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RESEARCH ARTICLE

Formulation and quality control of semi-solid containing harmless bacteria by-products: chronic wounds pro-healing activity

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Abstract

Chronic wounds are those that remain in a chronic inflammatory state and fail to follow normal healing process. Infection is one of the most important causes of chronicity. A frequent pathogen isolated from chronic infections is *Pseudomonas aeruginosa*; refractory to therapy and host immune attack in its biofilm phenotype. *Lactobacillus plantarum* cultures supernatants (LAPS) interfere with its pathogenic capacity. In addition, LAPS showed bacteriostatic and bactericide properties and is neither cytotoxic nor an inductor of necrosis-apoptosis. LAPSs chemical composition was determined; allowing us to propose a correlation between its constituents and their biological activity. This article shows a pharmaceutical dosage form designed by using LAPS as an API with pro-healing activity and its quality control. Pharmacotechnical and anti-microbial assays were adapted to demonstrate that the vehicle used does not modify LAPS activities. Selected formulation (F100) showed fair mechanical and technological properties. From the *in vitro* release assays was found an adequate release from the carrier matrix and maintains its anti-pathogenic activity for 6 months. We propose F100 for chronic wounds treatment. The use of harmless bacteria by-products, such as LAPS, to antagonize infectious pathogens that have ability to form biofilm is an efficient and economic approach to treat infected chronic wounds.

Keywords

Bacteria by products, chronic wounds, formulation, quality control

History

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Introduction

Chronic wounds are those that remain in a chronic inflammatory state and therefore fail to follow normal healing process patterns[1–4], being infection one of the most important causes of the chronicity[3]. A frequent pathogen isolated from chronic infections is *Pseudomonas aeruginosa*, a Gram-negative opportunist organism[2]. This bacterium is extremely refractory to therapy and host immune attack when living in biofilm phenotype[5]. Bjarnsholt et al. suggested that the lack of proper wound healing is partially caused by inefficient eradication of pathogens like *P. aeruginosa*[6].

Lactobacillus plantarum ATCC 10241 cultures supernatants (LAPS) interfere with the *P. aeruginosa* pathogenic capacity by inhibiting *in vitro* adhesion, quorum sensing, biofilm and virulence factors like elastase, pyocyanin and rhamnolipids[7,8]. In addition, LAPS showed bacteriostatic and bactericide properties and a great biofilm disrupting capacity[5,8]. LAPS is neither cytotoxic nor an inductor of necrosis-apoptosis when evaluated using polymorphonuclear leukocytes in *ex vivo* model, and key cells in chronic wounds, and inflammatory response *in vivo* on a mouse model[9]. The chemical composition of LAPS was determined; allowing us to propose a correlation between the chemical constituents and their biological activity[10].

Given the direct and indirect pro-healing properties and safety demonstrated by LAPS, the design of pharmaceutical dosage form to facilitate their administration in the treatment of infected chronic wounds has become a necessity. The presence of chronic wound exudates whose volume increases in heavily colonized or infected ulcers[11,12] represents another barrier for healing[13,14]. These exudates are rich in matrix metalloproteinases (MMPs) and pro-inflammatory cytoquines, which contribute to chronification[15]. The formulation designed should contribute to the control of low and medium volume of wounds exudates, adequate bioadhesion and allowing the patient to remain ambulatory.

The aims of this work were to design a pharmaceutical dosage form and ensure its quality control by using LAPS as an active pharmaceutical ingredient (API), with pro-healing activity that contribute to diminish the volume of wounds exudates and display adequate bioadhesion to mammalian cells. Since LAPS is an API of biological origin with many components, the formulation seeks

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to be as simple as possible. Cellulose and carbomer polymers were used as vehicle for LAPS. Pharmacotechnical assays were performed as required by Farmacopea Argentina Sixth Edition. Besides, several anti-microbial assays were performed to demonstrate that the vehicle used does not modify the LAPS activities.

Materials and methods

Gel material

Carbomer 940, hidroxyethylcellulose, methylparaben, glycerol and triethanolamine were donated from Fabriquimica-Buenos Aires, Argentina.

Bacterial strains and culture conditions

Pseudomonas aeruginosa ATCC 27853 was grown in Luria–Bertani (LB) medium and *L. plantarum* ATCC 10241 (initial $DO_{600} = 0.1$) in de Man Rogosa Sharpe (MRS) broth (Britania – Buenos Aires, Argentina) and kept at 37 °C for 24 h and 12 h, respectively. *Lactobacillus plantarum* reaches stationary phase and maximum concentration of bioactive metabolites in this time period[16].

L. plantarum supernatants

After 12 h of static cultivation, supernatants of *L. plantarum* were recovered after centrifugation (8000 rpm, 15 min) and subsequent filtration through 0.22-μm membranes. The pH was pH determinate as 5.22 ± 0.43.

Formulation design

Pre-formulation studies were carried out using nine different formulations (Table 1) in order to evaluate pharmacotechnical aspects such as: pH, extensibility, viscosity, organoleptic properties, stability and sanitary assays. Microbiological evaluation (sanitary assays) was developed as required on Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT) disposition N° 7667.

