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Research Article

First Steps towards the Pharmaceutical Development of Ovules Containing *Lactobacillus* Strains: Viability and Antimicrobial Activity as Basic First Parameters in Vaginal Formulations

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Abstract. In the majority of Latin-American countries, including Argentina, there is a limited availability of vaginal bioproducts containing probiotics in the market. In addition, the conventional treatments of genital tract infections in women represent a high cost to the public health systems. The future development of this type of bioproducts that employ specific lactobacilli strains would not only have a meaningful impact on women's health but would also represent a significant challenge to the pharmaceutical industry. The aims of the work described in this paper were (i) to study different pharmaceutical formulations of vaginal ovules containing *Lactobacillus fermentum* L23 and *L. rhamnosus* L60, to determine in which formulation lactobacilli viability was sustained for longer time and (ii) to evaluate if probiotic strains maintained both the antimicrobial activity and biofilm-producing ability after being recovered from the ovules. In this study, we developed and characterized three pharmaceutical formulations containing different glycerol amounts and specific lactobacilli strains. Three relevant parameters, cell viability, antimicrobial activity, and biofilm production, by lactobacilli recovered from the ovules were tested. Although the viability of L23 and L60 strains was mainly influenced by high ovule's glycerol proportion, they survived at 4 °C during the 180 days. Both lactobacilli's antimicrobial activity and biofilm-producing ability were maintained for all treatments. In conclusion, employing a much reduced number of components, we were able to select the most suitable pharmaceutical formulation which maintained not only lactobacilli viability for a long period of time but also their antimicrobial activity and biofilm-producing ability.

KEYWORDS: *Lactobacillus*; Probiotic; Vaginal ovule; Pharmaceutical formulation; Bioproduct.

INTRODUCTION

The vaginal microbiota of healthy women is dominated by different *Lactobacillus* species that play an important role not only in the maintenance of a healthy status but also in prevention of infections (1). Conventionally, long-term antibiotic and antifungal treatments are the most common methods for the managing of recurrent vaginal tract infections that are commonly difficult to cure. These antimicrobial treatments are not always effective. In fact, in recent years, it has become apparent that these problems still remain, probably due to bacterial and yeast antimicrobial resistance, recurrent infections, as well as side effects that have been reported worldwide (2). Under this context, biotechnological development of therapeutic alternatives using native and safe microorganisms from the vaginal niche has become of increasing interest. Nowadays, in some high-income countries, there are biotechnological products containing *Lactobacillus* species for treatment of vaginal infections. These include ovules, powders, douches, and tablets as the most common pharmaceutical presentations in the market (2–6). However, the use of powders, douches, and tablets has been associated with numerous cases of irritation, discomfort, and leakage at the application site and, for this reason, they have a reduced compliance levels among women. The vaginal ovule containing probiotic lactobacilli would represent the most accepted option. Those containing probiotics and/or their active substances with antimicrobial activity have certain advantages such as dose uniformity, absence of vaginal irritation, and high melting ability in the vaginal tract (7).

Compared to products developed against infections that affect the gastrointestinal tract, there are few research articles reporting the development of products containing probiotic

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vaginal microorganisms (8,9). During the last years, biotechnological development of vaginal products using safe microorganisms has gained renewed interest. In fact, there are several scientific reports claiming the use of probiotics as a complementary therapy with preventative approaches (5,10,11). Furthermore, in some countries, there is a growing tendency in some patients towards the use of vaginal products containing probiotics to naturally reinforce the conventional therapy (12).

