Pharmaceutical Development and Technology

http://informahealthcare.com/phd
ISSN: 1083-7450 (print), 1097-9867 (electronic)

Pharm Dev Technol, Early Online: 1–6
 DOI: 10.3109/10837450.2015.1011661

informa healthcare

RESEARCH ARTICLE

Novel topical formulation for ischemic chronic wounds. Technological design, quality control and safety evaluation

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Abstract

Ulceration of the foot in diabetes is common and disabling, and frequently leads to amputation of the leg. The pathogenesis of foot ulceration is complex, clinical presentation variable and management requires early expert assessment. Despite treatment, ulcers readily become chronic wounds. Chronic wounds are those that remain in a chronic inflammatory state failing a normal healing process patterns. This is partially caused by inefficient eradication of opportunistic pathogens like *Pseudomonas aeruginosa*. We propose its control or eradication will promote wound healing. *Lactobacillus plantarum* cultures supernatants (LAPS) shows antipathogenic and pro-healing properties. The main objective was to design two pharmaceutical dosage forms by using LAPS as active pharmaceutical ingredient and to perform its quality control, *in vitro* activity conservation tests and human trials (safety evaluation). Both selected formulations reach the technological quality expected for 120 days, shows adequate occlusive characteristics and proper adhesion to human skin. From the *in vitro* release assays were found that LAPS shows adequate release from matrix and maintain its antimicrobial and anti-biofilm activity. First human trials were developed and neither edema nor erythema on healthy skin voluntaries was found. We conclude that C80 and C100 are adequate for their use in future clinical trials to demonstrate a comprehensive therapeutic effectiveness in ischemic chronic wounds.

Keywords

Bacteria by products, *Lactobacillus plantarum*, *P. aeruginosa*, pharmaceutical biotechnology, pharmaceutical technology, quality control

History

Received 16 September 2014
Revised 8 January 2015
Accepted 17 January 2015
Published online 17 February 2015

Introduction

Ulceration of the foot in diabetes is common and disabling, and frequently leads to amputation of the leg. Mortality is high and healed ulcers often recur. The pathogenesis of foot ulceration is complex, clinical presentation is variable and management requires early expert assessment. Interventions should be directed at infection, peripheral ischemia and abnormal pressure loading caused by the peripheral neuropathy and limited joint mobility[1]. Despite treatment, ulcers readily become chronic wounds[1]. Chronic wounds are those that remain in a chronic inflammatory state and therefore fail to follow normal healing process patterns[2–5], being infection one important cause of the chronicity[4]. *Pseudomonas aeruginosa* is a Gram-negative opportunist organism frequently isolated from chronic wounds[5–7]. This bacterium is extremely refractory to therapy and host immune response when it forms biofilm[8]. Bjarnsholt et al. suggested that the lack of proper wound healing is partially caused by inefficient eradication of pathogens like *P. aeruginosa*[9].

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[10,11]. In addition, LAPS showed bacteriostatic and bactericide properties and a great biofilm-disrupting capacity on *P. aeruginosa*[6,11]. LAPS is neither cytotoxic nor an inductor of apoptosis–necrosis *ex vivo* on polymorphonuclear leukocytes and *in vivo* in a mouse model[12,13]. The chemical composition of LAPS was determined, allowing us to propose a correlation between the chemical constituents and their biological activity[14]. Given LAPS pro-healing properties, it is necessary to design a pharmaceutical dosage form to facilitate its administration in the treatment of ischemic chronic wounds. Because of this, the aims of this work were to design a pharmaceutical dosage form by using LAPS as an active pharmaceutical ingredient (API), to ensure its quality and to evaluate safety administration on healthy volunteers. Furthermore, we expect by a correct selection of excipients to achieve an adequate occlusive characteristics and proper adhesion to mammalian cells.

Materials and methods

Cream's ingredients

Cetostearyl alcohol (Ceral PW), vaseline, methylparaben and glycerol were donated from Fabriquímica (Buenos Aires, Argentina).