Physicochemical evaluation at 0 and 180 d

Gel stability studies were made at day 0 ($F100_{(0)}$) and after a period of 6 months ($F100_{(180)}$). In the selected formulation case, LAPS was use as API and as vehicle (non-water was added, for the semi-solid formulation). Variation of its physical and chemical properties and anti-pathogenic capacities were analyzed. Each assay was made using two negative controls: carbomer gel 1.6% (C_{940}) and a semi-solid formulation using the same carbomer concentration formulated with MRS broth as vehicle (C_{MRS}).

Table 1. Composition of all formulations designed (pre-formulation).

F	LAPS (mL)	HEC (g)	Cm (g)	Pb (g)	TEA (mL)	W_d (mL)
F10	250	16	–	2	–	722
F20	250	20	–	2	–	718
F25	250	–	16	2	1.7	730
F35	250	–	18	2	1.7	728
F45	250	–	20	2	1.7	726
F55	500	–	16	2	1.7	480
F100	972	–	16	2	1.7	–
F118	972	–	18	2	1.7	–
F120	972	–	20	2	1.7	–

Formulation code (F), *Lactobacillus plantarum* supernatants (LAPS), hidroxy ethyl cellulose (HEC), Carbomer (Cm), Parabens (Pb), triethanolamine (TEA), Distillate water (W_d).

Homogeneity assays

$F100_{(0)}$ and $F100_{(180)}$ were applied as a thin layer on polypropylene slides. The homogeneity was tested by visual appearance after application. In parallel, 3 g of the same formulations were used for centrifugation assays (15 min, 3500 rpm) and separation in phases was observed[17]. Under these conditions the homogeneity of $F100_{(0)}$ and $F100_{(180)}$ was qualitatively qualified as follows: very good (no phase separation), good (appearance of small volume of supernatant), regular (phase separation with slight appearance of clotted) and poor (separation of the phases with appearance of pellet).

pH measurements

The pH of $F100_{(0)}$ and $F100_{(180)}$ was measured in a pHmeter (Broadley James Corporation, Irvine, CA) by dipping the glass electrode into the gel[17].

Rheological studies

The viscosity of $F100_{(0)}$ and $F100_{(180)}$was determined at 25 °C (stored temperature), 32 °C (healthy skin temperature) and 37 °C (injury skin temperature) by using a Cannon viscometer with spindle N° 8 from 3 rpm to 60 rpm (Cannon Instrument Company, LV2000 model, Pennsylvania, EEUU)[17].

Spreadability assays

The spreadability of $F100_{(0)}$ and$F100_{(180)}$ was evaluated using a extensometer consisted in two plates, the lower plate holds the sample (0.5 g) and the upper plate (28 g) exerts forces to the sample[18]. Force is generated by adding known weigh in the upper plate (5 g) at pre-determinate times. Each formulation was measured at constant temperature in triplicate.

In-vitro release assays

In vitro *passive permeation studies*

This assay was conducted using vertical type Franz diffusion cells[19–21] having a receptor compartment capacity of 10 mL. Cellulose membranes (D9527 avg. flat width 43 mm (1.7 *in.*) Sigma Aldrich Chemical CO., St. Louis, MO) were mounted between the half-cells in contact with receptor fluid (0.9% NaCl) and were equilibrated for 1 h. The area available for diffusion was 1.8 cm^2. The fluid in the receptor compartment was maintained at 32 ± 0.5 °C (skin temperature). Semi-solid formulation (0.2 g) was placed in the donor compartment. The entire assembly was kept on a magnetic stirrer (100 rpm) and at each time four cells were removed from the system, aliquots (2 mL) of the receptor phase at specific time intervals (2, 4, 6 and 8 h). The OD_{300} of the solution was measured in a UV spectrophotometer (Thermo Spectronic Genesys 10 UV-Rochester, NY). Cumulative amounts of LAPS that permeated the diffusion unit surface (cm^2) were plotted against time (h). The results were expressed as mean ± standard deviation (SD) ($n = 16$).

Membrane retention assays

At each time, cellulose membranes were washed with NaCl solution (0.85 %), cut in pieces, and placed on tubes with 10 mL of NaCl solution. Stirred for 40 min at 32 °C and then 2 mL of receptor fluid was withdrawn[21]. The OD_{300} of the receptor fluid was measured in a UV spectrophotometer. Cumulative amounts of LAPS (mg) retained on the unit diffusion surface (cm^2) was plotted against time (h)[22]. The results were expressed as mean ± SD ($n = 16$).

Anti-pathogenic properties conservation assays;

Agar diffusion assays

Pseudomonas aeruginosa was grown in LB broth and then about 10^5 CFU mL^{-1} were transferred to LB agar. Wells of 7 mm of diameter were made in LB agar. To test the inhibitory effect of LAPS vehiculized, aliquots (200 µL) of LAPS, $F100_{(0)}$ and $F100_{(180)}$ were placed in the wells. Gentamicin 8 µg/mL (commercial topical gel concentration) and C_{940} and C_{MRS} were used, respectively, as positive and negative controls. The plates were kept at 4 °C for 1 h to allow the diffusion and then incubated for 24 h at 37 °C. The diameter of the inhibition zone was measured. All experiments were performed in triplicate and the results were expressed as mean ± SD.

