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Viability of vaginal probiotic lactobacilli during refrigerated and frozen storage

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Abstract

The viability of six different strains of probiotic vaginal *Lactobacillus* was examined in two different cryoprotective media, during refrigerated versus frozen storage, and using two traditional types of stock cultures for starting the biomass production. Freezing at −20°C and −70°C had much less adverse effect on viability than did storage at 7°C, and the reduction in viability was greater at −20°C than at −70°C. The strains showed variation in the extent of the viability losses during both types of storage. Milk-yeast extract (MYE) was shown to be the more suitable protective medium to maintain viability of the strains during the storage. The vaginal *Lactobacillus* strains are most stable in MYE at −70°C with only a small decrease of the viability observed under these conditions. The viable cell counts of *Lactobacillus paracasei* CRL 1251 and CRL 1289, *L. crispatus* CRL 1266 and *L. salivarius* CRL 1328 remained around 1×10^8 CFU/mL after 24 months of storage at −70°C, or up to 18 months for *L. acidophilus* CRL 1259.

Keywords: Vaginal lactobacilli; Frozen and refrigerated storage

1. Introduction

Lactobacilli are anaerobic and facultative Gram-positive bacilli, and comprise the dominant micro-organisms of the healthy human vagina. Their function is to maintain an environment that limits the growth of pathogenic micro-organisms [1]. There is a growing interest in the use of lactobacilli of human origin as probiotics against urogenital tract infections [2,3]. The beneficial and technological characteristics of lactobacilli must be studied in order to select strains for probiotic purposes [4,5].

To start the biomass production at an industrial scale, a stock culture is required. This culture can be used directly as starter culture, or it can be activated by successive subcultures to reach the sufficient concentration for the inoculation of fermentation tank. From the industrial standpoint, it is of primary interest to maintain the highest viability of the stock cultures for use in the plant as the same production lot over long periods of time [6,7]. Such cultures can be liquid (stored refrigerated), frozen (stored at different sub-zero temperatures) or freeze-dried [6]. Freezing and freeze-drying are commonly used for the preservation and storage of lactic acid bacteria for the production of concentrated starter cultures for the food industry [8], but there is little information available for organisms isolated from other ecological niches, such as the vagina, and for pharmaceutical applications.

The storage conditions, particularly refrigeration, can stress the cells and produce an adverse effect on viability [9]. The stress by freezing may occur at any stage of the process: cooling the cells and suspension medium from ambient temperatures to the freezing point, formation of extracellular and intracellular ice upon further cooling, concentration of solutes, storage and thawing [10]. The cryoprotective agents such as glycerol, sugar or milk inhibit intracellular formation of ice during freezing and therefore the cell injury is lessened [11–13].

The selection and production of starter cultures in the food industry has been extensively studied [6]. Moreover, the influence of frozen and refrigerated storage on the viability of several lactobacilli strains in fermented [14] or non-fermented milk [9,15,16] was reported. However, until now, the preservation of vaginal probiotic lactobacilli by different methods (liquid, frozen or lyophilized) has not been examined, except

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the recent evaluations of the viability of *Lactobacillus* strains in lyophilized powder and tablets for vaginal administration [3,17]. The authors emphasized the optimization of the production process of tablets containing different strains of lactobacilli rather than on the process of preparation of starter cultures for production of the biomass.

Lactobacillus strains have been isolated from normal human vaginas and selected for potential probiotic use because of characteristics such as hydrogen peroxide and lactic acid production and presence of bacteriocin-like substances [18–21]. The conditions that influence the growth of selected micro-organisms [22] as well as on the bacteriocin, hydrogen peroxide and lactic acid production were also reported [23–25].

The objective of the present study was to determine the viability of six potentially probiotic vaginal lactobacilli during refrigerated and frozen storage, in two cryoprotective media for different periods of time.

2. Material and methods

2.1. Micro-organisms

Lactobacilli isolated from vaginal swabs of women from Tucumán, Argentina were used throughout this study [18]. These strains were selected previously for their probiotic properties (Table 1).