Our research group has widely studied and characterized two human isolates, *Lactobacillus fermentum* L23 and *L. rhamnosus* L60, as probiotic strains through several *in vitro* tests and *in vivo* experimental models that demonstrate their potential to be usefully exploited in the pharmaceutical development of vaginal products (9,13–16). One of the most relevant probiotic properties that were demonstrated for these *Lactobacillus* strains is their high adherence capacity to vaginal epithelial cells. This capacity has been demonstrated by different tests such as auto-aggregation; surface hydrophobicity; and mechanisms of exclusion (co-aggregation), competition, and displacement of pathogens demonstrated by *in vitro* experiments (13,17,18). Furthermore, the synthesis of bioactive metabolites with antimicrobial activity is another important probiotic property found in both strains. Inhibitory activity was shown for a wide range of microorganisms such as *Gardnerella vaginalis*, *Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Streptococcus agalactiae*, *Enterococcus* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, enterobacteria (*Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., *Serratia* spp., *Citrobacter* spp., *Acinetobacter* spp.), other gram-negative non-fermentative bacilli (*Pseudomonas* spp.), and different *Candida* spp. (11,19–23). We have previously described that the bioactive metabolites produced by these lactobacilli strains are released in their cell-free supernatants (CFSs) (11,24). In fact, the inhibitory activity of the neutralized CFS (NCFS) of L23 is exclusively produced by its bacteriocin L23. In the case of the NCFS of L60, the inhibitory activity is caused by two metabolites, hydrogen peroxide (H_2O_2), and bacteriocin L60 (17,19,25). It is noteworthy that both bacteriocins L23 and L60 were the main biometabolites responsible for the antimicrobial activity found against the variety of microorganisms tested (11,15,22,24–27). Both lactobacilli strains have the ability to produce biofilm, as well as to inhibit the formation of pathogenic biofilms. In previous *in vivo* pre-clinical experimental models, we have demonstrated not only the high vaginal colonization ability of these lactobacilli strains but also their preventive and curative effects in murine models of vaginal infection by different bacterial pathogens (9,16,17,19). Finally, all this scientific background we had gathered for these lactobacilli strains led us to perform the first evaluations at laboratory scale towards the development of vaginal ovules. In this study, we focused on the design and development of a vaginal pharmaceutical bioproduct containing these specific strains, *L. fermentum* L23 and *L. rhamnosus* L60, which exhibit strong probiotic characteristics. The most remarkable aspect of this work is the use of these specific bacteriocin-producing lactobacilli with a wide previously demonstrated antimicrobial activity (9,11,15,17,22,25). In fact, there are a limited number of ovules containing lactobacilli commercially available in South America whose antimicrobial effect is based exclusively on the organic acids released by those lactobacilli strains (28–30). The aims of this work were i) to study different pharmaceutical formulations of vaginal ovules containing *L. fermentum* L23 and *L. rhamnosus* L60, to determine in which one the lactobacilli viability was maintained for a longer time and (ii) to evaluate if lactobacilli strains retained both properties, the antimicrobial activity and the biofilm-producing ability after being recovered from the ovules.

MATERIALS AND METHODS

Lactobacillus Strains, Culture Conditions, and Materials

L. fermentum strain L23 (GenBank accession number GQ455406.1) and *L. rhamnosus* L60 (GenBank accession number EF495247.1), hereafter termed "L23" and "L60," respectively, were grown in Man Rogosa-Sharpe (MRS) broth or agar plates (Britania) for 24 h at 37 °C in a 5% CO_2 atmosphere. Strains were stored at − 20 °C in MRS broth containing 30% (*v*/*v*) glycerol (11,17,25).

Selection of Optimal Proportion of Glycerol Content for Ovule Preparation

A preliminary *in vitro* test to select which glycerol proportions were more adequate to preserve lactobacilli viability was performed. Glycerol anhydrous (1,2,3 Propanotriol 99.5% p.a.) was purchased from Cicarelli, Santa Fe, Argentina. L23 and L60 overnight MRS cultures were seeded in different glycerol proportions (80, 60, 50, 30, and 16%), and every 10 days of storage, cultures in MRS broth were performed. The best suspension to maintain *in vitro* viability of the strains through time was chosen for ovule preparation.

Preparation and Characterization of Pharmaceutical Formulations

Two successive cultures of L23 and L60 strains were made in MRS broth incubated at 37 °C under microaerobic conditions for 24–48 h. At late exponential phase, these cells were harvested by centrifugation (870×*g*, 20 min) and bacterial mass was recovered into sterile tubes. Formulations were prepared by the fusion method, melting the excipients at 50–60 °C and cooling down to 37 °C, to then add the lactobacilli biomass. After homogenizing the preparation, this mixture was poured into plastic molds. Glycerol (Cicarelli, Santa Fe, Argentina) and gelatin (Oxoid, Waltham, USA) quality was in agreement with the Farmacopea Argentina (FA) (31). Thus, formulations No. 1, No. 2, and No. 3 ("F1", "F2", and "F3", respectively) were obtained (Table I). Certain characteristics for ovules recommended by FA, such as surface texture, consistency, absence of air bubbles, content uniformity (lactobacilli distribution), and the ability to melt at body temperature were evaluated. By an adaptation of FA recommendations (protocols <310> and <400>, vol. I; vol. III), Kale et al. (32) and Kaewsrichan et al. (3), fusion time test (dissolution or disintegration) was performed.

Vaginal Ovules Containing *Lactobacillus* Strains

Six ovules of each lactobacilli strain were placed in a small basket divided into compartments with metallic mesh at the bottom and immersed in a water bath at 37 ± 0.5 °C for 15 min. The time period needed for complete dissolution was recorded. This procedure was done in triplicate on sets of six ovules of each *Lactobacillus* strain for formulation F1, F2, and F3.