Effect on growth

In 96-well polystyrene microtiter plates (Costar Corning Inc., Union City, CA), were added 40 µL of LB and, respectively, 80 µL of each sample (LAPS, $F100_{(0)}$, $F100_{(180)}$), gentamicin (8 µg/mL) (as anti-microbial positive control), MRS (control for medium components) and PBS (as anti-microbial negative control). *Pseudomonas aeruginosa* was cultured overnight in LB broth and then 80 µL of this suspension ($OD_{600} = 0.150$) were added in the wells and incubated for 24 h at 37 °C. In order to conduct this assay using semi-solid samples, an adaptation of the techniques for liquid samples was performed and the formulations were diluted 1:2 in MRS. The OD_{600} was measured in a microplate reader. The percentages of stimulation or inhibition were calculated using PBS as 0%. Results were expressed as percentage mean ± SD ($n = 8$)[8].

Effect on biofilm

The content of the wells of the above assay (see ''Effect on growth'' section) was discarded and the wells were washed thrice with saline. The remaining attached biomass (biofilm) was stained during 15 min with 200 µL of crystal violet (0.4%). Cell-attached dye was solubilized with 200 µL of DMSO (Sigma-Aldrich Co., Buenos Aires, Argentina) and the OD_{570} 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 PBS as 0%. Results were expressed as percentage mean ± SD ($n = 8$)[8].

Effect on pre-formed biofilm

For this assay, 80 µL of an overnight *P. aeruginosa* culture in LB ($OD_{600} = 0.150$) and 120 µL of LB were placed in 96-well polystyrene microtiter plates and incubated during 24 h at 37 °C. The content of the wells was discarded and the remaining attached biomass (biofilm) was washed thrice with sterile saline. Then, 120 µL of LB broth and 80 µL of each sample (LAPS, $F100_{(0)}$, $F100_{(180)}$), gentamicin (8 µg/mL) (as anti-microbial positive control), MRS (control for medium components) and PBS (as disruption negative control) were added in the wells and incubated for 6 h at 37 °C. The content of the wells were discarded and the wells were washed thrice with saline. The remaining attached biomass (biofilm) was stained for 15 min with 200 µL of crystal violet (0.4%). Cell-attached dye was solubilized with 200 µL of DMSO (Sigma-Aldrich Co., USA) and the OD_{570} was measured in a microplate reader. The measured OD is directly proportional to the biomass (biofilm) remaining after disruption. The results were expressed as mean ± SD ($n = 3$), considering PBS as 0% of biofilm disruption[8].

Scanning electron microscopy

Biofilms of *P. aeruginosa* ATCC 27853 were grown in 96-well microtiter plates, as described in *Effect on biofilm* and *Effect on pre-formed biofilm* section, with a piece of Permanox™ slide (NalgeNunc International, Penfield, NY) in each well. The samples obtained from biofilm inhibition and biofilm disruption assays were fixed in 2.5% glutaraldehyde for 2.5 h, washed with 100 mM cacodylate buffer pH 7.2, treated for 2 h with 2% osmium tetroxide, washed again with 100 mM cacodylate buffer pH 7.2 and dehydrated in increasing concentrations of acetone. The Permanox slides were dried by the CO_2 critical point technique (CPD 030 Balzers, Liechtenstein), fixed on aluminum stubs, covered with gold film and examined in a JEOL JSM-6060 scanning electron microscope[23].

Statistical analysis of data

Data analysis results were expressed as a mean ± SD. The student *t*-test was performed and $p < 0.05$, $p < 0.01$ and $p < 0.001$ was considered statistically significant.

Results

Formulation design

Based on preliminary experiments (Table 2), was selected the formulation that achieved the mechanical properties required for a semi-solid and ensured the maintenance of the LAPS properties. The tests previously performed showed that F100 formulation (Carbomer (1.6%) + LAPS at 100%) demonstrate the best characteristics for the purposes of this study. In this sense, this formulation was chosen for further studies.

Physicochemical evaluation of F100 at 0 and 180 d

Table 3 shows the values of physicochemical properties determined for F100 at 0 and 180 d ($F100_{(0)}$ and $F100_{(180)}$, respectively) after production (aging test). The pH values of the formulations ranged from 5.2 for $F100_{(0)}$ to 5.9 for $F100_{(180)}$. Viscosity was measured at three different temperatures (25 °C, 32 °C and 37 °C) and in all cases the values were significantly different from the controls, $F100_{(0)}$ and $F100_{(180)}$. The rheological assay showed that the semi-solid formulation designed, displayed

Table 2. Pharmacotechnical aspects assayed: Extensibility (E), Viscosity (V), pH determination (pH) and microbiological evaluation (M).

F	E (mm^2)	V (cP)	pH	M (UFCg^{-1})
F10	4901.7 ± 7.03[a]	4578.6 ± 4.1[a]	4.6[a]	28
CF10	4536.4 ± 8.32	5445.4 ± 3.2	6.7	14
F20	4704.1 ± 1.45[a]	4875.2 ± 10.2[a]	4.4[a]	21
CF20	4599.0 ± 5.17	5624.1 ± 2.1	6.8	14
F25	1694.5 ± 3.43[a]	2250.1 ± 3.8[a]	5.02[a]	16
CF25	1538.2 ± 4.11	2475.7 ± 2.4	5.9	20
F35	1423.2 ± 5.21[a]	2254.4 ± 3.6[a]	5.02[a]	45
CF35	1334.6 ± 4.44	2472.5 ± 4.2	6.0	12
F45	1410.8 ± 8.11	2231.1 ± 4.2	5.05[a]	3
CF45	1400.7 ± 7.45	2245.4 ± 2.7	6.2	14
F55	1544.5 ± 9.23	2251.4 ± 9.1	5.1[a]	3
CF55	1500.1 ± 7.12	2266.4 ± 7.8	5.9	12
F100	1964.5 ± 8.23	2262.2 ± 3.9	5.7	2
CF100	1755.6 ± 9.11	2372.3 ± 4.4	5.5	10
F118	1858.2 ± 11.30	2248.7 ± 5.8	5.4[a]	7
CF118	1715.6 ± 5.14	2251.9 ± 2.6	5.9	11
F120	1806.4 ± 7.31[a]	2247.0 ± 1.8	5.9	3
CF120	1654.4 ± 4.23	2256.9 ± 2.8	6.2	14