2.2. Growth conditions and preparation of stock cultures

Before the experiments, all the strains were stored in milk-yeast extract (MYE) (13% non-fat milk, 0.5% yeast extract and 1% glucose) at −20°C, and were propagated three times in LAPTg broth (1.5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0.1% Tween 80) [27] at 37°C. The last culture was harvested in stationary phase by centrifugation at 10 000 *g* for 10 min at 4°C. The cell pellets were washed twice with saline solution (0.85% NaCl) and re-suspended in each cryoprotective medium at a cell concentration of 10^8–10^9 CFU/mL (colony forming units). Two cryoprotective media were used: MYE and LAPTg supplemented with 30% (vol/vol) glycerol (LAPTg-glycerol). After mixing, the resulting suspensions were dispensed into screw-capped cryogenic vials (1 mL) and stored refrigerated (7°C) or frozen (−20°C and −70°C). The experiments on the refrigerated and frozen strains were performed at different times.

Table 1
Lactobacillus strains used in the storage experiments

Strains	Probiotic properties
L. paracasei CRL 1251	Production of H_2O_2 [20]
L. acidophilus CRL 1259	Inhibition of urogenital pathogens through acid production [25]
L. crispatus CRL 1266	Production of H_2O_2 [19,24]
L. paracasei CRL 1289	Production of H_2O_2 [20,24]
L. acidophilus CRL 1294	Autoaggregating abilities [26]
L. salivarius CRL 1328	Production of bacteriocin [21,23]

CRL, Centro de Referencia para Lactobacilos Culture Collection.

2.3. Analytical methods

Viable counts of vaginal lactobacilli were determined immediately after mixing the micro-organisms with the cryoprotective media, and after 1, 3, 7, 30, 60, 90 and 150 days for refrigerated storage, and after 3, 6, 12, 18 and 24 months for frozen storage. The determination of the number of CFU/mL for each time and storage condition was performed in duplicate. The plate dilution method, with peptone water (0.1% peptone) as dilution medium and LAPTg agar as culture media, was used.

2.4. Statistical evaluation

Viability results were analysed by the one-way ANOVA, taking $P<0.05$ as value statistically significant differences among means.

3. Results

3.1. Refrigerated storage

The surviving micro-organisms during refrigerated storage for different periods of time are shown in Table 2. The survival rates of vaginal lactobacilli were significantly higher in MYE than in LAPTg-glycerol. For this reason, the last medium was not tested at −70°C.

The viable counts of *Lactobacillus acidophilus* CRL 1259 and CRL 1294, *L. crispatus* CRL 1266 and *L. salivarius* CRL 1328 in MYE remained stable during 7 d of storage at 7°C but declined after this period. However, for *L. paracasei* CRL 1251 and CRL 1289 the number of viable cells in MYE decreased markedly before 7 d. After 1 d of refrigeration, the viable counts of *L. paracasei* CRL 1289 decreased by about 1 log cycle, and the survival rate was 17%. The loss of viability between 7 and 30 d in MYE was higher for *L. paracasei* CRL 1289, *L. acidophilus* CRL 1294 and *L. salivarius* CRL 1328 than for *L. paracasei* CRL 1251, *L. acidophilus* CRL 1259 and *L. crispatus* CRL 1266. The viable counts of all six *Lactobacillus* strains continued to decline beyond 30 d. After 150 d of refrigerated storage in MYE only cells of *L. salivarius* CRL 1328, *L. acidophilus* CRL 1259 and CRL 1294 remained viable.