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Table 1. Composition of all formulations designed for pre-formulation studies.

F	LAPS (mL)	CPW (g)	Vs (g)	Pb (g)	G(mL)	W_d (mL)
C20	20	9.0	2.0	0.2	2.5	66.3
C25	25	9.0	2.0	0.2	2.5	61.3
C80	80	12.0	2.0	0.4	1.5	4.1
C100	100	12.0	2.0	0.4	1.5	–
C	–	9.0	2.0	0.2	2.5	86.3
CC	–	12.0	2.0	0.4	1.5	84.1

F, formulation code; LAPS, *Lactobacillus plantarum* supernatants; CPW, Ceral PW; Vs, vaseline; Pb, parabens; G, glycerol; and W_d, distillate water.

Bacterial strains and culture media

Pseudomonas aeruginosa, ATCC 27853, and *L. plantarum*, ATCC 10241, were grown in Luria-Bertani (LB) and in de Man Rogosa Sharpe (MRS) broth, respectively (Britania, Buenos Aires, Argentina).

L. plantarum supernatants

Lactobacillus plantarum reaches stationary phase and maximum concentration of bioactive metabolites in 12 h[14]. After this period of static cultivation at 37 °C, supernatants of *L. plantarum* were recovered after centrifugation (8000 rpm, 15 min) and subsequent filtration through 0.22 μm membranes.

Formulation development

Since LAPS is an API of biological origin with many components, the final product design sought to be as simple as possible. Pre-formulation studies were carried out using four different formulations (with increasing concentrations of LAPS) and its controls (Table 1). The pharmacotechnical aspects evaluated were as follows: pH, extensibility, apparent viscosity, organoleptic properties, stability and sanitary assays. Stability assays were performed as required in stability studies chapter of Farmacopea Argentina, eighth Edition. Microbiological evaluation (sanitary assays) was developed as required on Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT) disposition no. 7667.

Physicochemical evaluation at 0 and 120 d

Formulations stability studies (aging test) were made at day 0 ($C80_0$ and $C100_0$) and after a period of four months ($C80_{120}$ and $C100_{120}$). The semisolid products were maintained at 25 °C and 40% of relative humidity, which represents the annual average for Tucuman Argentina. In C100 case, LAPS was used as API and also as vehicle (no water was added, for the semi-solid formulation). Variation of their physical and chemical properties and antipathogenic capacities were analyzed. Each assay was made using negative control: basic cream (CC).

Homogeneity assays

All formulations and control were applied as a thin layer on polypropylene slides. The homogeneity was determined by visual appearance after application. In parallel, 3 g of the same formulations were used for centrifugation assays (15 min, 3500 rpm) and phase's separation was observed[15]. Under these conditions, the homogeneity 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 was measured in a pH meter (Broadley James Corporation, Irvine, CA) by dipping the glass electrode into the semisolid[15].

Rheological studies

The apparent viscosity was determined at 25 °C (stored temperature), 32 °C (healthy skin temperature) and 37 °C (wound bed temperature)[16] by using a Cannon viscometer with spindle N° 8 from 3 rpm to 60 rpm (Cannon Instrument Company, LV2000 model, Pennsylvania, EEUU)[15].

Spreadability assays

The spreadability was evaluated using a extensometer consist of two plates, the lower plate holds the sample (0.5 g) and the upper plate (28 g) exerts forces to the sample[15]. Force was generated by adding a weight of 5 g on the upper plate at pre-determinate times. Each formulation was measured at constant temperature (25 °C) in triplicate.

Microbiological evaluation (sanitary assays)

Dilutions (1:9; formulations and controls) were inoculated on selective mediums Triptone soy agar for bacteria and Sabouraud agar for yeast and fungi (Britania) and incubated for three days at 37 °C for bacteria and seven days at 25 °C for yeast and fungi. Mean results were expressed as CFU g^{-1} as required on Farmacopea Argentina eighth Ed.