All formulations tested (F) were compared to its respective control (CF).
[a]Significantly different from control ($p < 0.05$).

Table 3. Physicochemical properties for aging test: pH, Viscosity (V): centipoise (cP), Spreadability (S), Homogeneity (H).

	pH	V (cP) 25 °C	V (cP) 32 °C	V (cP) 37 °C	S (mm^2)	H
$Control_0$	5.7	2372.25 ± 4.4	2368.3 ± 1.3	2364 ± 2.5	1656.3 ± 0.2	VG
$F100_0$	5.2[a]	2252.3 ± 3.9[b]	2255.0 ± 0.8[b]	2258.3 ± 1.9[b]	1964.5 ± 0.03[b]	VG
$Control_{180}$	5.8	2387.23 ± 2.8	2390.5 ± 0.6	2391.2 ± 1.6	1538.2 ± 0.6	VG
$F100_{180}$	5.9	2292.04 ± 1.2[b]	2291.1 ± 1.4[b]	2295.01 ± 2.3[b]	1745.6 ± 0.1[b]	VG

Results represent the average of values obtained from measurements at 0 ($F100_0$) and after 6 months of shelf life ($F100_{180}$) ± SD.
Qualitative scale: VG (very good), G (good), B (bad).
[a]Significantly different from control ($p < 0.01$).
[b]Significantly different from control ($p < 0.001$).

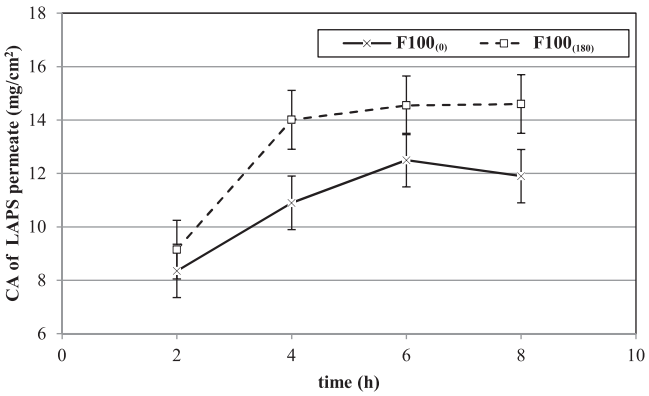

Figure 1. Cumulative amount (CA) of LAPS (mg/cm^2) permeated through cellulose membrane from $F100_{(0)}$ and $F100_{(180)}$. The data shown represent the mean ± SD of 16 replicates.

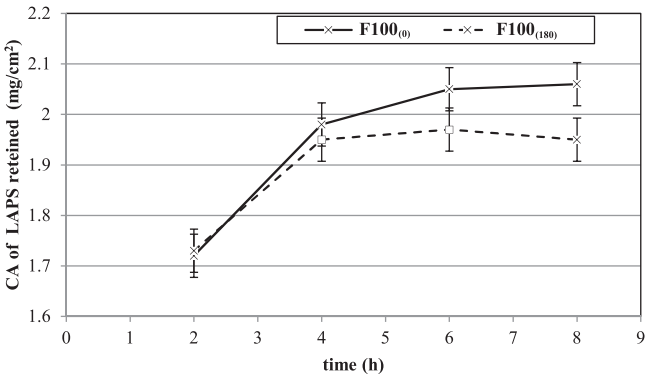

Figure 2. Cumulative amount (CA) of LAPS (mg/cm^2) retained on cellulose membrane from $F100_{(0)}$ and $F100_{(180)}$. The data shown represent the mean ± SD of 16 replicates.

thixotropic behavior. Spreadability test were performed to $F100_{(0)}$ and $F100_{(180)}$ showed a very good spreadability.

In-vitro release assays

The *in vitro* passive permeation studies were performed during 8 h, investigating the formulations $F100_{(0)}$ and $F100_{(180)}$. Figure 1 shows the passive permeation values of LAPS (mg/cm^2) through cellulose membrane during time (h). It was not observed significantly difference between $F100_{(0)}$ and $F100_{(180)}$ when the release profile reached the stationary state at 6 h. Figure 2 shows the values of LAPS cumulative amount in mg/cm^2 in membrane retention assays. The retained amount values measured also presented no significantly difference between $F100_{(0)}$ and $F100_{(180)}$, corroborating with the permeation results. In addition, the total amount of LAPS released from $F100_{(0)}$ and $F100_{(180)}$ polymeric matrix was calculated using the concentrations average sum