Viability of Lactobacilli Strains Recovered from the Ovules

Pharmaceutical formulations containing both strains were stored at 4 and 25 °C, and examined every 20 days during 180 days, to determinate bacterial viability over time. At each time, viable cell count was determined by the plate dilution method in triplicate. Then, 0.8 g of the ovules was weighed and incubated at 37 °C in 7.2 mL of MRS broth for 15 min, for dissolution. Serial dilutions were performed and seeded in MRS agar plates. Bacterial counts were expressed as CFU mL^{-1} (3,7).

To confirm the uniform distribution of lactobacilli, samples from the bottom, middle, and surface of the ovules of each formulation were taken to measure the bacterial counts. Variations in lactobacilli counts from sampled fractions were evaluated to estimate the bacterial distribution in the ovules.

Detection of Antimicrobial Activity

Lactobacilli strains were recovered from the ovules to test the antimicrobial activity of their active metabolites by two techniques, streak-diffusion, and agar well diffusion methods as previously described by Ruíz et al. (11,22). Although the antimicrobial activity of these lactobacilli strains has been demonstrated on a wide variety of pathogenic microorganisms, our studies were originally initiated on numerous *E. coli* strains (n = 200) isolated from different human infections (13,20). Based on these previous reports, a sensitive *E. coli* strain was selected as indicator microorganism. A second control was incorporated using *E. coli* ATCC 25922.

Biofilm Production

The biofilm-producing ability of the lactobacilli was tested by crystal violet staining, using a modification of the technique described by Terraf et al. (33). From each formulation, samples of 0.5 g were taken, seeded into MRS broths, and incubated overnight. Lactobacilli cultures were adjusted to tube No. 1 of McFarland turbidity scale (3×10^8 CFU mL^{-1}). Aliquots of 200 μL were transferred to sterile hemolysis tubes and incubated at 37 °C in microaerobic conditions for 6 and 24 h. Then, culture medium was carefully removed, and 25 μL of crystal violet 1% was added and left at room temperature for 15 min. To remove unattached cells and excess dye, three washes with 200 μL of phosphate-buffered saline (PBS; 0.736 g ClNa, 0.313 g PO_4KH_2, 0.465 g $PO_4Na_2 \cdot 12H_2O$, 100 mL H_2O, pH 6) was performed. Dye attached to biofilm on the walls of the tube was solubilized two times using 200 μL of 70% ethanol. Optical density (OD) at 540 nm on UV spectrophotometer was measured (34).

Statistical Analysis

All tests were performed in triplicate and mean ± SD was calculated. Bacterial counts were log-transformed. The obtained average values for each *Lactobacillus* strain were independently compared over time for all tests. All results were analyzed by ANOVA and then the Tukey test was performed. Statistical differences were considered as significant at $P \leq 0.05$ using InfoStat Software, version 2008, GrupoInfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

RESULTS

Preliminary *In Vitro* Lactobacilli Viability Test at Different Glycerol Proportions

In the bacterial suspensions containing 50% glycerol, L23 and L60 strains remained viable during 40 and 20 days, respectively. However, in those containing 30 and 16% glycerol, L23 and L60 strains were recovered viable for the duration of this *in vitro* experiment (180 days) (data not shown).

Ovules' Physical Characterization and Lactobacilli Content Distribution

The surface texture and consistency of the ovules were macroscopically observed. A smooth surface and uniform consistency without air bubbles were found in all cases. Bacterial counts for L23 and L60 strains were approximately 1×10^7 and 1×10^6 CFU mL^{-1}, respectively, at the three sampled fractions from the ovules (one set of six ovules of each strain for each formulation F1, F2, and F3 were tested). This demonstrated that lactobacilli were homogeneously distributed in the ovules. All formulations melted completely at 37 °C at three different times: 15, 10, and 2 min for F1, F2, and F3, respectively.

Lactobacilli Viability Test after Recovery from the Ovules

Ovules from F1 containing 50% glycerol showed a count at T_0 of 9.6×10^6 CFU mL^{-1} for L23 and 2×10^7 CFU mL^{-1} for L60. This formulation stored at 4 °C did not allow the recovery of lactobacilli after 40 days, confirming the loss of lactobacilli viability in F1. Viability results of F2 and F3 are shown in Fig. 1. Average counts of probiotic lactobacilli at day 90 were 2.7×10^4 and 8×10^2 CFU mL^{-1} for L23 and L60, respectively in F2, and approximately 3×10^5 CFU mL^{-1} for both strains contained in F3. At day120 of storage, lactobacilli counts in F2 were 1.5×10^3 and 1×10^3 CFU mL^{-1} for L23 and L60, respectively. In the case of F3, viable cell counts of L23 and L60 were 5.4×10^2 and 1.1×10^3 CFU mL^{-1}, respectively. Both lactobacilli strains in F2 and F3 remained viable until the end of the experiment (180 days), although low counts during the last time period were found. Average counts of lactobacilli at day 180 were 2×10^1 and 3.1×10^1 CFU mL^{-1} for L23 and L60, respectively in F2, while in F3, they were 1.7×10^1 and 6.3×10^1 CFU mL^{-1}, respectively. For F2 and F3, average bacterial counts of each *Lactobacillus* strain over time showed significant differences ($P < 0.05$).

