J. Gen. Appl. Microbiol., **58**, 71–81 (2012)

Full Paper

Effect of lyophilization and storage temperature on the activity of salivaricin CRL 1328, a potential bioactive ingredient of a urogenital probiotic product

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(Received July 30, 2011; Accepted October 1, 2011)

Bacteriocins are antimicrobial peptides with potential applications as therapeutic agents for the treatment of microbial infections. The aim of this work was to investigate the effect of different protectors on the activity of salivaricin CRL1328, a bacteriocin produced by *Lactobacillus salivarius* CRL1328, during the lyophilization process and subsequent storage at different temperatures for 18 months using statistical models. Different protectors such as mannitol, Tween 80, polyethylene glycol 8000 (PEG), monosodium glutamate (MSG), reconstituted skim milk, sucrose and ascorbic acid were used for the lyophilization and storage of salivaricin. The biplot of principal component analysis was used for the interpretation of the interactions between the different factors studied. The antimicrobial activity of salivaricin was dependent mainly on temperature, and also on the time of storage and protector assayed. The stability of salivaricin was higher at −20°C and 4°C than 25°C and decreased during the time of storage; however, salivaricin was active after 18 months of storage at 25°C. Sucrose, mannitol plus sucrose, PEG plus sucrose and MSG were the most effective agents in protecting the bacteriocin during the lyophilization process. Effective maintenance of the activity of the bacteriocin was observed by storage with sucrose and ascorbic acid at −20°C as well as with PEG plus sucrose at 4°C and −20°C. The results obtained suggest that sucrose alone or combined with PEG can effectively maintain the activity of salivaricin during lyophilization and storage. This study provides useful information for the potential application of salivaricin as a bioactive principle for a pharmaceutical formulation.

Key Words——antimicrobial peptide; bacteriocin storage; *Lactobacillus salivarius* CRL 1328, salivaricin CRL 1328, urogenital infection biopharmaceutical product

Introduction

Urogenital tract (UGT) infections constitute a serious social and public health problem because of their great prevalence (around 1 billion people worldwide every year) and the high health care costs involved (Reid and Bruce, 2003). Conventional therapies used against UGT infections involve the use of appropriate antimicrobials according to the etiological agent. Antimicrobials represent one of the greatest advances in the therapy of infectious diseases of the twentieth century. However, they have some limitations as well as undesirable characteristics such as the possibility of causing side effects, hyper-sensitivity or emergence of antibiotic resistant strains (Oduyebo et al., 2009; Piper

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et al., 2009). Moreover, the use of these substances frequently produces an imbalance in the indigenous microbiota that, if not properly re-established, can participate in the recurrence of infections or even cause secondary ones due to the absence or diminution of the indigenous microbiota (Bradshaw et al., 2006). Because of these problems, the need to develop new drugs and alternative therapies arises. A promising alternative is the use of bacteriocins, defined as ribosomally synthesized antimicrobial peptides produced by bacteria. Bacteriocins are widely applied as bioprotectors in foods (De Vuyst and Leroy, 2007; Khan et al., 2010; Rodgers et al., 2003) and are also used in veterinary medicine for the prevention of mastitis (Ryan et al., 1999; Twomey et al., 2000). Due to their antimicrobial action against a variety of clinically important pathogens, they have been proposed for use as bioactive principles in novel pharmaceutical formulations (Piper et al., 2009). Some studies have demonstrated the in vivo protective effect of the bacteriocin ABP118, produced by *L. salivarius* UCC118, against challenge with *Listeria monocytogenes* in a murine experimental model (Corr et al., 2007). Furthermore, in vitro and in vivo studies showed that lactocin 160, a bacteriocin produced by *Lactobacillus rhamnosus*, does not irritate vaginal epithelial tissue (Dover et al., 2007). However, there are no descriptions of the inclusion of a bacteriocin in a pharmaceutical product designed to prevent urogenital infections in women.

Salivaricin CRL 1328 is a bacteriocin produced by *Lactobacillus salivarius* CRL 1328, a lactic acid bacterium isolated from a woman's vagina (Ocaña et al., 1999). This bacteriocin is active against potentially urogenital pathogenic bacteria such as *Enterococcus faecalis*, *E. faecium* and *Neisseria gonorrhoeae* (Ocaña et al., 1999). It is constituted by two structural peptides and acts by dissipating the proton motive force of the sensitive pathogen strains (Vera Pingitore et al., 2009a, b).