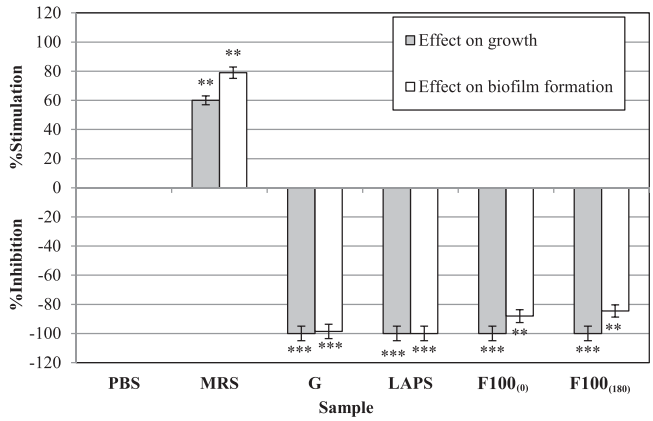

Figure 3. Effect on growth (grey bars) and biofilm formation (white bars) of *P. aeruginosa* produced by different samples: PBS (0% inhibition and 0% stimulation) MRS (control for LAPS), gentamicin 8 µg/mL (G), LAPS, $F100_{(0)}$ and $F100_{(180)}$. Significant differences were observed when compared to control (PBS): $^{**}p < 0.01$; $^{***}p < 0.001$.

of passive permeation and retained on membrane assays for the stationary state the values were 14.55 ± 1.4 mg/cm^2 for $F100_{(0)}$ and 16.57 ± 1.8 mg/cm^2 for $F100_{(180)}$, whose results were not significantly different.

Anti-pathogenic properties conservation assays

Inhibition zone for *P. aeruginosa* ATCC 27853 in the presence of LAPS, $F100_{(0)}$ and $F100_{(180)}$ were measured. The obtained values were 20 ± 1 mm, 19 ± 2 mm and 18 ± 3 mm, respectively, for LAPS, $F100_{(0)}$ and $F100_{(180)}$. The positive control (gentamicin) presented a zone inhibition of 30 ± 2 mm while negative controls (PBS, MRS, $FC_{(0)}$ and $FC_{(180)}$) showed no inhibition zone.

Figure 3 shows effect on planktonic growth (grey bars) and biofilm formation (white bars) of *P. aeruginosa* produced by different samples. MRS stimulate both planktonic and biofilm growth. Gentamicin and LAPS inhibit completely both parameters while $F100_{(0)}$ and $F100_{(180)}$ retain the LAPS growth inhibitory capacity and partially lose the biofilm inhibitory capacity.

The samples effect on *P. aeruginosa* biofilm formation was also observed by SEM using the hydrophobic polymer Permanox as substratum (Figure 4). In the presence of PBS, images showed a dense and uniform pseudomonal biofilm covering the polymeric surface. In this case the biofilm obtained shows a three-dimensional structure and a normal amount of exopolysaccharides in the matrix (Figure 4, PBS: 30 000×). *Pseudomonas aeruginosa* cells show normal bacillus structure surrounded by matrix represented as a roughness cell surface (Figure 4, PBS: 30 000×). With MRS (negative control for LAPS), *P. aeruginosa*