Table I. Different Composition of Pharmaceutical Formulations

	Active principle	Excipients		Reverse osmosis water (% *v*/*v*)
	Lactobacillus 10^8 CFU mL^{-1} (%)	Glycerin (% *v*/*v*)	Gelatin (%)	
F1	12.5	50.0	10.0	27.5
F2	12.5	30.0	12.5	45.0
F3	12.5	16.0	11.5	60.0

F1 formulation no. 1, *F2* formulation no. 2, *F3* formulation no. 3

When the experiment was carried out at 25 °C, lactobacilli remained viable in F1, F2, and F3 for a period of 30 days of storage, and their counts were significantly lower than at 4 °C (data not shown).

Detection of Antimicrobial Activity by Streak-Diffusion Method

After recovery of both L23 and L60 strains from F2 and F3, the antimicrobial activity of their bioactive metabolites was retained during the 180 days. The average sizes of the inhibition zones produced by L23 were 23.4 mm ± 1.6 and 25.8 mm ± 1.9 in F2 and F3, respectively. This inhibitory activity was due to the joint action of organic acids and bacteriocin. In case of the L60 strain from F2 and F3, averages sizes of the inhibition zones of its bioactive metabolites (organic acids, H_2O_2, and bacteriocin) were 23.0 mm ± 2.0 and 25.9 mm ± 1.7, respectively. For F2 and F3, antimicrobial activity of both lactobacilli strains did not show significant differences through time ($P > 0.05$). For both lactobacilli strains in F1, antimicrobial activity was detected only until day 20 (Table II).

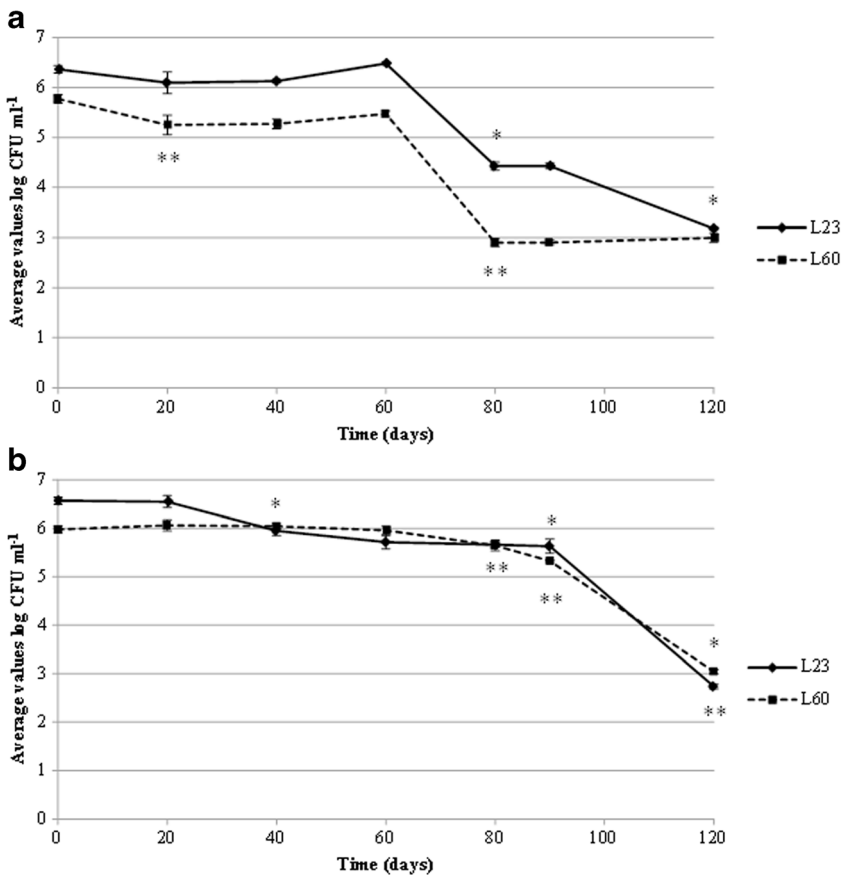

Fig. 1. Survival of *Lactobacillus* strains contained in ovules stored at 4 °C. **a** Formulation no. 2. **b** Formulation no. 3. *Indicates significant difference comparing mean values of log CFU mL^{-1} of L23 strain through time according to the Tukey test ($p < 0.05$). **Indicate significant difference comparing mean values of log CFU mL^{-1} of L60 strain through time according to the Tukey test ($p < 0.05$)