The technological properties of the bioactive compounds in a pharmaceutical product are one of the most important subjects for pharmaceutical companies, since a suitable form of the product and its long stability are highly desirable. A properly lyophilized formulation should maintain physically and chemically stable antagonistic effects after manufacture as well as during shipping and long-term storage.

The design of experiments and the evaluation of their results can be performed and analyzed in different ways. The application of factorial designs to obtain data and models of analysis of variance (ANOVA) is an optimal strategy used in many experimental protocols (Juárez Tomás et al., 2002; Ko et al., 2011). However, when an experimental model involves the effects of two or more factors, the structure of the statistically significant interaction effects presents a major challenge for interpretation. The Principal Component Analysis (PCA) biplot is an effective graphic technique in classical multivariate analysis to understand the structure of the data. The biplot displays the observations and variables and it simultaneously represents the relationships between them (Park et al., 2008).

Therefore, in view of the fact that there is lack of information regarding bacteriocin stability during lyophilization and storage and that the data obtained from the evaluation of many protective agents and storage conditions should be carefully analyzed, the aims of this work were: a) to study the effects of different protective agents on salivaricin CRL 1328 activity during the lyophilization process, and b) to determine the stability of salivaricin CRL 1328 during storage at different temperatures. The use of PCA to interpret a complex structure in the interaction between the different agents studied is also proposed.

Materials and Methods

Bacterial strains. Bacteriocin-producing *L. salivarius* CRL 1328 (from the CERELA culture collection), originally isolated form a human vagina, was used in the experiments (Ocaña et al., 1999). The uropathogenic strain *Enterococcus faecalis* MP97, obtained from the Instituto de Microbiología of the Universidad Nacional de Tucumán, Argentina, was used as the indicator strain. The microorganisms were stored in milk-yeast extract (10% non-fat milk, 0.5% yeast extract and 1% glucose) at −20°C.

Media and salivaricin CRL 1328 production conditions. *L. salivarius* CRL 1328 and *E. faecalis* MP97 were cultured in LAPTg broth (1.5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0.1% Tween 80) (Fournaud et al., 1964) at 37°C for 24 h and subcultured twice at 37°C for 12 h in the same medium. The third subculture of *L. salivarius* CRL 1328 was centrifuged (8,000 × *g*, 10 min, 20°C) and the supernatant was sterilized by filtration through a cellulose acetate membrane (0.22 μm pore size; Millipore, USA).

Bacteriocin activity detection. The agar-well diffu-

sion method (Jack et al., 1995) was used to detect the inhibitory activity of the supernatant fluid of the *L. salivarius* CRL 1328 culture serially diluted in deionized water. Aliquots (25 μl) of each dilution were poured into the 4 mm holes preformed in LAPTg agar plates (LAPTg 1% agar) which contained the uropathogenic *E. faecalis* MP97 strain (10^6 CFU ml^{-1}). 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 (AU). One AU was defined as the reciprocal of the highest dilution that produced an inhibition halo.

Protein concentration determination. The protein concentration of the samples was assessed to express bacteriocin activity as AU per μg of protein. The protein detection method described by Bradford (1976) was used, with bovine serum albumin as a standard. Absorbance at 595 nm was determined in a microplate reader (Molecular Device, USA).