In vitro release assays

In vitro *release studies*

This assay was conducted using vertical type Franz diffusion cells[17,18] having a receptor compartment capacity of 10 mL. The system consistent on 16 cells was placed in a plastic container as support and then was inserted in an orbital shaker. Constant temperature is achieved by using a stove. Cellulose membranes (D9527 avg. flat width 43 mm (1.7 inches) (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. This equilibration period allows that the whole system reach the assay temperature[18]. For these studies, the entire system was maintained at 32 ± 0.5 °C (skin temperature). The area available for diffusion was 1.8 cm^2. 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 optical density at 300 nm (OD300) of the solution was measured in a UV spectrophotometer (Thermo Spectronic Genesys 10 UV, Rochester, NY). Tests were repeated four times on four consecutive days. 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) ($n = 16$).

API retention in membrane assays

At each time, cellulose membranes were washed with NaCl solution (0.9%), cut into pieces and placed on tubes with 10 mL of NaCl solution. Then, tubes were stirred (40 min at 32 °C) and 2 mL of receptor fluid was withdrawn[18]. The optical density at 300 nm (OD300) of the receptor fluid was measured in a UV spectrophotometer. The test was repeated four times on four consecutive days. Cumulative amounts of LAPS (mg) retained on

the unit diffusion surface (cm^2) were plotted versus time (h). The results were expressed as mean ± SD ($n = 16$).

Antipathogenic properties conservation assays

Antimicrobial assays were performed to demonstrate that the vehicle used does not modify the LAPS activities.

Agar diffusion assays

P. aeruginosa was grown overnight in LB broth, and 100 µL were spread on LB agar to form a uniform layer. Wells of 7 mm of diameter were made in LB agar, and aliquots (200 µL) of LAPS, $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$ were placed into them. Gentamicin 8 mg/mL and CC 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. Inhibition zone diameter was measured by using a Venier caliper. 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 placed, respectively, 80 µL of each sample (LAPS, $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$) and controls (gentamicin 8 mg/mL as antimicrobial positive control; MRS as control for medium components and phosphate-buffered saline (PBS) as antimicrobial negative control). Then, 40 µL of LB and 80 µL of an overnight culture of *P. aeruginosa* (optical density at 600 nm; OD600 = 0.150) were added in all wells in LB broth. The plates were incubated for 24 h at 37 °C, and OD600 was measured in a microplate reader. 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 percentages of stimulation or inhibition were calculated using PBS as 0%. Results were expressed as percentage mean ± SD ($n = 8$)[19].

Effect on biofilm

The wells content of the above assay (see ''*Effect on growth*'') was discarded, and wells were washed thrice with saline. 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., Buenos Aires, Argentina), and the optical density at 570 nm (OD570) of the resulting solution was measured in a microplate reader. The measured OD570 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 ($n = 8$)[19].

Effect on pre-formed biofilm

For this assay, 80 µL of an overnight *P. aeruginosa* culture in LB (OD600 = 0.150) and 120 µL of LB broth were placed in 96-well polystyrene microtiter plates and incubated for 24 h at 37 °C. The content was discarded, and the wells were washed thrice gently with sterile saline. The attached biomass (biofilm) was treated for 6 h at 37 °C with a mixture of 120 µL of LB broth and 80 µL of each sample (LAPS, $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$) and controls (gentamicin 8 mg/mL as antimicrobial positive control; MRS as control for medium components and PBS as antimicrobial negative control). The remaining attached biomass (post-disruption biomass) 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.) and OD570 was measured in a microplate reader. The results were expressed as mean ± SD ($n = 8$), considering PBS as 0% of biofilm disruption[19].

Safety evaluation of $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$: response to healthy skin voluntaries application

There is no foreseeable risk from the use of LAPS. It is possible to asses in a clinical test, the safety of their ingredients simply by evaluating the appearance of irritation and/or allergy. The corrosion test on healthy skin model[20] is a methodology validated by the National Drug and Medical Technology (Resolution No 288/90 ANMAT) in Argentina. This trial involved healthy volunteers (with skin integrity in both forearms). To evaluate $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$, a panel of 30 volunteers was used. Each cream (0.5 g) was applied on the forearm, and the occurrence of edema and/or erythema at 20 min and 24 h after application, was evaluated. All volunteers gave their prior informed consent and confirmed that they had not received any antiallergic medication for at least 15 d before the study. 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 the following: itching, burning, pain, numbness and redness.

