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RESEARCH ARTICLE

Design of novel urogenital pharmabiotic formulations containing lactobacilli, salivaricin CRL 1328 and non-microbial compounds with different functionalities

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Abstract

Context: The administration of pharmabiotics is a promising alternative to antimicrobial drugs for the treatment and/or prevention of female urogenital infections.
Objective: To design pharmabiotic formulations including bioactive ingredients of microbial origin combined with non-microbial substances and then to evaluate the stability of the combinations during freeze-drying and storage.
Materials and methods: Different formulations including *Lactobacillus gasseri* CRL 1263, *Lactobacillus salivarius* CRL 1328, salivaricin CRL 1328 (a bacteriocin) and non-microbial compounds (lactose, inulin and ascorbic acid) were assayed, and the ingredients were freeze-dried together or separately. The formulations were stored in gelatin capsules at 4 °C for 360 d.
Results: The viability of lactobacilli was affected to different extents depending on the strains and on the formulations assayed. *L. salivarius* and ascorbic acid were successfully combined only after the freeze-drying process. Salivaricin activity was not detected in formulations containing *L. gasseri*. However, when combined with ascorbic acid, lactose, inulin or *L. salivarius*, the bacteriocin maintained its activity for 360 d. The selected microorganisms proved to be compatible for their inclusion in multi-strain formulations together with lactose, inulin and ascorbic acid. Salivaricin could be included only in a *L. salivarius* CRL 1328 single-strain formulation together with non-microbial substances.
Conclusions: This study provides new insights into the design of urogenital pharmabiotics combining beneficial lactobacilli, salivaricin CRL 1328 and compounds with different functionalities.

Keywords

Bacteriocin, lactic acid bacteria, pharmabiotic, probiotic, urogenital infection, vaginal formulation

History

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Introduction

Urogenital tract infections (UGTI) are among the most common infections affecting women of all socioeconomic levels around the world. There is a strong correlation between the maintenance of the indigenous vaginal microbiome and the health of the host[1,2]. Therefore, in some situations, UGTI are a consequence of modifications of the vaginal microbiome, where pathogens become dominant and replace protective lactic acid bacteria (LAB). The conventional treatment of a wide variety of etiological agents responsible for UGTI implies the application of specific antimicrobial drugs (i.e. antifungal, antibiotic, antiparasitic and antiviral substances). However, promising alternatives such as probiotics and pharmabiotics have emerged to restore the urogenital microbiome in order to facilitate and promote the growth of beneficial microorganisms and decrease the overgrowth of pathogens[3].

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Probiotics are defined as ''live microorganisms which, when administered in adequate amounts, exert a beneficial physiological effect on the host health''[4]. Currently, several urogenital probiotics products containing LAB are available on the international market (e.g. Gynoflor®, Medinova, Zürich, Switzerland; Lactin V, Osel Inc., Mountain View, CA; Normogin, Laboratory Baldacci, Pisa, Italy; Ecovag® Flora, Bifodan A/S, Hundested, Denmark; Lactinex®, Omega Laboratory, Buenos Aires, Argentina; Fem-Dophilus®, Jarrow Formulas, Los Angeles, CA, etc.). On the other hand, pharmabiotics include ''live or dead microorganisms as well as microbial constituents and metabolites (e.g. bacteriocins), which can beneficially interact with the host''[5]. To the best of our knowledge, pharmabiotic formulations for urogenital applications containing autochthonous microbial cells and bacteriocins have not yet been developed.

The main goal of our research group is the design of novel probiotics/pharmabiotics to restore the human urogenital microbiome for the prevention of female UGTI. Several human vaginal lactobacilli have been previously isolated from women from Tucumán (Argentina), characterized and selected for their beneficial, functional and technological properties[6,7]. Furthermore, the basic, genetic, mechanistic and technological studies of

salivaricin CRL 1328, a bacteriocin produced by vaginal *Lactobacillus salivarius* CRL 1328, were carried out[8–11]. Salivaricin CRL 1328 is active against urogenital pathogens including *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Enterococcus faecium* and *Staphylococcus saprophyticus*.

Our research team is studying the design of urogenital pharmabiotic formulations that contain beneficial microorganisms combined with other microbial and different functional ingredients of non-microbial origin, all of them carefully selected to increase the functionality and stability of the final product. Besides bacteriocins (ribosomally synthesized antimicrobial substances of microbial origin), some other substances with remarkable functional characteristics could be included, as vitamins, prebiotics, protective carbohydrates, antioxidants and bioadhesives. During the design of a new pharmabiotic formula, the compatibility of all the compounds of interest or constituents must be evaluated in order to avoid antagonistic effects and negative interferences between them. Furthermore, the appropriate method to quantify the viable microorganisms or the activity and bioavailability of the bioactive compounds over the process and storage time needs to be optimized and standardized. On the other hand, the pharmaceutical industry requires low production and storage costs together with the design of stable products capable of retaining their properties during their shelf-life. In this sense, being lyophilization one of the most successful methods to preserve the stability of bioactive compounds and microorganisms during long-term storage.

The main pharmaceutical forms of drug administration in the urogenital tract include gels, creams, vaginal suppositories and capsules[12]. Gelatin capsules offer some adequate characteristics for the formulation of the product, such as accurate dosing and low cost. These capsules allow the maintenance of freeze-dried microorganisms and their subsequent release in the vaginal environment under adequate conditions of humidity and temperature. Furthermore, this type of dosage has proved to be well tolerated in clinical trials of vaginal application in women aged 15–35 years for three months[13].

The objective of this work was to design novel urogenital pharmabiotic formulations containing a high number of viable beneficial lactobacilli combined with salivaricin CRL 1328 and substances with different functionality (lactose, inulin and ascorbic acid). To achieve this goal, the compatibility and resistance during the lyophilization process of vaginal lactobacilli and salivaricin CRL 1328 and the stability of the formulations during subsequent storage in gelatin capsules for vaginal application were evaluated.

Materials and methods

Microorganisms and growth conditions

The microorganisms used in this study were *L. salivarius* CRL [Centro de Referencia para Lactobacilos (CERELA) Culture Collection, Tucumán, Argentina] 1328 (a strain that inhibits urogenital pathogens by lactic acid and salivaricin CRL 1328 production) and *Lactobacillus gasseri* CRL 1263 (a strain that produces H_2O_2 and inhibits urogenital pathogens by lactic acid production)[8,11]. These organisms were previously isolated from human vagina and deposited in the CERELA Culture Collection[6]. Compatibility between the *Lactobacillus* strains was previously evaluated[11]. Vaginal pathogenic *E. faecalis* MP97 (from the Instituto de Microbiología of the Universidad Nacional de Tucumán, Argentina) was used as an indicator for the quantification of salivaricin activity. All the microorganisms were stored in milk–yeast extract (% (w/v): 13 nonfat milk, 0.5 yeast extract and 1 glucose) at −70 °C. Prior to the assays, the strains were grown in LAPTg (% (w/v): 1 yeast extract, 1.5 meat peptone, 1 tryptone and 1 glucose, 0.1% (v/v) Tween 80, pH 6.5; ingredients were obtained from Britania Laboratories, Buenos Aires, Argentina) broth for 24 h at 37 °C under static conditions and then subcultured twice in LAPTg broth for 12 h at 37 °C.