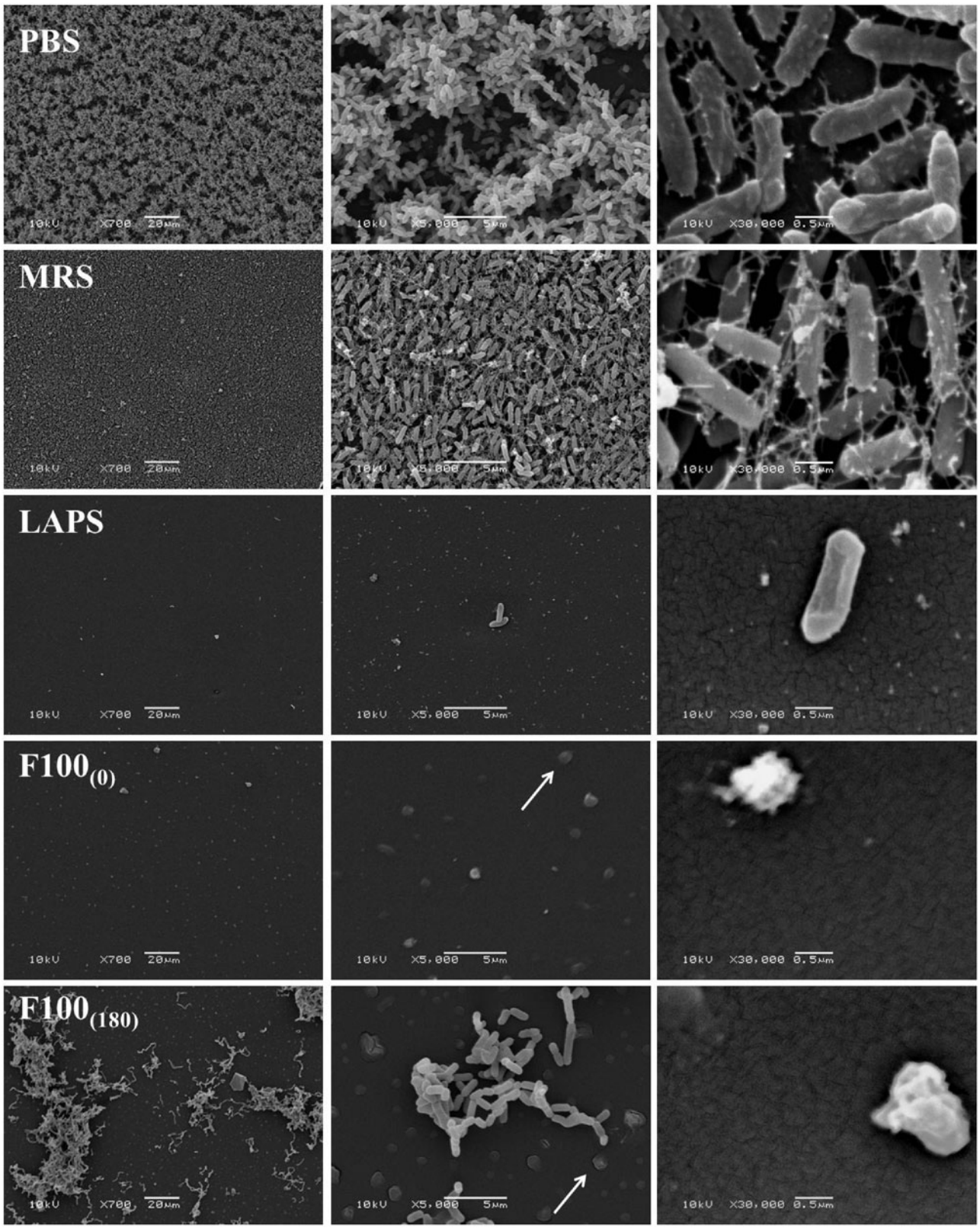

Figure 4. *Pseudomonas aeruginosa* biofilm formation observed by scanning electron microscopy (SEM). Each line shows biofilm formation in presence of PBS (negative control), MRS (control for LAPS), LAPS, $F100_{(0)}$ and $F100_{(180)}$. Optical magnification: 700×; 5000× and 50 000×. Scale bars: 20 µm, 5 µm and 0.5 µm, respectively. Arrows shows formulation micro-particles.

biofilm covered completely the Permanox surface but lost much of its three-dimensional structure forming a flat biofilm (Figure 4, MRS: 700× and 5000×) with a matrix hyper production (Figure 4, MRS: 5000× and 30 000×). In LAPS and $F100_{(0)}$ presence neither attachment nor biofilm formation was observed at the surface of Permanox (Figure 4, 700×; 5000×; 30 000×). With $F100_{(180)}$ the biofilm displayed a significant reduction in the number of adherent bacteria and in the size of aggregates, which were reduced to small clusters or even single cells (compared to negative control) (Figure 4, 700 and 5000×). Therefore, SEM images were very consistent to the results obtained by crystal violet assay.

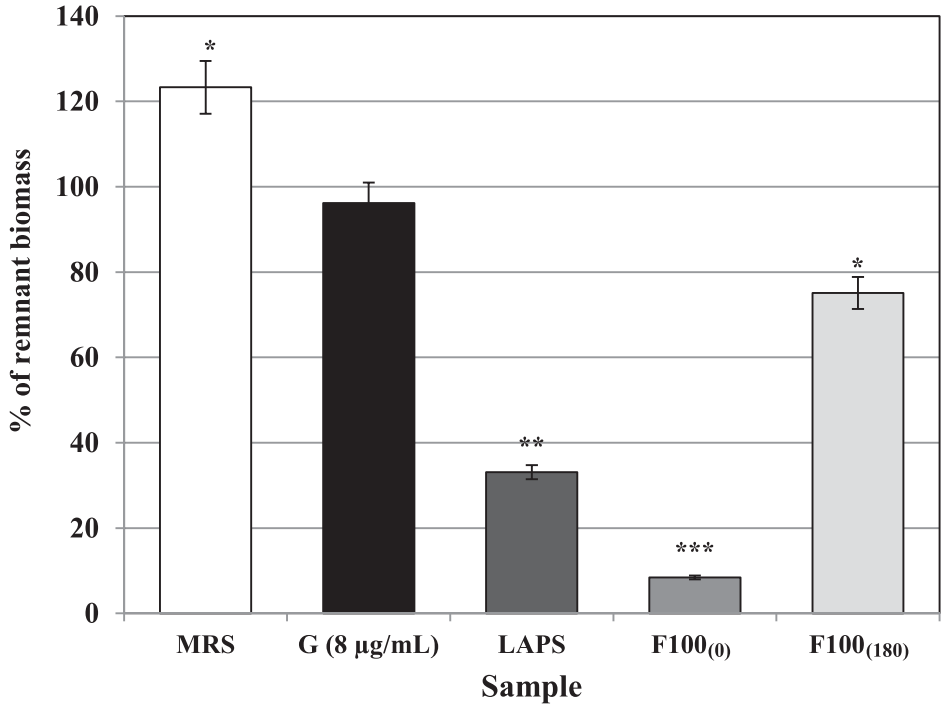

Figure 5. Remnant biomass of *P. aeruginosa* after disruption treatments: MRS (control for LAPS), gentamicin 8 µg/mL (G), LAPS, $F100_{(0)}$ and $F100_{(180)}$. Significant differences were observed compared to negative control (PBS): $*p<0.05$; $**p<0.01$; $***p<0.001$.