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).

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 $p < 0.001$ were considered statistically significant.

Results and discussion

LAPS is a biological origin API with a complex composition. Formulation must be simple, adequate for bioadhesion and easy to apply, allowing patient care on an outpatient level. Thus, the selection criteria were based in that the formulation should maintain an adequate pH, viscosity, occlusive properties, bioadhesion and preservation of LAPS's antipathogenic properties[19].

Formulation development

Table 1 lists the composition of all formulations designed for pre-formulation. Based on preliminary experiments (Table 2), two formulations that achieved the mechanical properties required for a semi-solid (support maximum concentration of LAPS and ensured the maintenance of the LAPS properties) were selected (C80 and C100).

Physicochemical evaluation at 0 and 120 d (aging test)

Table 3 lists the physicochemical properties for aging test. When pH was evaluated, significant differences between controls and C80 and C100 at 0 and 120 d were found. This could be due to the acidic nature of LAPS (pH = 5.22 ± 0.43). It was reported that these values are in the acceptable range for topical skin

formulation (4.0–7.2 depending on race, age, sex and environmental factors)[21]. This would be useful in the treatment of chronic wounds as it was demonstrated that wound healing is more effective at low pH (pH = 5.0 or lower). Alkaline environments in a wound (mean values of pH = 7.42) are related to chronicity[22].

The apparent viscosity of C80 and C100 was significant lower than control (Table 3). This could be due to the high volume of LAPS used to formulate them. All formulations maintain their bioadhesiveness and semi-solid characteristics. Furthermore, non-significant differences on apparent viscosity values between temperatures (25 °C and 37 °C) were found, showing rheological stability at shelf temperature for at least four months.

When spreadability was tested, only $C80_{120}$ and $C100_{120}$ showed significant differences against control CC_{120}; this could be due to water evaporation on CC_{120}. Non-significant differences were found on formulation tested at 0 or at 120 d. Homogeneity was categorized as very good for C80 and good for C100 in all times tested.

C80 and C100 showed less than 200 CFU g^{-1} for bacteria and less than 300 CFU g^{-1} for fungi, as recommended in Farmacopea Argentina (Table 3). This is due to the presence of several antibacterial compounds previously reported for LAPS[14]. All the physicochemical properties for aging test analysis obtained support our formulation choice.

In vitro release assays

It is well known that vehicles employed 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 primarily to verify proper release from the carrier matrix as well as to predict time of application of treatment for human trials. Synthetic membranes are preferred to skin tissue as they are easier resourced, less expensive and structurally simpler[23]. This means that large-scale studies can be undertaken more readily as well as the mechanisms can be deconvoluted[24,25]. The *in vitro* release study on $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$was performed. Figure 1 shows the cumulative amount of LAPS (mg/cm^2) during time (h) in all samples tested. API released reaches maximum concentration at 6 h. However, significant differences were found between $C80_0$ and $C80_{120}$ (2.90 ± 0.10 and 3.79 ± 0.36 mg/cm^2, respectively). The same situation was observed when $C100_0$ and $C100_{120}$ were compared

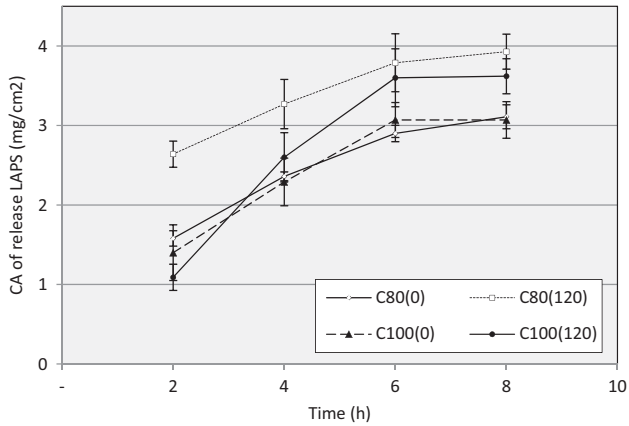


Figure 1. Cumulative amount (CA) of LAPS (mg/cm^2) from $C80_{(0)}$ and $C80_{(120)}$. The data shown represent the mean ± SD of 16 replicates.