Lyophilization process and storage of samples at different temperatures. To assess salivaricin activity after the lyophilization process and subsequent storage, the following pharmaceutical excipients were evaluated: 5% sucrose, 4% monosodium glutamate (MSG), 4% mannitol + 1% sucrose, 5% reconstituted skim milk (RSM), 0.5% polyethylene glycol 8000 (PEG) + 5% sucrose, 0.4% polysorbate 80 (Tween 80) and 2% ascorbic acid. Each protector was dissolved in the *L. salivarius* CRL 1328 supernatant containing the active salivaricin CRL 1328 to obtain the desired concentration. These solutions were sterilized by filtration through a cellulose acetate membrane (0.22 μm pore size; Millipore, USA). To prepare freeze-dried samples, 15 ml of each solution was added to sterile petri dishes. The dishes were frozen at −20°C and dried in a chamber-type freeze-drier (Leybold, Germany) for 16 h at 0.3 mbar. After lyophilization, the resulting powder was dispensed into screw-capped vials and salivaricin activity was determined to evaluate its stability during the lyophilization process. To determine the above stability during the subsequent storage of the dried bacteriocin, samples were kept at 25°C, 4°C and −20°C for 18 months. Protein concentration and inhibitory activity were determined to obtain specific activity (AU μg^{-1} protein). Due to the high protein concentration in samples with RSM and the interference produced by Tween 80 with the Coomassie blue dye used for the protein quantification assay, in both cases the specific activity was determined as AU in reference to the net weight of the dry powder in milligrams (AU mg^{-1} powder). Because of these technical limitations, the results obtained with Tween 80 and RSM had to be evaluated in a different set. All the experimental assays for every time and storage condition were performed in duplicate.

Statistical analysis. For the design of the lyophilization process protocol, a complete factorial design 8×2: eight lyoprotectors and two sampling times (before lyophilization and after lyophilization) was used. A two-way analysis of variance (ANOVA) test was used to determine the protective effect of different lyoprotectors used for salivaricin lyophilization. Tukey's test was applied for the pairwise comparison of the results obtained from the assays with different protective agents.

To determine the stability of salivaricin CRL 1328 during storage, a complete factorial design $3\times8\times7$ (temperature × protector × storage time) was applied. The factor temperature includes 3 different levels (−20°C, 4°C and 25°C); the protectors studied were 8 levels (sucrose, MSG, mannitol + sucrose, RSM, PEG + sucrose, Tween 80, ascorbic acid and control) and storage time includes 7 levels (0, 30, 90, 150, 240, 390 and 540). Each one of the 168 possible combinations that resulted from the design was experimentally determined.

The ANOVA test was used to evaluate the results considering all the lyoprotectors used. The experimentally determined variable was the specific activity of salivaricin CRL 1328, which was expressed as AU μg^{-1} protein with the exception of the samples with the protectors RSM and Tween 80, because of the technical limitations explained above. In the last experiments, bacteriocin activity was expressed as AU mg^{-1} powder. Therefore, a second mathematical expression for the analysis of variance was applied to quantify the importance of RSM and Tween 80. In both cases, a model of main effects and interactions between two and three factors was applied.

On the basis of the complexity of the significant interactions, PCA was used to evaluate the relationships between protectors, temperature and storage time, and the results were displayed in biplot graphics. The dependent variables used were the specific activity (experimental data) in each one of the six times assayed (initial time was not included) for all the 18 combinations of temperature and protectors applied for the first model and for the 9 combinations used for the

second model. This technique allowed us to detect the relationships between the effects of the treatments on the specific activity variable for different time periods. The relative positions of the samples in the generated plot indicate the differences between the protectors combined with the temperatures.

Results

Resistance of salivaricin to the lyophilization process

The evaluation of the inhibitory activity of the protective agents indicated that none of them exert any type of antimicrobial property per se against the indicator strain *E. faecalis* MP97. The effect of the two groups of protective agents on salivaricin activity after the lyophilization process were analyzed in different sets to facilitate the interpretation of the results.

First set of experiments. The combination of the different protectors and salivaricin before the freeze-drying process showed a slight variation in the specific activity of salivaricin (Table 1). During the freeze-drying process, bacteriocin activity proved to be more stable in the presence of the different lyoprotective agents compared to the control, with significant differences between them (Table 1). The main protector effects were observed with sucrose, MSG, mannitol + sucrose and PEG + sucrose, with no significant differences among them. The decrease in bacteriocin activity was higher with ascorbic acid than with the other lyoprotectors, but smaller than the control without protective agents, as summarized in Table 1.

Second set of experiments. Before the freeze-drying process, the activity of salivaricin decreased in those samples supplemented with RSM (35.3±1.5 AU mg^{-1} powder) but increased in the samples with Tween 80 (144.5±2.1 AU mg^{-1} powder) compared with the control (104.3±1.8 AU mg^{-1} powder). After lyophilization, the salivaricin specific activity of RSM samples showed no significant changes compared with pre-lyophilization values. However, Tween 80 samples showed a decrease in the specific activity from 145 to 119 AU mg^{-1} powder (Table 1).