Pharmabiotic formulations under evaluation

Two experiments were performed (Table 1). The first design of experiments was carried out to determine the following: (a) the compatibility between the different bioactive ingredients of microbial origin (BIMO) and compounds of non-microbial origin (non-microbial compounds, N-MC) when combined before the lyophilization process and (b) the stability of formulations during subsequent storage. In this assay, three BIMO [biomass of *L. gasseri* CRL 1263, *L. salivarius* CRL 1328 and salivaricin CRL 1328 (Sal), either as a single BIMO or all of them combined] were freeze-dried in the presence of the following N-MC, which were added either individually or combined: 8% (w/v, final concentration in the suspensions before freeze-drying) lactose (L, a pharmaceutical excipient also used as a lyoprotector for the preservation of LAB)[14,15], 8% (w/v) inulin (I, a heterogeneous mixture of polymers of fructose, widely used as a prebiotic compound to favor the growth of beneficial microorganisms)[16,17] and 2.5% (w/v) ascorbic acid also named vitamin C (C, an antioxidant substance) (Table 1). A total of 16 different combinations were assayed.

From the results obtained in this first assay, a second design of experiments including eight combinations was carried out in order to evaluate the stability of the formulations when salivaricin and vitamin C were combined with the other ingredients of the

Table 1. Ingredient combinations of the pharmabiotic formulations assayed in the designs of experiments.

Assay	Combination number	Ingredients in formulations*
First design of experiments	1	CRL 1263-L
	2	CRL 1263-I
	3	CRL 1263 -C
	4	CRL 1263-LIC
	5	CRL 1328-L
	6	CRL 1328-I
	7	CRL 1328-C
	8	CRL 1328-LIC
	9	Sal-L
	10	Sal-I
	11	Sal-C
	12	Sal-LIC
	13	CRL 1328-CRL 1263-Sal-L
	14	CRL 1328-CRL 1263-Sal-I
	15	CRL 1328-CRL 1263-Sal-C
	16	CRL 1328-CRL 1263-Sal-LIC
Second design of experiments	1	(CRL 1263-L)-Sal
	2	(CRL 1263-Sal-L)
	3	(CRL 1328-L)-Sal
	4	(CRL 1328-Sal-L)
	5	(CRL 1328-L)-C
	6	(CRL 1328-CRL 1263-LI)-Sal
	7	(CRL 1328-CRL 1263-LI)-C
	8	(CRL 1328-CRL 1263-LI)-Sal-C

In the second design of experiments, the ingredients indicated in brackets were lyophilized together and later combined with the other ingredients in the formula.

*Ingredients: CRL 1328, *Lactobacillus salivarius* CRL 1328; CRL 1263, *Lactobacillus gasseri* CRL 1263; Sal, salivaricin CRL 1328; L, lactose; I, inulin; C, vitamin C (ascorbic acid); LIC, lactose, inulin and vitamin C; and LI, lactose and inulin.

formula, either before or after freeze-drying (Table 1). Two replications of the two experiments were performed.

Freeze-drying of pharmabiotic formulas and storage in gelatin capsules

The bacterial cells from the third subculture of lactobacilli were centrifuged (6000 *g* for 10 min at 4 °C), washed twice with saline and concentrated into a smaller volume to obtain 10^9–10^{10} CFU mL^{-1}. On the other hand, salivaricin [320 Arbitrary Units per milliliter (AU mL^{-1})] was combined with 0.5% polyethylene glycol 8000 and 5% sucrose as bacteriocin lyoprotectors[18]. When preparing multi-strains formulations, *L. gasseri* CRL 1263 and *L. salivarius* CRL 1328 concentrated cultures were combined in equal proportions. Later, the concentrated cultures and salivaricin were homogeneously mixed with the other ingredients in the formula (Table 1) as described above. The suspensions obtained were placed in Petri dishes and frozen at −20 °C for 24 h prior to the freeze-drying process. As cited before, in some combinations of the second design of experiments, salivaricin and ascorbic acid were lyophilized separately and later combined with the corresponding ingredients (Table 1).

Lyophilization was carried out in a chamber-type freeze-drier (Lyovac GT2, Leybold, Cologne, Germany) for 16 h at 0.3 mbar (1 bar = 100 kPa), which yielded products with <1% residual moisture. Each of the designed combinations of the lyophilized powders was filled into size 00 gelatin capsules (approximately 0.95 cm^3) and stored at 4 °C in sealed containers (Parafilm® M, Wertheim, Germany) and under dark conditions for different time periods.

Cell viability

The number of viable microorganisms was evaluated at different times during the lyophilization process (before freeze-drying and after freeze-drying) and storage (at 60, 120, 180 and 360 d). Viable cells were determined by the plate dilution method described above, using LAPTg agar (for single-strain formulations) or LAPTg agar supplemented with antibiotic to selectively quantify the *Lactobacillus* strains in the multi-strain formulations. The results were expressed as log CFU g^{-1}. Since *L. salivarius* CRL 1328 is vancomycin resistant[19], the antibiotic susceptibility of *L. gasseri* CRL 1263 was studied to determine the number of viable cells of each of the strains assayed when combined in the mixtures. The susceptibility to vancomycin was determined by the plate dilution test, according to the technique described by Ocaña et al.[19].

Quantification of salivaricin activity

Salivaricin activity was quantified in all bacteriocin-containing formulas under study before and after freeze-drying and during storage. The agar plate diffusion method was used to quantify salivaricin[20]. Aliquots (25 µl) of each diluted sample were poured into 4 mm holes performed in LAPTg agar [LAPTg 1% (w/v) agar] plates, which contained the pathogenic *E. faecalis* MP97 strain (10^6 CFU mL^{-1}) used as an indicator. The plates were incubated for 2 h at room temperature and then for 24 h at 37 °C. The results were expressed as Arbitrary Units per gram (AU g^{-1}). One AU was defined as the reciprocal of the highest dilution that produced an inhibition halo of at least 1 mm around the hole.

Statistical evaluation

Estimation of the survival parameters

An exponential decay model was adjusted to describe the survival modifications of the number of viable cells during freeze-drying and storage. The exponential decay model includes three parameters, as shown in the following equation:

$$\log \text{ CFU g}^{-1} = A + De^{(-bt)} \quad (1)$$

where A stands for the number of viable cells (log CFU g^{-1}) at the end of storage, D for the differences between the higher and lower number of microorganisms (as log CFU g^{-1}), b for the rate of cell viability decay (d^{-1}) and t for time (expressed in days) of storage. Survival parameters were estimated by nonlinear regression.

To calculate the standard errors of the survival parameters estimated, the bootstrapping technique was applied using repeated samples from the original data set[9,21]. The number of bootstrap samples chosen was 100.

The nonlinear mixed model was used to determine the statistical significance of the effects of the different formulations on the survival parameters. The differences between mean values were considered significant when $p < 0.05$. Since each microorganism showed a different behavior, the results obtained for each one were analyzed individually.