Some strains were viable in higher numbers than others in LAPTg-glycerol up to 7 d of refrigerated

Table 2
Influence of refrigerated storage in MYE or LAPTg-glycerol on the viability of vaginal *Lactobacillus* strains

Condition of storage	Time (days)	Micro-organisms					
		L. paracasei CRL 1251	*L. acidophilus* CRL 1259	*L. crispatus* CRL 1266	*L. paracasei* CRL 1289	*L. acidophilus* CRL 1294	*L. salivarius* CRL 1328
MYE	0	$8.93^{a,1}$	$8.98^{a,1}$	$9.05^{a,1}$	$8.64^{a,1}$	$8.60^{a,1}$	$8.70^{a,1}$
	1	$8.82^{ab,1}$	$8.97^{a,1}$	$9.00^{a,1}$	$7.87^{b,1}$	$8.59^{a,1}$	$8.69^{a,1}$
	3	$8.51^{b,1}$	$8.96^{a,1}$	$9.00^{a,1}$	$7.95^{bc,1}$	$8.60^{a,1}$	$8.69^{a,1}$
	7	$8.53^{b,1}$	$8.92^{a,1}$	$8.94^{a,1}$	$7.98^{bc,1}$	$8.62^{a,1}$	$8.65^{a,1}$
	30	$8.57^{b,1}$	$8.81^{a,1}$	$8.65^{b,1}$	$7.34^{c,1}$	$7.16^{b,1}$	$7.85^{b,1}$
	60	$7.29^{c,1}$	$6.84^{b,1}$	$6.43^{c,1}$	$6.55^{d,1}$	$6.41^{c,1}$	$7.53^{c,1}$
	90	$1.78^{d,1}$	$5.64^{c,1}$	$4.14^{d,1}$	$5.26^{e,1}$	$5.50^{d,1}$	$6.02^{d,1}$
	150	0.00	$2.70^{d,1}$	0.00	0.00	$2.25^{e,1}$	$4.02^{e,1}$
LAPTg-glycerol	0	$8.94^{a,1}$	$9.06^{a,1}$	$9.01^{a,1}$	$8.39^{a,1}$	$8.43^{a,1}$	$8.55^{a,1}$
	1	$8.72^{a,1}$	$8.88^{a,1}$	$9.00^{a,1}$	$7.92^{b,1}$	$8.36^{a,2}$	$8.53^{a,1}$
	3	$8.67^{a,1}$	$8.73^{ab,1}$	$8.74^{a,1}$	$7.82^{b,1}$	$8.33^{a,2}$	$7.82^{b,2}$
	7	$8.64^{a,1}$	$8.63^{b,2}$	$8.74^{a,1}$	$6.72^{c,2}$	$8.32^{a,2}$	$7.68^{b,2}$
	30	$5.54^{b,2}$	$6.58^{c,2}$	$7.55^{b,2}$	$3.67^{d,2}$	$5.40^{b,2}$	$4.74^{c,2}$
	60	$2.24^{c,2}$	0.00	$0.30^{c,2}$	0.00	$1.11^{c,2}$	$0.48^{d,2}$
	90	0.00	ND	0.00	ND	$1.40^{c,2}$	0.00

Each value represents the mean of Log CFU/ml of two trials.
ND: Non-determined.
[a,b,c,d,e]Different superscripts mean significant differences between sampling times, for each strain and at one condition of refrigerated storage ($P<0.05$).
[1,2]Different superscripts mean significant differences between both conditions of refrigerated storage (MYE and LAPTG-glycerol) at the same sampling time and for the same strain ($P<0.05$).

storage. The viable counts obtained on 30 d in LAPTg-glycerol were about 1–3 log cycles lower than those obtained in MYE.

3.2. Frozen storage

The extent of the decrease of the viability during 24 months of frozen storage, at the two assayed temperatures (−20°C and −70°C) and in both cryoprotective media, was different for each *Lactobacillus* strain (Table 3). In general, the reduction of viability was greater at −20°C than at −70°C during the period evaluated.

L. salivarius CRL 1328 showed the least loss in viability during 24 months of storage in MYE at −70°C, or up to 18 months at −20°C. No significant differences were observed in the viability of *L. crispatus* CRL 1266 and *L. acidophilus* CRL 1259 for up to 18 or 24 months, respectively, during the storage in MYE at −70°C. *L. acidophilus* CRL 1294, *L. paracasei* CRL 1251 and CRL 1289 exhibited the greatest reductions in viability after 3 months of frozen storage at −20°C and −70°C both in MYE and LAPTg-glycerol. However, *L. paracasei* CRL 1251 and CRL 1289 did not show a significant decrease in viability in MYE at −70°C during the period from 3 to 24 months.