Salivaricin stability during storage

First set of experiments. A progressive reduction in the specific activity was detected in all the samples during the time of storage (540 days). This decrease was lower during the last days of storage (Fig. 1).

The ANOVA test showed that the temperature, protective agent and storage time were the factors that affected the specific activity of salivaricin. The main effects were significant, the differences produced by the

Table 1. Specific activity of salivaricin before and after the lyophilization process.

Protectors	Specific activity before lyophilization	Specific activity after lyophilization	Difference of activity*
First set of experiments	AU μg^{-1} protein		
Sucrose	4.8±0.1	4.9±0.1	-0.1^{a}
MSG	4.3±0.1	3.9±0.1	0.4^{ab}
Mannitol + sucrose	4.7±0.2	4.8±0.1	-0.1^{a}
PEG + sucrose	5.0±0.1	4.6±0.1	0.5^{ab}
Ascorbic acid	5.1±0.3	4.0±0.1	1.1^{b}
Control	4.5±0.2	2.4±0.1	2.2^{c}
Second set of experiments	AU mg^{-1} powder		
RSM	35.3±1.5	33.4±1.4	1.8^{d}
Tween 80	144.5±2.1	119.0±1.4	25.5^{e}
Control	104.3±1.8	56.9±1.6	47.4^{f}

*Differences in specific activity between samples before and immediately after the lyophilization process (Day 0 of storage) are indicated in this column. Different superscripts indicate significant differences ($p<0.05$) between the samples lyophilized with different lyoprotectors. Each sample was assayed in triplicate. The specific activity is expressed in AU μg^{-1} protein for the first set of experiments and in AU mg^{-1} powder for the second set of experiments as explained in the material and methods section. MSG, monosodium glutamate; PEG, polyethylene glycol 8000; RSM, reconstituted skim milk; Tween 80, polysorbate 80.

storage temperatures being higher than those of the lyoprotectors or the storage time (data not shown). The interpretation of the interaction effects is very complex because they do not show easily detectable behavioral patterns. However, the PCA biplot methodology enabled us to establish the associations between the experimental factors assayed and the levels of specific activity detected in each of the combinations. The biplot associated with this PCA analysis showed the differences between salivaricin activity after 30, 90, 150, 240, 390 and 540 days of samples stored with different protective agents and the control at the three temperatures assayed (Fig. 2). The first component (PC1) accounts for 60.4% of the variability of the data and was positively associated with the activity at 540 days (96%), 390 days (85%), 240 days (92%) and 150 days (65%). The second component (PC2) accounts for 19.8% of the variability of the data and was positively associated with the activity observed at 30 days (91%). At 90 days storage, the specific activity did not show a strong relationship with either of the two principal components; hence it was not relevant for the analysis (Fig. 2).

According to the results of Tukey's test, which was used for the evaluation of interactions between protective agents, temperature and storage time, and the relative positions of the samples in the biplot (Fig. 2), the following considerations can be expressed:

—The lowest values of the specific activity of salivaricin were obtained at 25°C and were detected in those samples assayed with MSG, mannitol + sucrose, PEG + sucrose and sucrose. At this temperature, the specific activity recovered was not significantly different from the control. The samples assayed with ascorbic acid maintained a higher specific activity at 25°C.

—At 4°C the highest specific activities of salivaricin was obtained by storing the samples with PEG + sucrose. At −20°C the highest values of salivaricin activity