Evaluation of salivaricin CRL 1328 stability during freeze-drying and storage

To analyze the effects of the different formulations under study and of the storage times on salivaricin activity, a mixed model of analysis of variance was applied. Dunnett's T3 *post-hoc* test was used to identify the conditions in which there were statistically significant differences between the salivaricin activity data. The differences between mean values were considered significant when $p < 0.05$.

Results

First design of experiments applied to pharmabiotic formulations

Resistance of vaginal lactobacilli to the lyophilization process

The addition of vancomycin to the culture medium (10 µg mL^{-1}) was used for the differential counting of *L. salivarius* CRL 1328 and *L. gasseri* CRL 1263 viable strains since *L. gasseri* CRL 1263 was susceptible to this antibiotic (minimum inhibitory concentration = 2 µg mL^{-1}). After freeze-drying, the number of viable cells of each strain was dependent on the type of formulation (Table 2). *Lactobacillus gasseri* CRL 1263 was the most resistant strain under all evaluated conditions. The lowest decreases in *L. gasseri* viable cells (0.54–0.67 log CFU g^{-1}) were recorded in the formulations containing all the N-MC (CRL 1263-LIC and CRL 1328-CRL 1263-Sal-LIC) and in the ones including the three BIMO combined only with lactose (CRL 1328-CRL 1263-Sal-L).

Lactobacillus salivarius CRL 1328 was able to resist to the freeze-drying process in four of the eight formulations under evaluation. The lowest viability loss was observed with lactose alone (CRL 1328-L, decrease in viable cells = 0.45 log CFU g^{-1}). Interestingly, the conditions that showed no viable cells were those with ascorbic acid added (Table 2). These results indicate that ascorbic acid was injurious to *L. salivarius* under the conditions assayed in this design of experiments, and that a different strategy was then applied in order to include both ingredients in the same formulation.

Resistance of lactobacilli to storage conditions

Microbial cell survival of the lyophilized samples stored for 360 d and periodically analyzed is summarized in Figure 1. The survival parameters estimated applying the exponential model are listed in

Table 2. Resistance to the lyophilization process of vaginal lactobacilli included in different pharmabiotic formulations (first design of experiments).

Formulation ingredients*	Log CFU g^{-1} before freeze-drying†	Log CFU g^{-1} after freeze-drying‡	Viability decrease¶
	Lactobacillus gasseri CRL 1263		
CRL 1263-L	10.93	9.59	1.34
CRL 1263-I	10.79	9.54	1.25
CRL 1263-C	10.75	9.50	1.24
CRL 1263-LIC	10.73	10.06	**0.67**
CRL 1328-CRL 1263-Sal-L	10.67	10.05	**0.63**
CRL 1328-CRL 1263-Sal-I	10.93	8.88	2.06
CRL 1328-CRL 1263-Sal-C	10.74	9.46	1.28
CRL 1328-CRL 1263-Sal-LIC	10.43	9.88	**0.54**
	Lactobacillus salivarius CRL 1328		
CRL 1328-L	10.30	9.85	**0.45**
CRL 1328-I	10.16	7.19	2.97
CRL 1328-C	10.20	0.00	10.20
CRL 1328-LIC	9.72	0.00	9.72
CRL 1328-CRL 1263-Sal-L	9.74	8.05	1.69
CRL 1328-CRL 1263-Sal-I	9.83	7.85	1.98
CRL 1328-CRL 1263-Sal-C	9.98	0.00	9.98
CRL 1328-CRL 1263-Sal-LIC	9.58	0.00	9.58

Values in bold type correspond to the minimal decreases in viable cells.
*Ingredients: CRL 1328, *Lactobacillus salivarius* CRL 1328; CRL 1263, *Lactobacillus gasseri* CRL 1263; Sal, salivaricin CRL 1328; L, lactose; I, inulin; C, vitamin C (ascorbic acid); and LIC, lactose, inulin and vitamin C.
†Number of viable *Lactobacillus* cells (as log CFU g^{-1}) before freeze-drying.
‡Number of viable *Lactobacillus* cells (as log CFU g^{-1}) after freeze-drying.
¶Differences between the number of viable cells before and after lyophilization (as log CFU g^{-1}).

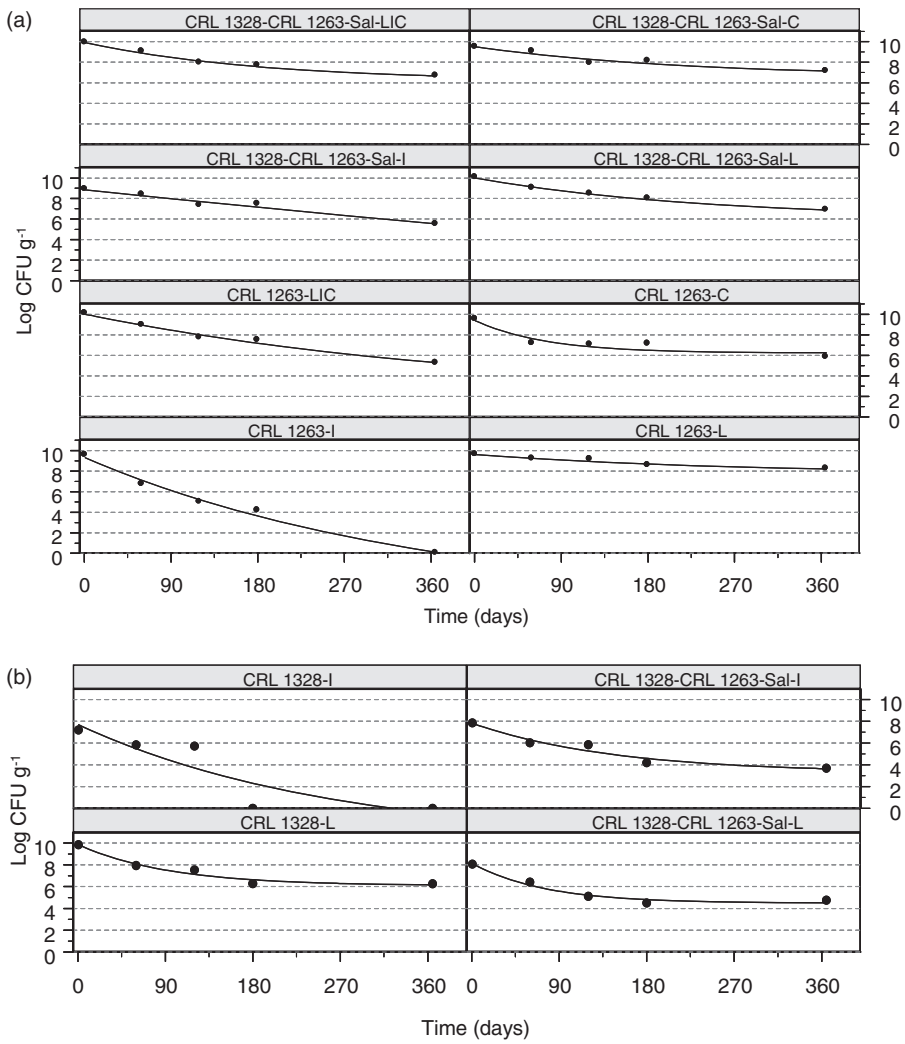

Figure 1. Number of viable cells of freeze-dried vaginal lactobacilli during storage for 360 d at 4 °C in different pharmabiotic formulations (first design of experiments). (a) Viability of *Lactobacillus gasseri* CRL 1263. (b) Viability of *Lactobacillus salivarius* CRL 1328. Ingredients in formulations: CRL 1328, *Lactobacillus salivarius* CRL 1328; CRL 1263, *Lactobacillus gasseri* CRL 1263; Sal, salivaricin CRL 1328; L, lactose; I, inulin; C, vitamin C (ascorbic acid); LIC, lactose, inulin and vitamin C. Lines represent the adjustment to experimental data (• symbols) using the exponential decay model (see Materials and methods). The first data in each graph represent the log CFU g^{-1} of each combination after lyophilization (initial storage time).