Table II. Average Sizes of Inhibition Zones Produced by *L. Fermentum* L23 and *L. Rhamnosus* L60 Recovered from F1, F2, and F3

	Inhibition zone in mm (average ± SD)					
	Formulation No. 1		Formulation No. 2		Formulation No. 3	
	L23	L60	L23	L60	L23	L60
C[a]	20.0 ± 1.0a	20.3 ± 1.5ab	20.3 ± 1.2ab	20.7 ± 1.2a	20.3 ± 1.5a	21.7 ± 0.6a
0 d	23.3 ± 1.5a	19.7 ± 0.6a	22.0 ± 2.0a	20.7 ± 2.1a	30.7 ± 2.3c	29.0 ± 1.7c
20 days	21.3 ± 1.5a	23.3 ± 1.2b	25.0 ± 2.0ab	23.0 ± 2.6a	30.6 ± 1.2c	27.6 ± 1.2bc
40 days	–	–	21.3 ± 1.2ab	21.0 ± 2.6a	28.0 ± 2.0bc	37.7 ± 2.1bc
60 days	–	–	26.3 ± 1.5b	25.7 ± 1.5a	25.3 ± 3.1abc	24.0 ± 2.6ab
80 days	–	–	22.0 ± 2.0ab	23.0 ± 2.0a	26.3 ± 1.5bc	25.3 ± 2.5abc
90 days	–	–	22.7 ± 0.6ab	20.7 ± 1.5a	22.7 ± 1.5ab	24.7 ± 0.6abc
120 days	–	–	24.0 ± 2.0ab	25.3 ± 1.5a	23.7 ± 2.1ab	25.7 ± 1.5abc
180 days	–	–	23.7 ± 2.1ab	25.0 ± 1.7a	25.0 ± 2.0abc	23.0 ± 1.0ab

Mean values with different letter indicate significant difference according to the Tukey test ($p < 0.05$)
– there was no growth of the recovered strains after 40 days
[a] Controls (C) were performed using overnight MRS cultures of L23 and L60 strains

Detection of Antimicrobial Activity by Agar Well Diffusion Method

Different inhibition zones of CFS and NCFS containing the bioactive metabolites produced by L23 and L60 strains were obtained (Table III). Inhibition zones corresponding to CFSs from L23 and L60 recovered from F2 ranged between 25 and 21 mm. The corresponding antimicrobial zones of NCFSs from both strains ranged between 20 and 17 mm. Similar results were obtained with F3. When comparing the sizes of the inhibition zones produced by the CFSs of L23 and L60, no significant differences were found ($P > 0.05$). When the NCFSs of L23 and L60 strains were compared, similar results were found ($P > 0.05$). Therefore, antimicrobial activity was not affected by the composition of the ovules.

Antimicrobial activity of strains recovered from pharmaceutical formulations was mainly produced by bacteriocins production throughout the 180 days. The inhibitory activity due to organic acids and H_2O_2 was meaningfully lower than that attributed to bacteriocins (Fig. 2). For both lactobacilli strains, there were significant differences between inhibitory activity due to organic acids *versus* bacteriocin ($P < 0.05$). In relation to the L23 strain, the proportional values of antimicrobial activity due to its bacteriocin ranged between 70 and 84% in F2 and between 69 and 84% in F3. For the L60 strain, antimicrobial activity attributed to its bacteriocin ranged between 73 and 86% and between 72 and 87% for F2 and F3, respectively. The chemical composition of the ovules did not affect the antimicrobial activity of organic acids and bacteriocins produced by L23 and L60 strains ($P > 0.05$).

Biofilm Production Ability

The biofilm-producing ability by the lactobacilli strains was estimated by OD values. Lactobacilli strains recovered from the ovules were able to maintain their biofilm-producing ability during the 180 days in comparison with the controls. For L23 recovered from F2 at 6 and 24 h of incubation, the average OD values were similar, with values of 0.060 ± 0.005 and 0.061 ± 0.006, respectively. Similar results were obtained at 6 and 24 h for L23 recovered from F3, with average OD values of 0.058 ± 0.005 and 0.060 ± 0.004, respectively. For L60 from F2, the average values were 0.061 ± 0.005 and 0.062 ± 0.004 after 6 and 24 h of incubation, respectively. Finally, the average OD values for L60 from F3 were 0.059 ± 0.004 and 0.060 ± 0.005, respectively (Table IV). There was no significant difference between the biofilm-producing ability of both lactobacilli strains through time ($P > 0.05$).