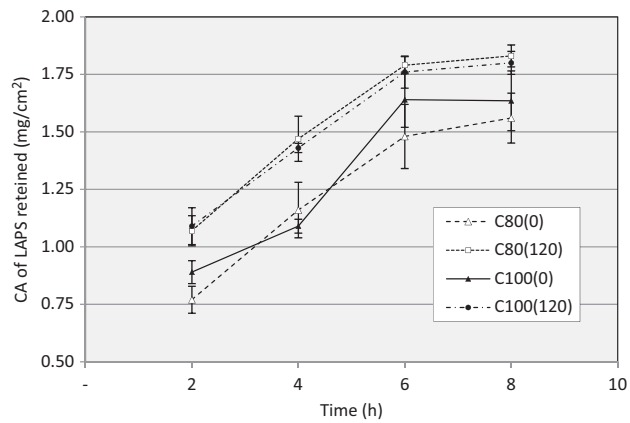


Figure 2. Cumulative amount (CA) of LAPS (mg/cm2) retained from $C80_{(0)}$, $C100_{(0)}$ and $C80_{(120)}$, $C100_{(120)}$. The data shown represent the mean ± SD of 16 replicates.

Table 2. Pharmacotechnical aspects assayed.

			M (UFCg^{-1})	
F	S (mm^2)	pH	Bacteria	Fungi
C20	54.98*	5.50*	0	11
C25	51.54*	5.01*	16	0
C80	41.84*	5.07*	10	6
C100	42.15*	5.71*	40	18
C	47.12	7.38	0	6
CC	45.34	7.00	10	0

Spreadability (S), pH determination (pH) and microbiological evaluation (M) by counting of colony forming units. All formulations tested (F) were compared to its respective control (CF).
*Significantly different from control ($p < 0.05$).

Table 3. Physicochemical properties for aging test.

							M (UFC g^{-1})	
	pH	V (cP) 25 °C	V (cP) 32 °C	V (cP) 37 °C	S (mm^2)	H	B	F
CC	7.00	2156 ± 7.8	2103 ± 1.6	2016 ± 6.2	45.34	VG	10	0
$C80_0$	5.07*	2253 ± 1.2**	2264 ± 3.5**	2128 ± 4.3**	41.84*	VG	10	6
$C100_0$	5.71*	2256 ± 5.7**	2255 ± 4.2**	2262 ± 7.1**	42.15*	G	40	18
CC_{120}	6.8	2160 ± 21	2153 ± 2.0	2034 ± 8.2	37.70*	VG	61	39
$C80_{120}$	4.65*	2250 ± 11**	2240 ± 5.7**	2156 ± 7.1**	45.03*	VG	0	8
$C100_{120}$	4.58*	2136 ± 9.1**	2098 ± 1.5**	2006 ± 9.2**	42.89*	G	29	52

Qualitative scale: VG, very good; G, good; and B, bad.
*Significantly different from control ($p < 0.01$).
**Significantly different from control ($p < 0.001$).
pH, viscosity at 3 rpm (V): centipoise (cP), spreadability (S), homogeneity (H) and microbiological evaluation (M) bacterial (B) and fungi (F) count.
The results represent the average of the values obtained from measurements performed at 0 ($C80_0$ and $C100_0$) and after four months of shelf life ($C80_{120}$ and $C100_{120}$) ± SD.