Table 3. Survival parameters estimated by the exponential decay model during the storage of freeze-dried vaginal lactobacilli in different pharmabiotic formulations (first design of experiments).

		Vaginal *Lactobacillus* strains	
Survival parameters*	Ingredients in formulations†	*Lactobacillus gasseri* CRL 1263	*Lactobacillus salivarius* CRL 1328
A (log CFU g^{-1})	CRL 1263-L	**7.64 ± 3.31**	
	CRL 1263-I	0.00 ± 1.41	
	CRL 1263-C	**6.23 ± 2.91**	
	CRL 1263-LIC	7.33 ± 2.92	
	CRL 1328-L		**6.09 ± 1.86**
	CRL 1328-I		0.00 ± 1.62
	CRL 1328-C		ND
	CRL 1328-LIC		ND
	CRL 1328-CRL 1263-Sal-L	6.05 ± 0.94	4.48 ± 1.06
	CRL 1328-CRL 1263-Sal-I	7.16 ± 0.59	3.26 ± 1.31
	CRL 1328-CRL 1263-Sal-C	**6.58 ± 2.12**	ND
	CRL 1328-CRL 1263-Sal-LIC	6.15 ± 1.36	ND
D (log CFU g^{-1})	CRL 1263-L	**1.97 ± 3.24**	
	CRL 1263-I	9.70 ± 3.33	
	CRL 1263-C	**3.19 ± 2.08**	
	CRL 1263-LIC	3.67 ± 2.52	
	CRL 1328-L		**3.79 ± 1.84**
	CRL 1328-I		7.89 ± 4.09
	CRL 1328-C		ND
	CRL 1328-LIC		ND
	CRL 1328-CRL 1263-Sal-L	3.96 ± 0.78	3.69 ± 1.61
	CRL 1328-CRL 1263-Sal-I	3.22 ± 0.47	4.58 ± 1.65
	CRL 1328-CRL 1263-Sal-C	**2.93 ± 1.96**	ND
	CRL 1328-CRL 1263-Sal-LIC	3.78 ± 1.37	ND
b (d^{-1})	CRL 1263-L	0.003 ± 0.009	
	CRL 1263-I	0.006 ± 1.486	
	CRL 1263-C	0.014 ± 0.399	
	CRL 1263-LIC	0.295 ± 0.136	
	CRL 1328-L		0.011 ± 0.007
	CRL 1328-I		0.007 ± 1.674
	CRL 1328-C		ND
	CRL 1328-LIC		ND
	CRL 1328-CRL 1263-Sal-L	0.004 ± 0.094	0.014 ± 0.399
	CRL 1328-CRL 1263-Sal-I	0.627 ± 0.199	0.007 ± 0.441
	CRL 1328-CRL 1263-Sal-C	0.005 ± 0.124	ND
	CRL 1328-CRL 1263-Sal-LIC	0.005 ± 0.173	ND

Values in bold type correspond to the minimal decreases in viable cells. ND, not determined; the survival parameters of *Lactobacillus salivarius* CRL 1328 in formulations containing ascorbic acid were not estimated since the values of viable cells after lyophilization were zero.

*Survival parameters: *A*, number of viable cells (as log CFU ml^{-1}) at the end of storage; *D*, difference between the highest and lowest number of microorganisms (as log CFU g^{-1}), *b*, rate of cell viability decay (d^{-1}). The data represent the mean values of survival parameters ± standard error.

†Ingredients: CRL 1328, *Lactobacillus salivarius* CRL 1328; CRL 1263, *Lactobacillus gasseri* CRL 1263; Sal, salivaricin CRL 1328; L, lactose; I, inulin; C, vitamin C (ascorbic acid); and LIC, lactose, inulin and vitamin C.

Table 3. The values of different parameters [*A* (cell viability after 360 d of storage), *D* (loss of viability) and *b* (rate of cell viability decay)] indicated that the number of viable cells of the two vaginal *Lactobacillus* strains showed a progressive reduction during storage that was dependent on the combination of BIMO and N-MC in the formulation (Table 3).

In both *L. gasseri* CRL 1263 and *L. salivarius* CRL 1328, the highest number of viable cells after 360 d of storage (*A* parameter) and the lowest reduction in viability (*D* parameter) were obtained in the formulations where lactose was the unique N-MC included CRL 1263-L and CRL 1328-L. Only in the case of *L. gasseri* CRL 1263 a lower decrease in viability was evidenced in some formulations including ascorbic acid [e.g. CRL 1263 in the presence of ascorbic acid alone (CRL 1263-C) or combined with CRL 1328 and salivaricin (CRL 1328-CRL 1263-Sal-C)] (Table 3). Thus, *A* and *D* values in these conditions were statistically different compared to the values estimated for the other formulations.

In contrast, a significant drop in the cell viability of both strains was observed in single-strain formulations where inulin was the unique compound added (CRL 1263-I and CRL 1328-I). In these conditions, the lowest values of *A* and the highest values of *D* were estimated. Only in the case of *L. gasseri* CRL 1263, when inulin was combined with other compounds, survival at the end of the storage period was higher (higher *A* and lower *D* in CRL 1263-LIC and CRL 1328-CRL 1263-Sal-LIC formulations) (Table 3).

The comparison of the survival parameters of the two strains stored under the same conditions evidenced that *L. gasseri* CRL 1263 was more resistant than *L. salivarius* CRL 1328 (Table 3).

Salivaricin CRL 1328 stability during lyophilization and storage

Eight different formulations containing salivaricin CRL 1328 were evaluated in order to determine the best conditions for the maintenance of the inhibitory activity of the bacteriocin.

In the formulas including the two *Lactobacillus* strains, salivaricin CRL 1328 activity was completely lost (CRL 1328-CRL 1263-Sal-L = 0 UA g^{-1}; CRL 1328-CRL 1263-Sal-I = 0 UA g^{-1}; CRL 1328-CRL 1263-Sal-C = 0 UA g^{-1} and CRL 1328-CRL 1263-Sal-LIC = 0 UA g^{-1}). However, the antimicrobial activity of salivaricin was maintained after lyophilization in those formulations where salivaricin was combined only with the N-MC (Figure 2). In those samples, salivaricin activity was not significantly affected by the storage time and remained stable or with a slight decrease for 360 d. AU g^{-1} values recorded in the formulation salivaricin-ascorbic acid (Sal-C) were significantly higher ($p < 0.001$) than those observed in the combinations of salivaricin with lactose (Sal-L) and with the three N-MC assayed (Sal-LIC).