DISCUSSION

The maintenance of suitable proportion of *Lactobacillus* species in the vagina is a key condition to prevent vaginal infections. The study and optimization of vaginal pharmaceutical formulations containing probiotic lactobacilli is a critical issue to ensure an adequate number of viable cells in the genital tract (35). One of the first steps after obtaining the ovules containing the lactobacilli was a macro-analysis on their physical properties. While Maggi et al. (36) employed double-layer ovules with different release time of its contents, in the present study, single-layer ovules were used whose contents (excipients, lactobacilli) were homogeneously distributed. This last type of ovules could constitute an interesting technological advantage because these single-layer ovules melt faster; therefore, lactobacilli would be easily delivered and a faster vaginal colonization would be ensured. In this study, the choice of excipients for the development of ovules was made because of their frequent usage in vaginal products containing drugs or living bacteria, as it was already reported by Kale et al. (32) and Zárate et al. (37).

Regarding ovule development containing probiotic lactobacilli, it requires to achieve not only the survival of these microorganisms but also the unaltered maintenance of their own probiotic properties during both stages, production, and storage (shelf-life) of the product (37,38). This first study was carried out using fresh living bacteria, and two factors, storage temperature and glycerol proportion, were considered as potential causes affecting bacterial viability in the ovules. Bacterial viability was maintained after storage at

Table III. Average Sizes of Inhibition Zones Produced by Non-Treated and Neutralized Cell-Free Supernatants of *L. fermentum* L23 and *L. rhamnosus* L60 Recovered from the Different Formulations

	Inhibition zone in mm (average ± SD)											
	Formulation no. 1				Formulation no. 2				Formulation no. 3			
	L. fermentum 23		*L. rhamnosus* 60		*L. fermentum* 23		*L. rhamnosus* 60		*L. fermentum* 23		*L. rhamnosus* 60	
	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS
C[a]	22.0 ± 1.0a	17.7 ± 0.6a	24.0 ± 1.0a	17.5 ± 0.5a	23.0 ± 1.1a	17.3 ± 1.2a	23.7 ± 1.5a	18.0 ± 2.6a	22.3 ± 1.1ab	17.5 ± 0.5a	24.7 ± 0.6a	18.3 ± 2.9a
0 day	22.6 ± 1.5a	18.0 ± 1.0a	23.0 ± 2.0a	18.3 ± 2.5a	22.8 ± 0.8a	18.0 ± 1.8ab	23.0 ± 3.5a	19.7 ± 3.2a	23.3 ± 1.5ab	16.3 ± 0.6a	23.7 ± 2.5a	19.7 ± 1.1a
20 days	23.0 ± 0.6a	18.7 ± 1.5a	22.7 ± 0.7a	18.7 ± 1.5a	25.7 ± 2.1a	18.0 ± 1.0ab	24.0 ± 4.0a	19.0 ± 2.6a	24.0 ± 1.0b	18.7 ± 0.6a	24.7 ± 1.5a	19.3 ± 1.2a
40 days	–	–	–	–	24.7 ± 1.5a	18.7 ± 1.5ab	24.0 ± 2.0a	20.0 ± 2.6a	23.7 ± 2.1ab	17.7 ± 1.2a	24.0 ± 2.0a	18.0 ± 2.6a
60 days	–	–	–	–	25.3 ± 2.5a	21.3 ± 0.6b	23.7 ± 2.9a	18.3 ± 3.2a	21.7 ± 0.6ab	18.3 ± 1.5a	23.7 ± 2.5a	20.7 ± 1.5a
80 days	–	–	–	–	23.7 ± 1.5a	18.3 ± 1.5ab	24.3 ± 1.5a	18.0 ± 2.0a	23.3 ± 2.1ab	18.7 ± 2.1a	24.0 ± 1.7a	19.3 ± 2.3a
90 days	–	–	–	–	22.3 ± 1.5a	18.0 ± 1.0ab	23.7 ± 3.2a	17.3 ± 1.5a	21.0 ± 0.7ab	16.0 ± 1.7a	24.0 ± 1.0a	17.3 ± 1.5a
120 days	–	–	–	–	23.0 ± 1.0a	19.0 ± 1.7ab	21.7 ± 3.1a	19.0 ± 2.6a	21.0 ± 1.0ab	17.3 ± 1.5a	23.7 ± 4.2a	17.7 ± 1.5a
180 days	–	–	–	–	21.3 ± 1.5a	17.3 ± 1.5a	22.7 ± 1.5a	18.0 ± 1.0a	20.0 ± 1.5a	16.1 ± 0.8a	24.0 ± 2.0a	17.7 ± 0.6a

Mean values with different letter indicate significant difference according to the Tukey test ($p < 0.05$)
CFS cell-free supernatant, *NCFS* neutralized cell free supernatant. – there was no growth of the recovered strains after 40 days
[a] Controls (C) were performed using overnight MRS cultures of L23 and L60 strains