(3.07 ± 0.35 and 3.62 ± 0.23 mg/cm^2, respectively) being higher concentration of LAPS released from $C80_{120}$ and $C100_{120}$. Figure 2 shows the values of cumulative amount of LAPS in mg/cm^2 in retention in membrane assays. No significant differences were found in the retained amount values measured for $C80_0$ compared with $C100_0$ and for $C80_{120}$ compared with $C100_{120}$ at 6 h (time in which maximum concentration of LAPS retained on membrane was reached). Total amount of LAPS released from the carrier matrix were calculated as the sum of the average values, being the concentration of $C80_0$ and $C100_0$, respectively, 4.46 ± 0.4 and 5.62 ± 0.32 mg/cm^2 and the concentration of $C80_{120}$ and $C100_{120}$ 4.71 ± 0.24 and 5.642 ± 0.5 mg/cm^2, respectively. When these results are compared with the agar diffusion assay (Table 4; diffusion from carrier matrix and antimicrobial effect), it is clear that LAPS shows no significant difference between the semi-solid and its liquid form. This confirms that LAPS is appropriately released from the semi-solid matrix. From these tests, LAPS-release profile was established for both formulations. It is proposed to repeat the application of C80 and C100 in wounds every 6 h.

Table 4. Agar diffusion assay.

	Inhibition (mm) *P. aeruginosa*	
	0 d	120 d
LAPS	31.0 ± 1*	29.0 ± 2
C80	29.0 ± 2*	29.5 ± 1
C100	29.5 ± 1*	28.5 ± 2*
CC	0	0
G	34.5 ± 1	31.5 ± 2

Inhibition halos (mm) for *P. aeruginosa* for CC, LAPS, $C80_{(0)}$, $C100_{(0)}$, $C80_{(120)}$ and $C100_{(120)}$. Gentamicin 8 μg/mL (G) was used as positive control.
*Significantly different from positive control gentamicin ($p < 0.01$).

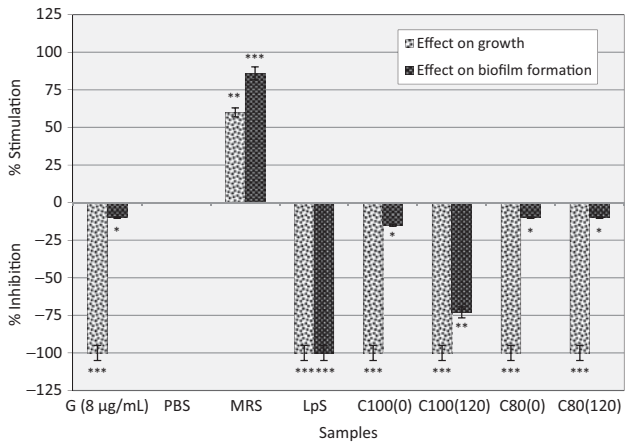


Figure 3. Effect on growth and biofilm formation of *P. aeruginosa* produced by tested samples: PBS (0% inhibition and 0% stimulation), MRS (control for LAPS), gentamicin 8 mg/mL (G), LAPS, $C80_{(0)}$, $C100_{(0)}$, $C80_{(120)}$ and $C100_{(120)}$. Significant differences were observed when compared to control (PBS):*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