Second design of experiments of pharmabiotic formulations

On the basis of the previous results, a new design of experiments of different formulations was carried out (Table 1). In this new design, some ingredients were lyophilized separately in order to prevent negative effects on the survival of *L. salivarius* CRL 1328 when lyophilized in the presence of ascorbic acid and on salivaricin activity lyophilized in the presence of lactobacilli.

Resistance of vaginal lactobacilli to the lyophilization process

The microorganisms assayed were resistant to the lyophilization process in all the combinations under study. For the two *Lactobacillus* strains, the highest survival rates after freeze-drying were obtained when each one of the strains was individually combined with lactose and salivaricin in formulations where salivaricin was added either before [(CRL 1263-Sal-L) and (CRL 1328-Sal-L)] or after lyophilization [(CRL 1263-L)-Sal and (CRL 1328-L)-Sal] (Table 4).

As the ascorbic acid negatively affected *L. salivarius* CRL 1328 viability during lyophilization (according to the previous design of experiments), the formulations containing this strain were freeze-dried without ascorbic acid, which was added later. This experimental strategy proved to be adequate to preserve cell viability in the different formulations including *L. salivarius* CRL 1328 and ascorbic acid (Table 4).

Survival of lactobacilli during storage

In the single-strain formulations, the highest viability during 360 d of storage of *L. gasseri* CRL 1263 and *L. salivarius* CRL 1328 was obtained when each one of the strain was freeze-dried in the presence of lactose and salivaricin [(CRL 1263-Sal-L) and (CRL 1328-Sal-L), respectively] (Figure 3 and Table 5). In these formulations, the number of viable cells (*A* parameter) was higher and the decrease in viability during the storage time (*D* parameter) was lower compared with the remaining conditions of the second design of experiments. The viability of *L. gasseri* cells ($A = 9.48 \pm 0.07$ log CFU g^{-1}; $D = 0.16 \pm 0.53$ log CFU g^{-1}) was higher than that of the *L. salivarius* strain ($A = 7.19 \pm 0.64$ log CFU g^{-1}; $D = 2.40 \pm 0.57$ log CFU g^{-1}) (Table 5).

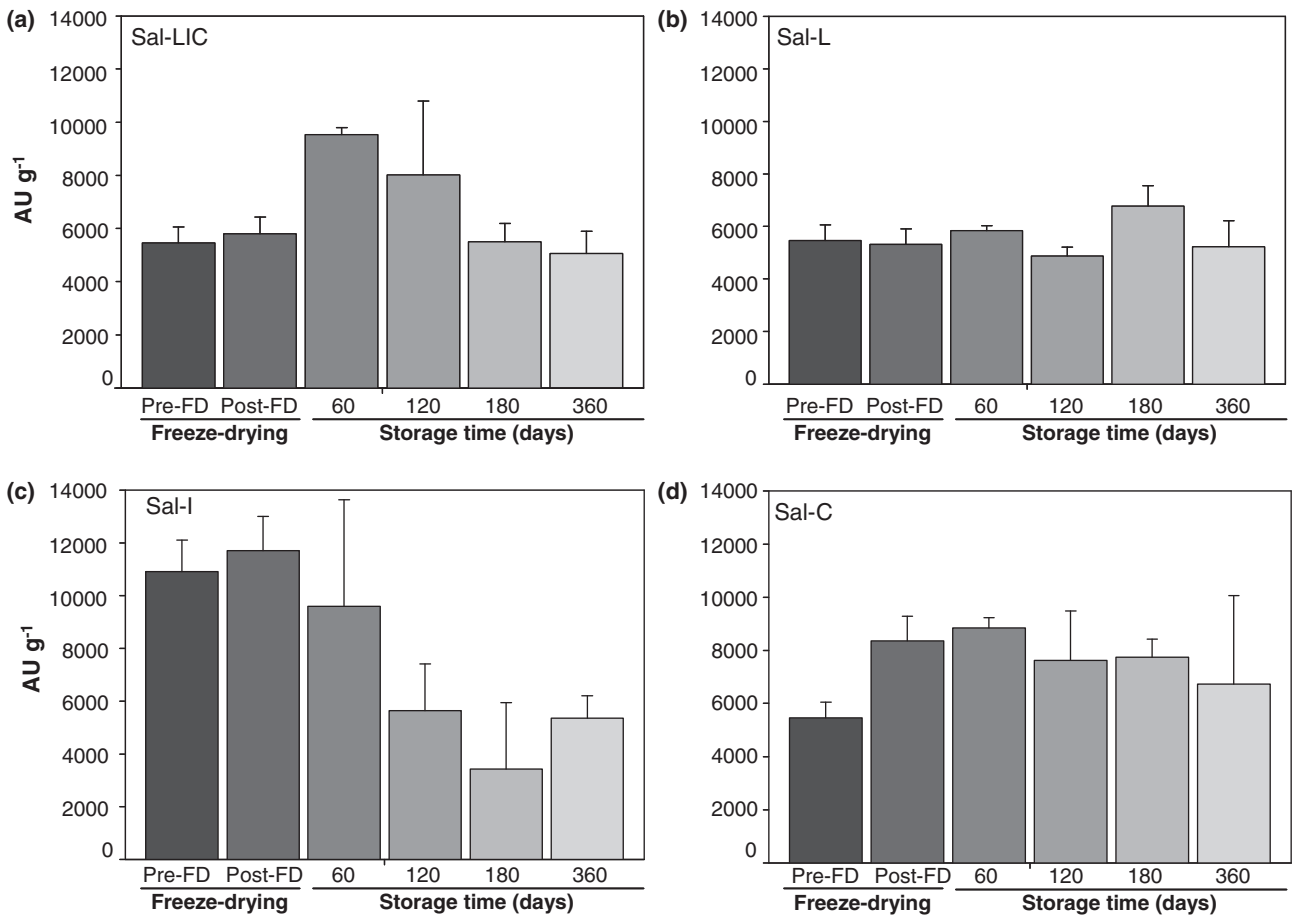

Figure 2. Antimicrobial activity of salivaricin CRL 1328 during freeze-drying and subsequent storage at 4 °C for 360 d in different formulations (first design of experiments): (a) Sal-LIC, salivaricin CRL 1328 combined with lactose, inulin and ascorbic acid; (b) Sal-L, salivaricin CRL 1328 combined with lactose; (c) Sal-I, salivaricin CRL 1328 combined with inulin and (d) Sal-C, salivaricin CRL 1328 combined with ascorbic acid. The data are plotted as the average values of salivaricin activity (AU g^{-1}) ± standard error during freeze-dried process (Pre-FD, before freeze-drying; Post-FD, after freeze-drying) and storage period.

Table 4. Resistance to the freeze-drying process of vaginal lactobacilli included in different pharmabiotic formulations (second design of experiments).

