS26	13,67	1,51

5. Anesthetic properties:

Numerous studies show that pain is present in the majority of chronic wounds [20, 35, 36]. Chronic wound-related pain constitutes a psychological stress or that triggers the hypothalamic-pituitary-adrenalax is promoting the production of vasopressin and cortisol [37]**.** Vasopressin is a potent vasoconstrictor compromising the delivery of oxygen and nutrients for wound healing. Cortisol reduces the immunoinflammatory response, suppresses cellular differentiation and proliferation, inhibits the regeneration of endothelial cells and delays collagen synthesis. In the presence of cortisol, T-cells become less responsive to the interleukin-1(IL-1) signalling for the production of growth factors that facilitate T-cell proliferation [38]. Therefore, pain is one of the most important challenges that must be faced in wound care treatment [39]. Because of this, the anesthetic capacity of LAPS26 was evaluated. The verbal scale allowed us to determine the sensations of volunteers in response to various stimuli (**Fig. 7**).

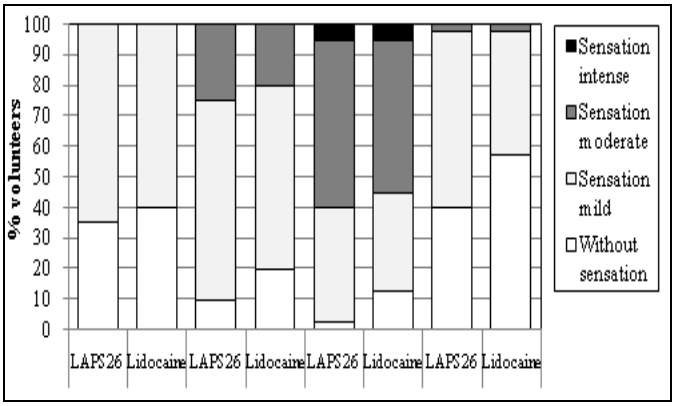

FIG.7: LAPS26 ANESTHETIC PROPERTIES TO VARIOUS SENSORY STIMULI. SENSATIONS IN HEALTHY VOLUNTEERS WERE ASSESSED THROUGH A VERBAL SCALE. PERCENTAGES REPRESENT THE AMOUNT OF VOLUNTEERS THAT REPORT EACH SENSATION

The results with LAPS26 were very similar to those obtained in presence of lidocaine (2% + epinefrin 1:200000). Between 97-100% of patients reported no sensation or mild sensation in response to distension or temperature stimuli. Besides, between 90-95% of patients reported mild or moderate sensation in response to pressure or non-penetrative needling stimuli. This means that LAPS26 (maximum concentration of yeast extract) have ananesthetic effects lightly inferior to lidocaine.

As previously described yeast extract (YE) (a main component of MRS broth) is a source of barbiturates precursors for *L. plantarum* [8].YEis a water-soluble extract from an autolysate of *Saccharomyces cerevisiae* cells. The final metabolite in *S. cerevisiae* pyrimidines metabolism is 5-methyl barbituric acid [40]. We propose that the enzymatic machinery of *L. plantarum* produces methylations in 5 – methyl - barbituric acid transforming it into 5, 5-diethyl-barbituric acid (DEBA) (**Fig. 8**). In fact, there is a correlation between the concentration of yeast extract in the culture media and the amount of DEBA found in the respective supernatant as is shown in **Table 3**.

TABLE 3: AMOUNT 5,5-DIETHYL-BARBITURIC ACID (DEBA) PRODUCED BY *L. PLANTARUM* IN TERMS OF YEAST EXTRACT CONCENTRATION IN CULTURE MEDIUM. YE (YEAST EXTRACT CONCENTRATION IN THE CULTURE MEDIUM), AAA (AVERAGE ABSOLUTE AREA OF THE CORRESPONDING PEAKS IN THE GAS CHROMATOGRAM), Y (DEBA CONCENTRATION IN THE EXTRACT CALCULATED THROUGH CALIBRATION CURVE), SC (DEBA CONCENTRATION IN THE SUPERNATANT), μ (BARBITURATE PRODUCTION RATE).

Supernatant	YE (g/L)	X=AAA	$Y = 6.10^{-5}X - 3.01$	SC (mg/L)	μ= (mg/L.h)
			Y (mg/L)		
LAPS	5	1995000	116,69	1,1669	0,0972
LAPS23	6	2000015	116,99	1,1699	0,0975
LAPS24	7	2050000	119,99	1,1999	0,1000
LAPS25	8	2085045	122,09	1,2209	0,1017
LAPS26	9	2172850	127,36	1,2736	0,1061

We propose that DEBA is responsible for the observed local anesthetic action as already described for other barbiturates [41]. Furthermore, it is possible that the presence of DEBA in *L. plantarum* supernatants could be beneficial in chronic wounds treatment because some barbiturates can promote wound healing via enhancing collagenization [42].