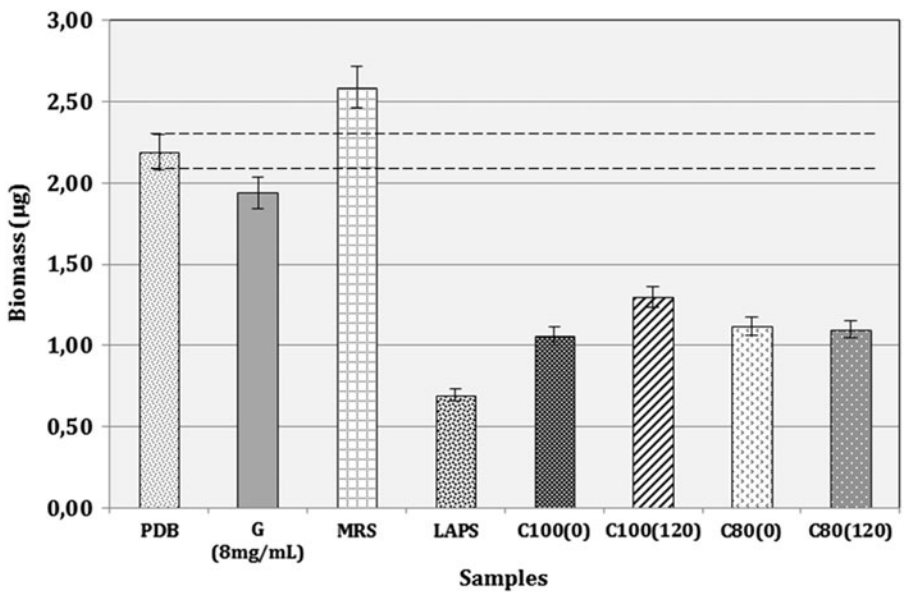
Antipathogenic properties conservation assays

When antimicrobial activity against *P. aeruginosa* was tested (Table 4), non-significant differences were found between LAPS, $C80_{(120)}$ and $C100_{(120)}$ compared to positive control. LAPS and all formulations tested maintain its antimicrobial activity against *P. aeruginosa* for 120 d. The percentage of growth of *P. aeruginosa* in presence of LAPS, $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$ (Figure 3) were significantly lower than PBS ($p < 0.001$). When biofilm formation was analyzed (Figure 4), LAPS and $C100_{120}$ showed 100% and 75% of inhibition, respectively; this was consistent with previous findings[11,12]. In this work, it was demonstrated through several assays that LAPS formulations preserve the antipathogenic properties until at least 120 d post manufacture. This could be due the chemical stability of LAPS components responsible for the antimicrobial activities like organic acids (lactic, acetic, butyric and benzoic acid), 5-methylhydantoin, mevanololactone and 2,5-piperazinedione[14]. Therefore, the manufacture process used does not affect nor destroy the antimicrobial compounds.

Safety evaluation of $C80_0$, $C100_0$, $C80_{120}$ and $C100_{120}$ response to volunteer's healthy skin application

Our results demonstrate that is no foreseeable risk from the use of LAPS as in all cases; no edema or erythema was observed. In addition, when patients were surveyed about their skin sensations, 100% of the voluntaries reported without sensation. Demonstration of the safe application of LAPS in healthy volunteers represents a breakthrough on the road to test the therapeutic efficacy of LAPS in humans.

Figure 4. Biofilm Biomass of *P. aeruginosa* (μg) after disruption treatments: PDB (Pre Disruption Biomass), gentamicin 8 mg/mL (G), MRS (control for LAPS), LAPS, $C80_{(0)}$, $C100_{(0)}$, $C80_{(120)}$ and $C100_{(120)}$. Significant differences were observed compared to negative control (PBS): range between dot spot line represent pre disruption biomass real values.



Conclusion

We conclude that C80 and C100 are adequate for their use in future clinical trials for demonstrating a comprehensive therapeutic effectiveness in ischemic chronic wounds.

Acknowledgements

The authors thank Fabriquimica SA Argentine for the donation of excipients for this work.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

This work was supported by Unit of Technological Bonding of National University of Tucumán and National Council of Scientific and Technical Research (CONICET).