Ingredients in formulations*	Log CFU g^{-1} before freeze-drying†	Log CFU g^{-1} after freeze-drying‡	Viability decrease¶
	Lactobacillus gasseri CRL 1263		
(CRL 1263-L)-Sal	9.02	8.98	**0.04**
(CRL 1263-Sal-L)	9.66	9.02	**0.64**
(CRL 1328-CRL 1263-LI)-Sal	10.52	8.82	1.70
(CRL 1328-CRL 1263-LI)-C	10.67	8.89	1.78
(CRL 1328-CRL 1263-LI)-Sal-C	10.50	8.92	1.59
	Lactobacillus salivarius CRL 1328		
(CRL 1328-L)-Sal	9.20	7.69	**1.51**
(CRL 1328-Sal-L)	9.24	8.45	**0.80**
(CRL 1328-L)-C	9.44	6.61	2.84
(CRL 1328-CRL 1263-LI)-Sal	9.57	7.27	2.30
(CRL 1328-CRL 1263-LI)-C	9.72	6.84	2.88
(CRL 1328-CRL 1263-LI)-Sal-C	9.55	6.62	2.92

The values in bold type correspond to the minimal decrease in viable cells.
*Ingredients**:** CRL 1328, *Lactobacillus salivarius* CRL 1328; CRL 1263, *Lactobacillus gasseri* CRL 1263; Sal, salivaricin CRL 1328; L, lactose; I, inulin; C, vitamin C (ascorbic acid); and LI, lactose and inulin. The ingredients indicated in brackets were lyophilized together and then combined with the others ingredients of the formulation.
†Number of viable *Lactobacillus* cells (as log CFU g^{-1}) before freeze-drying.
‡Number of viable *Lactobacillus* cells (as log CFU g^{-1}) after freeze-drying.
¶Differences between the number of viable cells before and after lyophilization (as log CFU g^{-1}).

In multi-strain formulations, the viability of both *Lactobacillus* strains lyophilized in the presence of lactose and inulin and then combined with either salivaricin [(CRL 1328-CRL 1263-LI)-Sal] or ascorbic acid [(CRL 1328-CRL 1263-LI)-C] was preserved for one year of storage. However, the least favorable multi-strain formulation for the preservation of microbial viability (lower *A* and higher *D*) was the one containing all the BIMO and N-MC assayed [(CRL 1328-CRL 1263-LI)-Sal-C, i.e. the formulation including freeze-dried microorganisms in the presence of lactose and inulin and then combined with both salivaricin and ascorbic acid] (Table 5).

Salivaricin CRL 1328 stability during lyophilization and storage

In the first design of experiments, no antimicrobial activity of salivaricin CRL 1328 was detected when it was freeze-dried combined with *L. salivarius* CRL 1328 and *L. gasseri* CRL 1263 microbial cells. Therefore, it was important to determine whether the bacteriocin was inactivated by one of the strains or both, and whether the combination bacteriocin-strains after lyophilization could reverse the negative effect on salivaricin activity. Consequently, two independent experimental modifications were performed: (1) the individual lyophilization of each of the strains with the bacteriocin and (2) the separate lyophilization of the strains and salivaricin followed by their combination after lyophilization (Table 1).

Bacteriocin activity was detected only in single-strain formulations containing *L. salivarius*, lactose and salivaricin added either before or after lyophilization [(CRL 1328-Sal-L) or (CRL 1328-L)-Sal, respectively] (Figure 4). No significant differences in AU g^{-1} values were found between the two experimental conditions mentioned or between storage times. In contrast, *L. gasseri* completely inhibited salivaricin activity when the two ingredients were combined before [(CRL 1263-Sal-L) = 0 AU g^{-1}] or after lyophilization [(CRL 1263-L)-Sal = 0 AU g^{-1}; (CRL 1328-CRL 1263-LI)-Sal = 0 AU g^{-1}; and (CRL 1328-CRL 1263-LI)-Sal-C = 0 AU g^{-1}]. These results suggest the existence of some type of interaction between *L. gasseri* CRL 1263 and salivaricin that originates the inactivation of the bacteriocin. Therefore, further studies are needed to study the mechanism of salivaricin inactivation in the presence of *L. gasseri*.

Selected pharmabiotic formulations

From the results obtained, the formulations containing stable components as interesting and novel alternatives to be included in the final pharmabiotic product are the following: (a) single-strain formulations: *L. gasseri* CRL 1263 combined with lactose (CRL 1263-L) or ascorbic acid (CRL 1263-C) (Figure 1 and Table 3); *L. salivarius* CRL 1328 combined with lactose and salivaricin CRL 1328 before freeze-drying (CRL 1328-Sal-L) (Figures 3 and 4 and Table 5); (b) multi-strain formulation: *L. gasseri* CRL 1263 and *L. salivarius* CRL 1328 freeze-dried in the presence of lactose and inulin, and later combined with ascorbic acid [CRL 1328-CRL 1263-LI)-C] (Figure 3 and Table 5). Different potential formulations resulting from new combinations of BIMO and N-MC will be evaluated in further studies.

Discussion

Different species and specific strains of lactobacilli colonize the urogenital ecosystem, participate in the ecological equilibrium and provide protection against pathogen entry. As described above, one of the mechanisms of competition is the production of antagonistic substances that includes bacteriocin production. Autochthonous *Lactobacillus* strains and/or their metabolites could be included as active ingredients for the design of pharmabiotic products to be used in the prevention and/or therapy of urogenital infections.

Since it is very difficult that the inclusion of a single-ingredient in a pharmabiotic product should meet all the requirements needed to act as an effective protector against the wide variety of urogenital pathogens, the design of products containing a combination of beneficial strains and antimicrobial compounds could be a novel and appropriate alternative[22]. In this way, the results would be a synergistic action between the different bioactive ingredients. In addition, other compounds could be included to complement the beneficial effects of