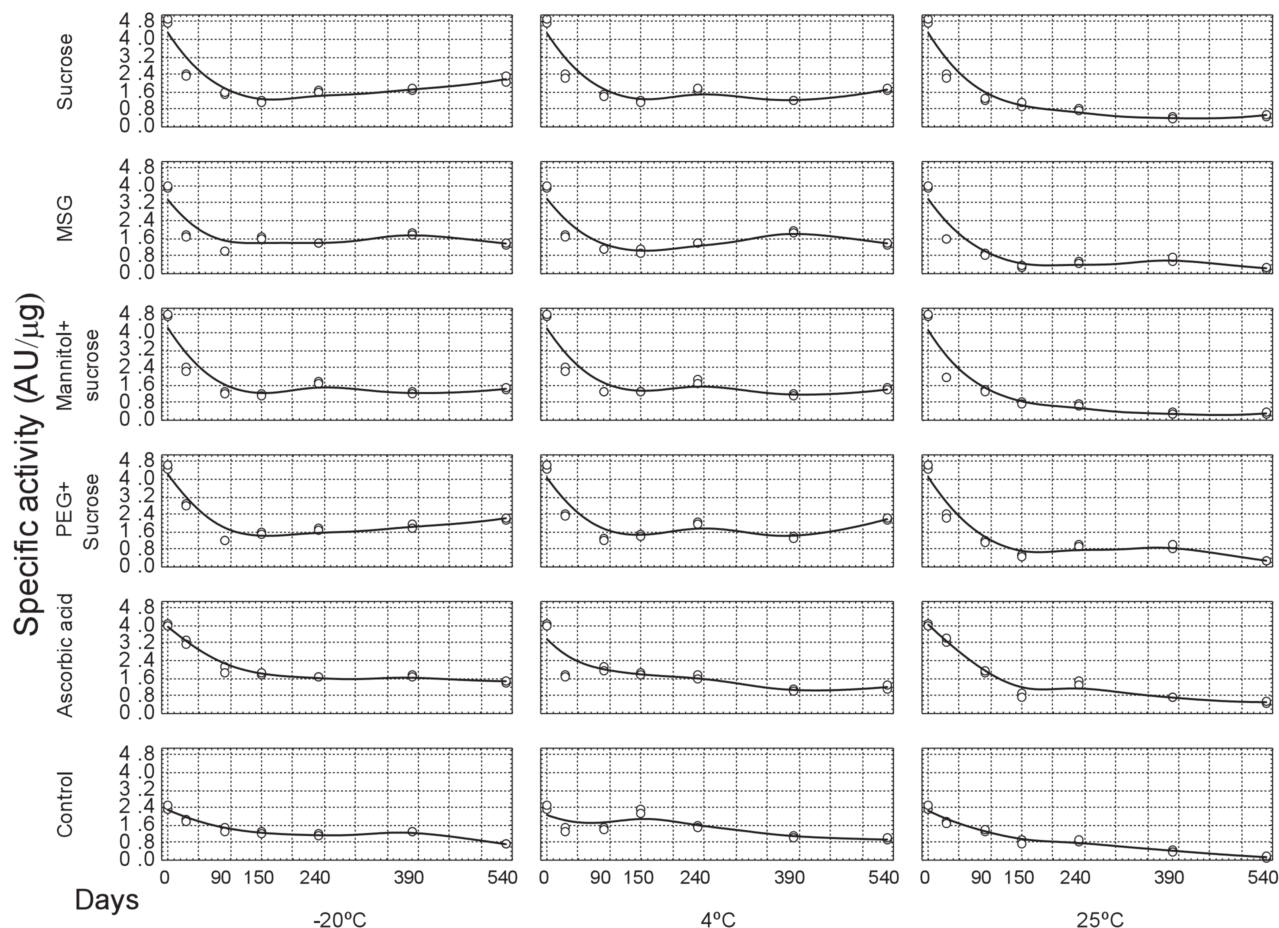

Fig. 1. Plot showing the specific activity of salivaricin (AU mg^{-1}) during eighteen months of storage at 25°C, 4°C and −20°C. The different protectors used were sucrose, MSG, mannitol + sucrose, PEG + sucrose, ascorbic acid and control (sample without protector).