References

1. Jeffcoate WJ, Harding KG. Diabetic foot ulcers. Lancet 2003;361:1545–1551.
2. Lazarus GS, Cooper DM, Knighton DR, et al. Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol 1994;130:489–493.
3. Harding KG, Morris HL, Patel GK. Healing chronic wounds. Br Med J 2002;324:160–163.
4. Stadelmann WK, Digenis AG, Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. Am J Surg 1998;176:26S–38S.
5. James GA, Swogger E, Wolcott R, et al. Biofilms in chronic wounds. Wound Rep Reg 2008;16:37–44.
6. Thomsen TR, Aasholm MS, Rudkjobing VB, et al. The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. Wound Rep Regen 2010;18:38–49.
7. Edwards R, Harding KG. Bacteria and wound healing. Curr Opp Infect Dis 2004;17:91–96.
8. Ramos AN, Peral MC, Valdez JC. Differences between *Pseudomonas aeruginosa* in a clinical sample and in a colony isolated from it: comparison of virulence capacity and susceptibility of biofilm to inhibitors. Comp Immunol Microbiol Infect Dis 2010;33:267–275.
9. Bjarnsholt T, Kirketerp-Moller K, Jensen PO, et al. Why chronic wounds will not heal: a novel hypothesis. Wound Rep Reg 2008;16:2–10.
10. Valdez JC, Peral M, Rachid M, et al. Interference of *Lactobacillus plantarum* on *Pseudomonas aeruginosa in vitro* and in infected burns. The potential use of probiotic in wound treatment. Clin Microbiol Infect 2005;11:472–479.
11. Ramos AN, Sesto Cabral ME, Noseda D, et al. Antipathogenic properties of *Lactobacillus plantarum* on *Pseudomonas aeruginosa*: the potential use of its supernatants in the treatment of infected chronic wounds. Wound Rep Regen 2012;20:552–562.
12. Ramos AN, Gobbato N, Rachid M, et al. Effect of *Lactobacillus plantarum* and *Pseudomonas aeruginosa* culture supernatants on polymorphonuclear damage and inflammatory response. Int Immunopharmacol 2010;10:247–251.
13. Valdez JC, Ramos AN, Fernández D, et al. Probiotics and their potential use in wound treatment. In: Alvarez S, ed. Probiotics: inmunobiotics and inmunogenics. Florida: Science publishers/CRC; 2013:296–333.
14. Ramos AN, Sesto Cabral ME, Arena ME, et al. Compounds from *Lactobacillus plantarum* culture supernatants with potential pro-healing and anti-pathogenic properties in skin chronic wounds. Pharm Biol 2015;53:350–358.
15. Vijaya Bhanu P, Shanmugam V, Lakshmi PK. Development and evaluation of Diclofenac Emulgel for topical drug delivery. Pharmacie Globale IJCP 2011;9:1–4.
16. Soldevilla J, Torra JE, Bou I. Atención Integral de las heridas crónicas. SPA ed. Madrid 2004;2:98–99.
17. Neves J, Bahia MF. Gels as vaginal drug delivery systems. Int J Pharmaceut 2006;318:1–14.
18. Sesto Cabral ME, Ramos AN, Cabrera CA, et al. Equipment and method for *in vitro* release measurements on topical dosage forms. Pharm Dev Technol 2015;20:619–625.
19. Sesto Cabral ME, Ramos AN, Macedo AJ, et al. Formulation and quality control of semi-solid containing harmless bacteria by-products: chronic wounds pro-healing activity. Pharm Dev Technol. 2015;20:911–919.
20. Pathan AK, Rahul T, Rukhsana AR. Formulation development and evaluation of silymarin gel for psoriasis treatment. J Inn Pharm Biol Sci 2014;1:21–26.
21. Matousek JL, Campbell KL. A comparative review of cutaneous pH. Veter Dermatol 2002;13:293–300.
22. Percival SL, McCarty S, Hunt JA, Woods EJ. The effects of pH on wound healing, biofilms, and antimicrobial efficacy. Wound Rep Reg 2014;22:174–186.
23. Riccia EJ, Lunardib LO, Nanclaresb DMA, Marchetti JM. Sustained release of lidocaine from Poloxamer 407 gels. Int J Pharmaceut 2005;288:235–244.
24. Shah SNH, Hussain T, Khan IU, et al. Formulation study of topically applied lotion: *in vitro* and *in vivo* evaluation. BI 2013;3:11–19.
25. Wester R, Maibach H. Percutaneous absorption of drugs. Clin Pharmacokinet 1992;23:253–266.