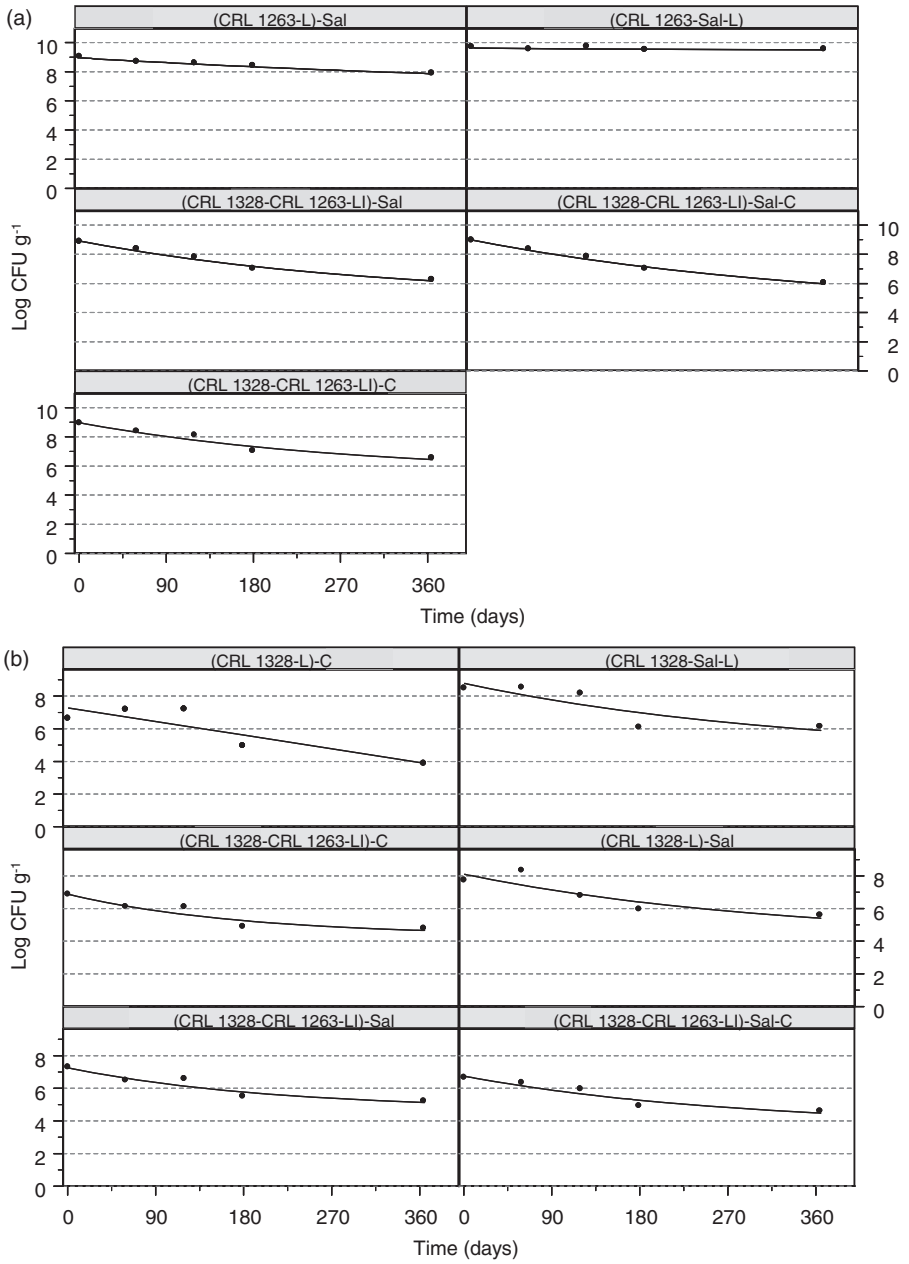

Figure 3. Viability of freeze-dried vaginal lactobacilli during storage for 360 d at 4 °C in different pharmabiotic formulations (second design of experiments). (a) Viability of *Lactobacillus gasseri* CRL 1263. (b) Viability of *Lactobacillus salivarius* CRL 1328. Ingredients in formulations: CRL 1328, *Lactobacillus salivarius* CRL 1328; CRL 1263, *Lactobacillus gasseri* CRL 1263; Sal, salivaricin CRL 1328; L, lactose; I, inulin; C, ascorbic acid; LI, lactose and inulin. The ingredients in brackets were lyophilized together and then combined with the other ingredients in the formulation. Lines represent the adjustment to experimental data (• symbols) using the exponential decay model (see Materials and methods). The initial points in each graph represent the log CFU g^{-1} in each combination after lyophilization (initial storage time).

pharmaceutical ingredients of microbial origin. For example, inulin (a prebiotic compound), ascorbic acid (an acidic and antioxidant compound) and powder lactose (a lyoprotector) could be combined with beneficial LAB[14,16,17]. Lactose could also act as a main source of nutrients for beneficial strains once in the host mucosa, supporting the growth of lactobacilli but not of pathogenic *E. faecalis* and *E. coli*[23].

In order to evaluate the incorporation of salivaricin CRL 1328 in a pharmabiotic product with autochthonous vaginal *Lactobacillus* strains, compatibility between the bacteriocin and microorganisms as well as compatibility among the different strains must be evaluated. Throughout *in vitro* assays of diffusion in agar plates, salivaricin has been shown to antagonize the growth of some microorganisms of related and unrelated species, but not the growth of *L. gasseri* CRL 1263[8,11]. In addition, *L. gasseri* CRL 1263 inhibited the growth of other vaginal lactobacilli strains, but not of *L. salivarius* CRL 1328 by the effect of different antagonistic substances (organic acids and hydrogen peroxide[11]. Therefore, the selected *L. salivarius* CRL 1328 and *L. gasseri* CRL 1263 strains used in this study are compatible.

The long-term storage of microorganisms is a challenge in the pharmaceutical area. Evaluation of the viability of the microorganisms in commercial products is critical during processing, packaging and storage. Several authors have shown the lack of correlation between the number and type of microorganisms on the product label and the further results of laboratory assessment. In particular, Drago et al. (2009) reported that 9 of 13 probiotic products commercialized in the United States did not correspond to the label claims[24].

The freeze-drying of LAB is usually employed to successfully maintain their viability during the development of pharmaceuticals and subsequent long-term storage and distribution[25,26]. The survival of freeze-dried bacteria depends on several factors such as specific strain, initial cell concentration, growth conditions, culture media, freeze-drying process and rehydration and storage conditions[27,28]. The results obtained in this study indicate that *L. gasseri* CRL 1263 was more resistant than *L. salivarius* CRL 1328 to both, the freeze-drying process and the subsequent storage in gelatin capsules. Significant differences in the resistance to freeze-drying and storage were described for different

Table 5. Survival parameters estimated by the exponential decay model during the storage of freeze-dried vaginal lactobacilli in different pharmabiotic formulations (second design of experiments).

Survival parameters*	Ingredients in formulations†	Vaginal *Lactobacillus* strains	
		Lactobacillus gasseri CRL 1263	*Lactobacillus salivarius* CRL 1328
A (log CFU g^{-1})	(CRL 1263-L)-Sal	6.43 ± 2.95	
	(CRL 1263-Sal-L)	**9.48 ± 0.07**	
	(CRL 1328-L)-Sal		4.13 ± 2.36
	(CRL 1328-Sal-L)		**7.19 ± 0.64**
	(CRL 1328-L)-C		5.79 ± 1.12
	(CRL 1328-CRL 1263-LI)-Sal	4.93 ± 2.39	4.70 ± 2.11
	(CRL 1328-CRL 1263-LI)-C	5.50 ± 2.98	4.32 ± 2.12
	(CRL 1328-CRL 1263-LI)-Sal-C	4.18 ± 1.98	3.68 ± 2.14
D (log CFU g^{-1})	(CRL 1263-L)-Sal	2.51 ± 2.89	
	(CRL 1263-Sal-L)	**0.16 ± 0.53**	
	(CRL 1328-L)-Sal		3.98 ± 2.10
	(CRL 1328-Sal-L)		**2.40 ± 0.57**
	(CRL 1328-L)-C		1.87 ± 1.05
	(CRL 1328-CRL 1263-LI)-Sal	3.98 ± 2.23	2.56 ± 2.14
	(CRL 1328-CRL 1263-LI)-C	3.47 ± 2.77	2.56 ± 2.06
	(CRL 1328-CRL 1263-LI)-Sal-C	4.80 ± 1.80	3.07 ± 1.95
b (d^{-1})	(CRL 1263-L)-Sal	0.002 ± 0.007	
	(CRL 1263-Sal-L)	0.006 ± 0.114	
	(CRL 1328-L)-Sal		0.003 ± 0.044
	(CRL 1328-Sal-L)		0.648 ± 0.442
	(CRL 1328-L)-C		0.825 ± 0.726
	(CRL 1328-CRL 1263-LI)-Sal	0.003 ± 0.076	0.005 ± 0.158
	(CRL 1328-CRL 1263-LI)-C	0.004 ± 0.076	0.006 ± 0.219
	(CRL 1328-CRL 1263-LI)-Sal-C	0.003 ± 0.003	0.004 ± 0.133

The values in bold type correspond to optimal survival parameters for each *Lactobacillus* strain.