For all the strains, except *L. salivarius* CRL 1328, the viability at −20°C decreased progressively between 3 and 24 months of storage. This decrease differed between both cryprotective media mainly for the strains CRL 1251, CRL 1266 and CRL 1289, since after 24 months of frozen storage at −20°C the viable counts in LAPTg-glycerol were 1 or 2 log cycles lower than in MYE.

4. Discussion

Excellent technological properties, such as a good growth yield and stability in both the production process of biomass and final product, are important requirements for a probiotic strain when designing a product for industrial purposes [6].

MYE and suspension media with glycerol were beneficial for improving survival of different bacterial groups, included lactic acid bacteria [11–13]. In our study, the results obtained after the refrigerated storage of the vaginal lactobacilli for different periods of time showed that the MYE is a more suitable cryoprotective medium than the LAPTg-glycerol. The experiments of storage at −20°C showed that the decrease of viability in MYE was equal or lower than in LAPTg-glycerol. El-Kest and Marth [11] reported that, during short-term frozen storage, milk components provided better protection to *L. monocytogenes* than did medium with glycerol. However, different performance was observed during long-term frozen storage. On the other hand, Aulet de Saab et al. [23] observed that MYE with 10%

Table 3
Influence of different conditions of frozen storage on the viability of vaginal *Lactobacillus* strains

Condition of storage	Time (months)	Micro-organisms					
		L. paracasei CRL 1251	*L. acidophilus* CRL 1259	*L. crispatus* CRL 1266	*L. paracasei* CRL 1289	*L. acidophilus* CRL 1294	*L. salivarius* CRL 1328
MYE (−70°C)	0	$9.27^{a,1}$	$8.93^{a,1}$	$8.96^{a,1}$	$8.22^{a,1}$	$7.22^{a,1}$	$8.44^{a,1}$
	3	$8.44^{b,1}$	$8.62^{a,1}$	$8.73^{a,1}$	$7.93^{b,1}$	$7.18^{ab,1}$	$8.42^{a,1}$
	6	$8.35^{b,1}$	$8.62^{a,1}$	$8.71^{ab,1}$	$7.93^{b,1}$	$7.15^{b,1}$	$8.39^{a,1}$
	12	$8.34^{b,1}$	$8.56^{a,1}$	$8.69^{ab,1}$	$7.93^{b,1}$	$7.06^{bc,1}$	$8.30^{a,1}$
	18	$8.33^{b,1}$	$8.53^{a,1}$	$8.57^{b,1}$	$7.93^{b,1}$	$6.75^{c,1}$	$8.23^{a,1}$
	24	$8.05^{c,1}$	$6.42^{b,1}$	$8.44^{c,1}$	$7.88^{b,1}$	$4.02^{e,1}$	$8.16^{a,1}$
MYE (−20°C)	0	$9.27^{a,1}$	$8.93^{a,1}$	$8.96^{a,1}$	$8.22^{a,1}$	$7.22^{a,1}$	$8.44^{a,1}$
	3	$7.52^{b,2}$	$8.57^{a,1}$	$8.66^{ab,1}$	$7.52^{b,2}$	$7.10^{b,1}$	$8.33^{a,1}$
	6	$7.47^{b,2}$	$8.57^{a,1}$	$8.60^{b,1}$	$7.51^{bc,2}$	$7.02^{b,2}$	$8.28^{a,2}$
	12	$7.46^{b,2}$	$7.73^{b,2}$	$8.16^{c,2}$	$7.31^{c,2}$	$6.89^{c,1}$	$8.23^{a,1}$
	18	$7.39^{b,2}$	$7.18^{c,2}$	$7.03^{d,2}$	$6.77^{d,2}$	$5.95^{c,1}$	$7.64^{b,2}$
	24	$6.35^{c,2}$	$4.76^{d,2}$	$5.38^{e,2}$	$6.07^{e,2}$	$3.81^{d,2}$	$7.05^{e,2}$
LAPTg-glycerol (−20°C)	0	$9.33^{a,1}$	$8.98^{a,1}$	$8.83^{a,1}$	$8.61^{a,1}$	$7.22^{a,1}$	$8.39^{a,1}$
	3	$7.66^{b,2}$	$7.66^{b,1}$	$8.73^{a,1}$	$7.90^{b,1}$	$6.89^{b,2}$	$8.35^{a,1}$
	6	$7.37^{b,2}$	$8.65^{bc,1}$	$8.71^{a,1}$	$7.54^{c,2}$	$6.81^{b,3}$	$8.33^{a,2}$
	12	$6.54^{c,3}$	$8.59^{cd,2}$	$6.67^{b,3}$	$7.46^{cd,2}$	$6.78^{b,1}$	$8.29^{a,1}$
	18	$6.05^{d,3}$	$8.23^{d,3}$	$6.33^{b,3}$	$6.57^{d,2}$	$4.81^{c,2}$	$7.83^{b,2}$
	24	$4.27^{e,3}$	$5.29^{e,2}$	$4.01^{c,3}$	$5.12^{e,2}$	$3.94^{d,1,2}$	$7.31^{b,2}$