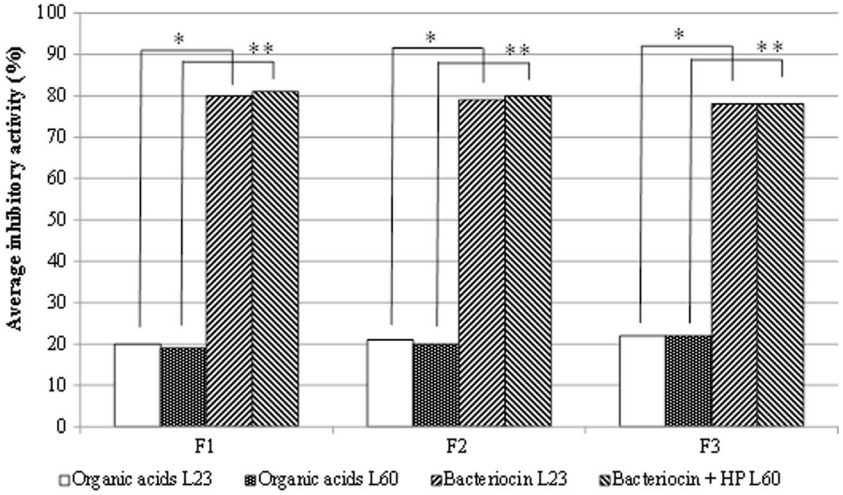

Fig. 2. Average percentage of inhibitory activity corresponding to each metabolite produced by L23 and L60 strains from pharmaceutical formulations. F1: Formulation No. 1, F2: Formulation No. 2, F3: Formulation No. 3. HP: hydrogen peroxide. *On columns indicates significant difference comparing mean values of inhibitory activity due to organic acids *versus* bacteriocin L23 of L23 strain. **On columns indicate significant difference comparing mean values of inhibitory activity due to organic acids *versus* bacteriocin L60 of L60 strain. These analysis were done employing the Tukey test ($P < 0.05$)

4 °C, but not at room temperature. This result was similar to those reported by Juárez Tomás et al. (6), who although evaluating other lactobacilli species found similar behavior during storage temperature. In a recent study by Albadran et al. (39), similar results to those obtained in this study were found, but these authors used lyophilized bacteria. Although so far our results using fresh living bacteria are promising, in future studies based on this report as well as on other recent ones employing freeze-dried methods (6,10,35,40), we estimate that more relevant results could be obtained. This drying method could represent a technological option that fulfills a double function, protecting microorganisms in the ovules during their shelf-life and being economically feasible, which could be approached by industry. Furthermore, the preliminary results of glycerol *in vitro* test suggested that the bacterial suspensions containing 30 and 16% of glycerol were the most adequate to preserve lactobacilli viability. Then, these findings were replicated when the test was done fusing ovules. Thus, it was demonstrated that the best formula to preserve viability of lactobacilli was F3. In relation to storage period, it is noteworthy to mention that lactobacilli viability in the ovules was maintained during a long period of time (180 days) at 4 °C, bearing in mind that the experiment began using fresh living bacteria cultures. The results obtained in our study are encouraging compared to those reported by Uehara et al. (41), who studied the viability of freeze-dried *L. crispatus* from vaginal suppositories during a shorter time period (60 days). Furthermore, when the antimicrobial activity of L23 and L60 strains was studied, we demonstrated that it was not affected for at least 180 days. Recently, Dhewa et al. (7), developed a symbiotic product which required the presence of prebiotics substances to improve the antimicrobial activity of their probiotics. In contrast, we found high antimicrobial activity in our probiotic strains, L23 and L60, without the previous addition of prebiotics. In this study, the values of inhibition zone produced by our *Lactobacillus* strains on *E. coli* growth were meaningfully higher than those found by Kaewnopparat et al. (42) and Saadatzadeh*et al.* (29) in similar tests evaluating other lactobacilli species. To detect the antimicrobial activity of our probiotic lactobacilli recovered from the ovules during the 180 days, two *in vitro* diffusion tests were performed. In this way, we could confirm that the antimicrobial activity of these lactobacilli was maintained throughout the study, being mainly due to the bacteriocin production by the L23 and L60 strains. While these bacteriocins were the main metabolites responsible for the antimicrobial activity produced by the lactobacilli strains, previous studies reported by Lin et al. (28) and Saadatzadeh et al. (29) using different *Lactobacillus* species have demonstrated that this activity was strictly due to organic acids.

Lactobacilli's biofilm formation ability is a relevant phenotypic property of these non-pathogenic bacteria, as it could be related to their capacity to adhere to the vaginal epithelium. Biofilms would promote vaginal colonization, thus achieving a more balanced ecosystem. This also has been associated with a competitive exclusion mechanism against pathogenic microorganisms, when these are in the vaginal niche (33,43–46). To detect *in vitro* biofilm production by our probiotic strains was an important first step, as a way to predict the possibility of an *in vivo* adhesion to epithelial cells. The results of this first study showed an intermediate level of biofilm-producing ability by L23 and L60 when the latter were recovered from the ovules, as compared to the controls.