Figure 5 shows the remnant biomass of *P. aeruginosa* after biofilm disrupting treatment. MRS increased the biomass formed in the wells, while gentamicin did not show biofilm disruption activity. LAPS and its formulations showed an important ability to disrupt pre-formed biofilms (LAPS: $67 \pm 5\%$; $F100_{(0)}$:92 ± 3; $F100_{(180)}$: 25 ± 4).

Figure 6 shows *P. aeruginosa* biofilm disruption observed by SEM. Once again images were consistent to the results obtained by crystal violet assay.

Discussion

LAPS is an active pharmaceutical ingredient (API) from biological origin with a complex composition. In this sense, the formulation must be simple, adequate for bioadhesion and be easy to apply, allowing patient care on an outpatient level, thus, the selection criteria were that the formulation should maintain an adequate pH value and viscosity, good adhesion and must preserve LAPS anti-pathogenic activity.

Topical gels were prepared by using Carbomer (cold mechanical method). LAPS was used as API and also as vehicle for the semi-solid formulation. The formulations pH values ranged from 5.2 to 5.9 at 180 d of assays, which are acceptable to avoid the risk of irritation upon skin[24]. The low value of pH presented by LAPS is due to the presence of organic acids such as lactic, acetic and butyric acids[10]. The high content of LAPS used to formulate F100 explain its lower pH (LAPS $pH = 5.22 \pm 0.43$) and viscosity (Table 3), but keep its bioadhesive and semi-solid characteristics. Both integral and injured skins support a minimum pH of 5.0 or less depending of several factors such as race, sex, age and physiological state[25]. Also, it was found that lower pH has a role to play in both the healing of and treatment of chronic and acute wounds[26]. Another aspect to consider is that $F100_{(180)}$ showed increased pH when compared to $F100_{(0)}$. It is suggested that this result could be due to the degradation of macromolecules during shelf by enzymes, such as esterase and DNAase, present on LAPS. Rheological analysis was performed on $F100_{(0)}$, $F100_{(180)}$, C_{940} and C_{MRS}, and was observed that the presence of the API did not modify the semi-solid rheological behavior. The rheological assay showed that the designed semi-solid formulation displays thixotropic behavior. This property of $F100_{(0)}$ and $F100_{(180)}$ can be considered as an additional advantage for easier application to large skin areas. Also, the temperatures (25, 32 and 37 °C) tested do not seem to influence the viscosity of $F100_{(0)}$ and $F100_{(180)}$ (Table 3). These findings demonstrate that $F100_{(0)}$ and $F100_{(180)}$ maintain, at the tested range of temperature, its rheological properties, whether is on shelf or during skin application. One important technological and therapeutic requirement is spreadability. It refers to the area to which the semi-solid topical form readily spreads during applications to skin or the affected zones. It was observed that the values of $F100_{(0)}$ and $F100_{(180)}$ were significantly different than their respective controls. They showed a smooth and homogeneous appearance, were easily spreadable with acceptable bioadhesion and fair mechanical properties.

It is well known it the literature that vehicles used in formulations intended for topical use can greatly influence the rate and extent of drug permeation across the skin. The *in vitro* passive permeation studies were performed with $F100_{(0)}$ and $F100_{(180)}$ primarily to verify proper release from the carrier matrix as well as to select time of application. Synthetic membranes are preferred to skin tissue as they are easier resourced, less expensive and structurally simpler [27]. This means large-scale studies can be more readily undertaken as well as the mechanisms can be deconvoluted[28,29]. Furthermore, synthetic membranes exhibit superior permeation and data reproducibility than *in vivo* since the variables such as skin age, race, sex and anatomical site are eliminated[30]. For $F100_{(0)}$ and $F100_{(180)}$, the tests were performed for 8 h and Figure 1 shows the passive permeation values of LAPS (mg/cm^2) through cellulose membrane during time (h). These values were not significantly different between $F100_{(0)}$ and $F100_{(180)}$ when the release profile reached the stationary state at 6 h. Figure 2 shows the values of cumulative amount of LAPS in mg/cm^2 in membrane retention assays. No significant difference were found on the retained amount values measured for $F100_{(0)}$ and $F100_{(180)}$. In addition, it