MYE: Milk-yeast extract.
Each value represents the mean of Log CFU/ml of two trials.
^{a,b,c,d,e}Different superscripts mean significant differences between sampling times, for each strain and at one condition of frozen storage ($P<0.05$).
^{1,2,3}Different superscripts mean significant differences between the three conditions of frozen storage at the same sampling time and for the same strain ($P<0.05$).

glycerol maintained higher viability of *H. influenza* both at −20°C and −70°C than did trypticase soy broth and brain heart infusion broth supplemented with glycerol.

In general, the micro-organisms tested showed viable populations between 10^7 and 10^8 CFU/mL in MYE up to 30 d or up to 12 months of storage at 7°C or at −20°C, respectively. The use of a liquid stock culture in industrial plants is only recommended where the transport from a central laboratory is possible, since shipment of frozen cultures is expensive, and requires skilled personnel to properly package the cultures such that unwanted thawing during transport is avoided. [6]. The relatively small influence of storage in MYE at −70°C on the viability of vaginal *Lactobacillus* studied indicate that these micro-organisms are stable under these conditions, depending on each particular strain.

The more detrimental effect of the storage at higher temperature (7°C compared with frozen storage, and −20°C compared with −70°C) agrees with previous reports on the stability of lactobacilli in dietary products [9,15]. These results support the observation that the refrigerated storage of *L. acidophilus* in non-fermented milk produces more damage than the frozen storage in liquid nitrogen at −196°C.

There were differences of stability among the six *Lactobacillus* strains during refrigerated or frozen storage. This fact precludes any generalization about typical behaviors. Differences between the survival during refrigerated or frozen storage of *L. acidophilus* and *L. casei* strains isolated from dietary products were reported by several authors [14,15]. Gómez Zavaglia et al. [28] correlated the fatty acid composition with the freeze-thaw cell resistance of eight strains of different *Lactobacillus* species isolated from dairy products (*L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *lactis*, *L. helveticus* and *L. acidophilus*). In this report, the authors concluded that the differences among thermophilic *Lactobacillus* species were largely due to different proportions of fatty acids present in the lipid fraction rather than different types of fatty acids.

5. Conclusions

Based on the differences observed in our strains, the different conditions of preservation of potentially probiotic micro-organisms should be determined in each case. Our results indicated that the more appropriate condition of stock culture preservation is frozen storage at −70°C in MYE, during 18–24 months. The use of frozen cultures requires low temperatures during

storage and shipment, if the stock culture is used directly as starter culture. However, the storage of the stock cultures in a central lab with preparation of a subculture that is shipped to the plant for preparation of the starter culture, is a practical alternative to eliminate the expense and complexity of shipping frozen materials. More research is needed on the resistance of selected lactobacilli to freeze-drying or spray-drying techniques.

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