*Survival parameters: *A*, number of viable cells (as log CFU ml^{-1}) at the end of storage; *D*, difference between the highest and lowest number of microorganisms (as log CFU g^{-1}), *b*, rate of cell viability decay (d^{-1}). The data represent the mean values of survival parameters ± standard error.

†Ingredients: CRL 1328, *Lactobacillus salivarius* CRL 1328; CRL 1263, *Lactobacillus gasseri* CRL 1263; Sal, salivaricin CRL 1328; L, lactose; I, inulin; C, vitamin C (ascorbic acid); and LI, lactose and inulin. The ingredients indicated in brackets were lyophilized together and then combined with the other ingredients in the formulation.

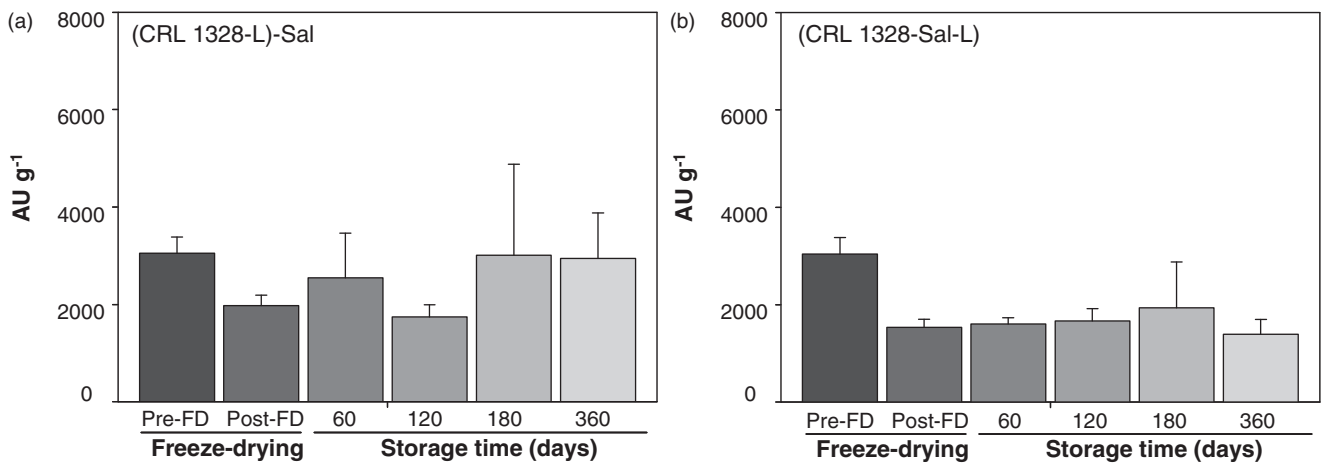

Figure 4. Antimicrobial activity of salivaricin CRL 1328 during freeze-drying and subsequent storage at 4 °C for 360 d in different formulations (second design of experiments): (a) (CRL 1328-L)-Sal, *Lactobacillus salivarius* CRL 1328 lyophilized together lactose (L) and then combined with salivaricin CRL 1328 (Sal), and (b) (CRL 1328-Sal-L), *Lactobacillus salivarius* CRL 1328 lyophilized together lactose and salivaricin CRL 1328. The data are plotted as the average values of salivaricin activity (AU g^{-1}) ± standard error during freeze-dried process (Pre-FD, before freeze-drying; Post-FD, after freeze-drying) and storage period.

strains of the same species. Therefore, experimental evaluation of the optimum storage conditions for each strain is required in all the formulations designed[29,30].

The nonlinear mixed model applied to the results showed that the pharmabiotic formulations including different combinations of BIMO and N-MC significantly affected the survival parameters of *Lactobacillus* strains during the storage period. The viability of both strains was higher when lactose was present in the mixture. In contrast, a significantly higher viability loss was evidenced when inulin was added individually, but the negative effect of inulin disappeared when the three N-MC were present in the mixture. Ascorbic acid favored the stability of *L. gasseri* CRL

1263, but was detrimental to *L. salivarius* CRL 1328 when lyophilized simultaneously with it. However, when *L. salivarius* and ascorbic acid were combined after the freeze-drying process, the survival rate of the bacteria increased significantly. These results indicate that one of the most critical steps to maintain the viability of the *L. salivarius* CRL 1328 cells is the lyophilization step and that this strain and ascorbic acid could be included in the same formula by adding the acid after freeze-drying.

Salivaricin CRL 1328 is a bacteriocin that maintains the inhibitory activity for long periods of time[18]. In this work, a significantly higher salivaricin activity during storage was observed in those samples with ascorbic acid. Furthermore, when combined with lactose, inulin or *L. salivarius* CRL 1328, salivaricin maintained its activity for 360 d. In contrast, salivaricin showed a complete loss of antimicrobial activity when combined with *L. gasseri* CRL 1263 either before or after the freeze-drying process. Although this microorganism is resistant to the antimicrobial effect of salivaricin (as observed in previous compatibility trials)[11], there seems to be some type of interaction responsible for the complete loss of salivaricin activity.

The mechanism of action of some bacteriocins described in the literature involves the recognition of the target site or receptor such as Lipid II for nisin (a type Ia bacteriocin) or mannose-PTS transport system in pediocin-type bacteriocins (type IIa bacteriocins) or G lactocin (type IIb bacteriocins)[31–33]. Once the molecule is anchored to the target cell, the inhibitory effect involves the permeabilization of the cell membrane. One hypothesis to explain the loss of salivaricin activity when combined with *L. gasseri* CRL 1263 is that, even though the bacteriocin recognizes a receptor/target site in the cell wall, it is retained on the surface and is not able to produce the antimicrobial effect. Another possibility is the non-specific adsorption of salivaricin on the lyophilized *L. gasseri* CRL 1263 cells, and further experiments should be performed to study this phenomenon.

Conclusions

Based on the last findings, a series of questions arise concerning the mechanism responsible for the inhibitory effect of *L. gasseri* CRL 1263 on salivaricin CRL 1328 activity and the occurrence of this phenomenon with different *Lactobacillus* strains and/or bacteriocins. Moreover, the results obtained in this study demonstrate the importance of this type of experiments to determine if the described situations are present when working in the design of combined formulas of microorganisms and bacteriocins. To this day, no pharmabiotic formulations containing bacteriocins and vaginal lactobacilli for the treatment and/or prevention of urogenital infections have been described in the literature. Further studies are being performed to deepen the technological characterization of pharmabiotic formulations that will allow their increased stability during long-term storage and to determine the mechanisms of interaction between the ingredients.

Declarations of interest

The authors report no declarations of interest.

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