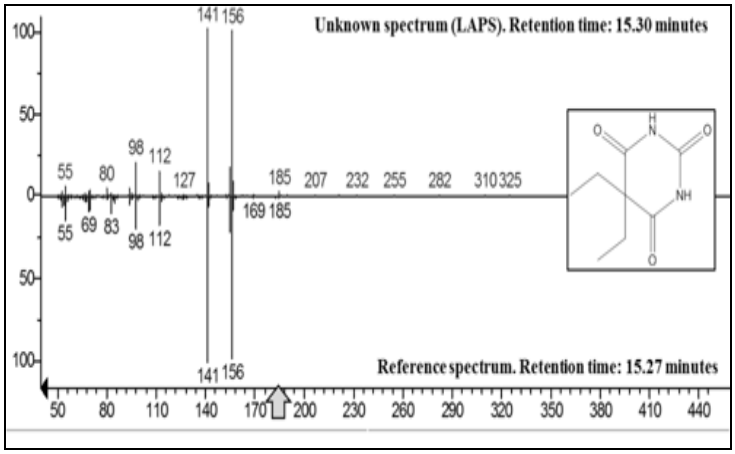

FIG. 8: COMPARATIVE GRAPH BETWEEN THE REFERENCE MASS SPECTRUM (BOTTOM) AND THE UNKNOWN SPECTRUM (ABOVE). THERE IS A 91% SIMILARITY BETWEEN BOTH SPECTRA AND RETENTION TIMES (GAS CHROMATOGRAM) ARE VIRTUALLY IDENTICAL IN THESE RUNNING CONDITIONS. BESIDES, THE ARROW SHOWS THE MOLECULAR ION OF 184 PRESENT IN BOTH SPECTRA. THEREFORE, THE MOLECULE WAS IDENTIFIED AS 5,5-DIETHYL-2,4,6(1H,3H,5H)-PYRIDINETRIONE (5,5-DIETHYL-BARBITURIC ACID).

9. Safety evaluation:

There is great safety in the application of the supernatants because no edema or erythema was observed with the application of LAPS12, LAPS19 and LAPS26. However, one male volunteer reported itching after 20 minutes of applying LAPS12 and LAPS19possibly due to high concentrations of lactic acid. With the application of LAPS29, two female patients showed edema and/or erythema at 24 h of application and redness/itching sensation. This could probably due to the presence of boric acid in the supernatant and must be taking into account in the future during culture media design.

CONCLUSION: The results (**Table 4**) showed that supernatants can be obtained with enhanced properties. Therefore, this will allow in the future a LAPS manufacture with a greater therapeutic effectiveness and even custom properties for each type of wound.

TABLE 4: COMPARATIVE TABLE OF RESULTS THE SYMBOLS (↑), (↓) AND (=) REPRESENTS RESPECTIVELY A HIGHER, LOWER OR SIMILAR ACTIVITY COMPARED TO THE ORIGINAL SUPERNATANT (LAPS).

LAPS	pH	Antimicrobialaction				Biofilminhibition				Biofilmdisruption				AI-2	DNAse	DEBA
		Pa	Sm	Sa	Se	Pa	Sm	Sa	Se	Pa	Sm	Sa	Se			
1	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	↓	=	↑	=	↑	=	=	=	=	↑	↑	↑	↑	-	-	-
3	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	↓	=	↑	=	↑	=	=	=	=	↑	↑	↑	↑	-	-	-
5	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	↓	=	↑	=	↑	=	=	=	=	↑	↑	↑	↑	-	-	-
7	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

9	↓	=	↑	=	↑	=	=	=	=	↑	↑	↑	↑	-	-	-
10	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	↓	=	↑	=	↑	=	=	=	=	=	=	=	=	-	-	-
13	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	↓	=	↑	=	↑	=	=	=	=	=	=	=	=	-	-	-
16	↓	=	↑	↓	=	=	=	=	=	=	=	=	=	-	-	-
17	↓	=	↑	=	↑	=	=	=	↓	=	=	=	=	-	↑	-
18	↓	=	↑	=	↑	=	=	=	=	=	=	=	=	-	↑	-
19	↓	↓	↓	↓	=	=	=	=	=	↑	↑	↑	↑	-	↑	-
20	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-
21	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-
22	↓	=	↑	=	↑	=	=	=	=	=	=	=	=	-	↑	-
23	↓	=	↑	=	↑	=	=	=	=	=	=	=	=	-	↑	↑
24	↓	=	↑	=	↑	=	=	=	=	=	=	=	=	-	↑	↑
25	↓	=	↑	=	↑	=	=	=	=	=	=	=	=	-	↑	↑
26	↓	=	=	↓	↓	=	=	=	=	↑	↑	↑	↑	-	↑	↑
27	↑	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-
28	↑	↓	↓	↓	↓	=	=	=	=	=	=	=	=	↑	-	-
29	↑	↓	↓	↓	↓	=	=	=	=	=	=	=	=	↑	-	-
30	↑	-	-	-	-	-	-	-	-	-	-	-	-	=	-	-
31	↑	-	-	-	-	-	-	-	-	-	-	-	-	=	-	-
32	↑	-	-	-	-	-	-	-	-	-	-	-	-	=	-	-

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CONFLICT OF INTERESTS: The authors declare that there is no conflict of interests regarding the publication of this paper.

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