CONCLUSION

In conclusion, this study allowed demonstrating that ovule's glycerol proportion and storage temperature are two variables that modify the viability of *L. fermentum* L23 and *L. rhamnosus* L60 in these formulations. However, although these ovules were formulated with a very limited number of excipients, our probiotic lactobacilli were viable for a long

Table IV. Biofilm Production by L23 and L60 Strains Recovered from the Ovules

	Optical density values (540 nm) (average ± SD)						
	Formulation no. 1				Formulation no. 2		
	L. fermentum 23		*L. rhamnosus* 60		*L. fermentum* 23		*L. rhamnosus* 60
	6 h	24 h	6 h	24 h	6 h	24 h	6 h
C[a]	0.058 ± 0.004a	0.060 ± 0.007a	0.058 ± 0.001a	0.059 ± 0.001b	0.058 ± 0.002a	0.059 ± 0.010a	0.062 ± 0.010a
0 day	0.058 ± 0.005a	0.061 ± 0.004a	0.061 ± 0.005a	0.064 ± 0.004a	0.061 ± 0.012a	0.064 ± 0.004a	0.058 ± 0.005a
20 days	0.054 ± 0.009a	0.058 ± 0.009a	0.062 ± 0.007a	0.065 ± 0.006a	0.061 ± 0.006a	0.064 ± 0.008a	0.060 ± 0.002a
40 days	–	–	–	–	0.060 ± 0.005a	0.059 ± 0.009a	0.062 ± 0.003a
60 days	–	–	–	–	0.061 ± 0.003a	0.063 ± 0.004a	0.059 ± 0.006a
80 days	–	–	–	–	0.059 ± 0.007a	0.062 ± 0.007a	0.060 ± 0.008a
90 days	–	–	–	–	0.057 ± 0.001a	0.058 ± 0.004a	0.058 ± 0.005a
120 days	–	–	–	–	0.056 ± 0.007a	0.059 ± 0.006a	0.062 ± 0.006a
180 days	–	–	–	–	0.058 ± 0.002a	0.060 ± 0.007a	0.063 ± 0.006a

	Optical density values (540 nm) (average ± SD)				
	Formulation no. 2	Formulation no. 3			
	L. rhamnosus 60	*L. fermentum* 23		*L. rhamnosus* 60	
	24 h	6 h	24 h	6 h	24 h
C[a]	0.063 ± 0.003a	0.058 ± 0.005a	0.062 ± 0.007a	0.060 ± 0.008a	0.061 ± 0.005a
0 day	0.058 ± 0.002a	0.056 ± 0.006a	0.061 ± 0.004a	0.057 ± 0.004a	0.058 ± 0.005a
20 days	0.061 ± 0.009a	0.056 ± 0.007a	0.058 ± 0.003a	0.059 ± 0.006a	0.060 ± 0.008a
40 days	0.061 ± 0.004a	0.058 ± 0.008a	0.061 ± 0.005a	0.058 ± 0.005a	0.060 ± 0.006a
60 days	0.061 ± 0.002a	0.061 ± 0.002a	0.061 ± 0.002a	0.060 ± 0.001a	0.061 ± 0.001a
80 days	0.062 ± 0.001a	0.058 ± 0.007a	0.058 ± 0.003a	0.059 ± 0.009a	0.059 ± 0.004a
90 days	0.063 ± 0.004a	0.060 ± 0.005a	0.061 ± 0.006a	0.061 ± 0.004a	0.062 ± 0.002a
120 days	0.063 ± 0.004a	0.060 ± 0.006a	0.060 ± 0.005a	0.059 ± 0.003a	0.060 ± 0.003a
180 days	0.060 ± 0.003a	0.058 ± 0.002a	0.060 ± 0.005a	0.060 ± 0.001a	0.059 ± 0.008a

Mean values with different letter indicate significant difference according to the Tukey test ($p < 0.05$)
– there was no growth of the recovered strains after 40 days
[a] Controls (C) were performed using overnight MRS cultures of L23 and L60 strains

period of time. In fact, their antimicrobial activity and biofilm-producing ability were also maintained without any change during the test time after recovery from the ovules. These two last properties are relevant for the development of a vaginal product. Furthermore, in this study, we highlight that still beginning with fresh cultures, these two specific lactobacilli strains with strong probiotic properties have a promising biotechnological potential. Future studies should be approached considering modifications in the formulations that were chosen in this study and employing freeze-drying methods to possibly ensure a higher viability level of lactobacilli contained in the ovules for a longer time period. We consider that these modifications could probably lead to a more feasible method to be reproduced at an industrial scale.

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