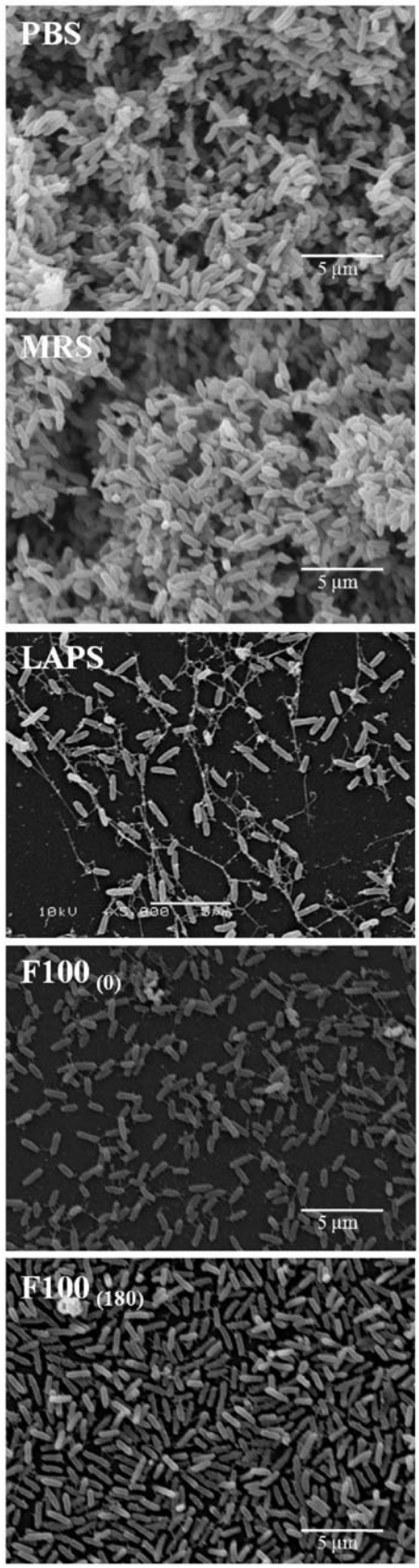

Figure 6. *Pseudomonas aeruginosa* biofilm disruption observed by scanning electron microscopy (SEM). Each line shows remnant biofilm after exposure to PBS (negative control), MRS (control for LAPS), LAPS, $F100_{(0)}$ and $F100_{(180)}$, respectively. Magnification: 5000× Scale bar: 5 µm.

was calculated the total amount of LAPS released from the polymeric matrix and no significant difference were found. When these results are compared with the agar diffusion assay (anti-microbial effect of $F100_{(0)}$ and $F100_{(180)}$), it is clear that LAPS shows no significantly difference between its semi-solid and its liquid form, being properly liberated from the semi-solid matrix. Since LAPS release profile is not different between the two formulations tested, it is proposed to repeat the application in wounds every 6 h.

LAPS interferes with the pathogenic capacity of *P. aeruginosa* inhibiting *in vitro* adhesion, quorum sensing and biofilm formation[8,9]. In addition, LAPS showed bacteriostatic and bactericide properties and a great biofilm disrupting capacity[5,9]. In this work it is demonstrated through several assays that LAPS formulations ($F100_{(0)}$ and $F100_{(180)}$) preserve these properties. The components present in LAPS responsible for the anti-microbial activities are organic acids such as lactic, acetic and butyric acid, hydrogen peroxide, bacteriocins such as plantaricins, benzoic acid, 5-methyl hydantoin, mevanololactone, and 2,5-piperazinedione[10]. On the other hand, components present in LAPS potentially responsible for the inhibition of quorum sensing (quorum quenching) of *P. aeruginosa* are the auto-inducers type 2 (AI-2)[10]. Therefore, the manufacture process used (cold mechanical method) does not destroy the anti-microbial compounds. In fact $F100_{(0)}$ and $F100_{(180)}$ formulations maintain almost completely the biofilm inhibition activity demonstrated by LAPS (Figure 3).

The inhibition of *P. aeruginosa* biofilm formation by LAPS, and its semi-solid formulations was confirmed by SEM (Figure 4). In the presence of MRS, *P. aeruginosa* biofilm lose much of its three-dimensional structure forming a flat biofilm with a matrix hyper-production possibly due to the presence of surfactants such as Tween 80. Surfactants interfere with the cohesiveness forces that allow *P. aeruginosa* to form biofilms with three-dimensional structures[31,32]. When exposed to LAPS, no adhering *P. aeruginosa* was observed. This can be explained due to the anti-microbial activity and the synergism of several components as cations, protons, Tween 80 and other surfactants as distearin, dipalmitin and 1-monolinolein[10]. The formulations showed an inhibitory activity of biofilm formation lower than LAPS (Figure 3). Probably, the increase of pH values and the interaction of the bioactive metabolites with some excipients partially interferes with LAPS activities.

In vivo P. aeruginosa biofilm matrix is composed of exopolysaccharides, proteins and DNA[9,33]. In this sense, our research group demonstrated in previous works that the components present in LAPS responsible for the disrupting activity on *P. aeruginosa* biofilms are DNAase, surfactants, chelating agents and cations[10]. The biofilm disruption assays performed in this work shows that $F100_{(0)}$ has a better disrupting activity than LAPS possibly because the disrupting components are stabilized in the formulation (Figure 5). However, after 180 d, the formulation drastically loses its disruptive ability (Figure 5) probably due to denaturation of the enzyme, which may act upon the distinct matrix components, such as DNAase. This is further supported with results obtained by SEM (Figure 6).

Conclusion

Pharmacotechnical aspects and the design proposed in this work reached the expected technological standards. The formulations using 100% of LAPS, showed a smooth and homogeneous appearance, were easily spreadable and demonstrate acceptable bioadhesion and fair mechanical properties. From the *in vitro* release, assays were found in adequate release from the carrier matrix. Furthermore, $F100_{(0)}$ is stable and maintains its anti-pathogenic activity for a period of 6 months $F100_{(180)}$. Based on

these findings, we propose for the proper treatment of infected chronic wounds F100 application. This new treatment provides *P. aeruginosa* anti-pathogenic activity as a combination of biocidal and anti-virulence actions. The use of harmless bacteria by-products, such as LAPS, to antagonize infectious pathogens that have ability to form biofilm is an efficient and economic approach to treat infected chronic wounds.

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Declaration of interest

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