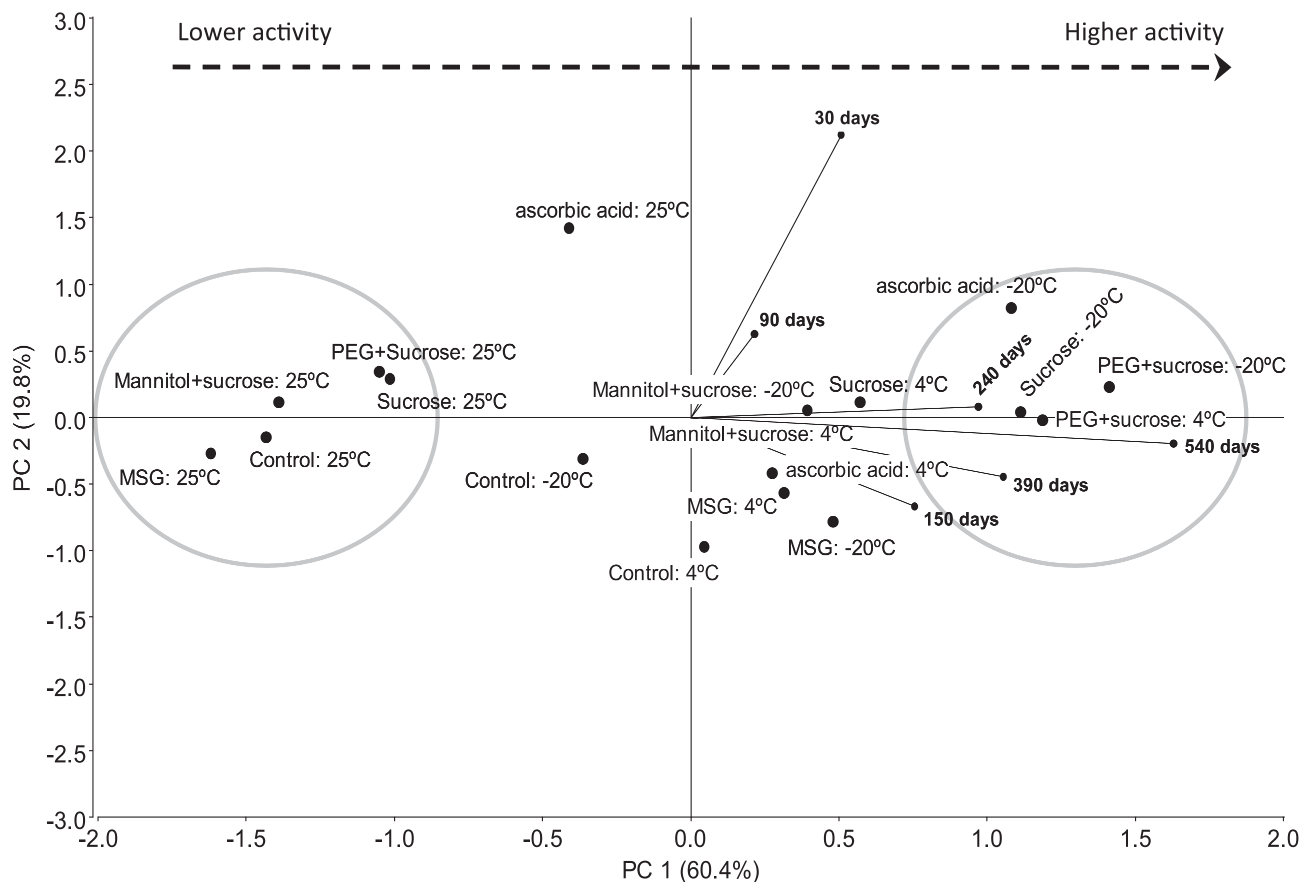

Fig. 2. Representation of the first two components of PCA.

The plot shows the specific activity (AU mg^{-1}) of salivaricin CRL 1328 during eighteen months of storage at 25°C, 4°C and −20°C. The different protectors used were sucrose, MSG, mannitol + sucrose, PEG + sucrose, ascorbic acid and control (sample without protector).

were obtained using PEG + sucrose, sucrose and ascorbic acid (these samples are located on the right of the biplot above the PC1 axis). At this temperature, after 30 days of storage, specific activity was higher with ascorbic acid than with the other protective agents assayed. On the other hand, the specific activity profiles obtained between 30 and 540 days using PEG + sucrose at 4°C and −20°C and sucrose at −20°C did not show significant differences during storage, these samples being very close to each other in the biplot graph. The samples lyophilized with mannitol + sucrose as protective agents and stored at −20°C and 4°C and those with sucrose stored at 4°C showed similar profiles during the time periods assayed.

—The samples assayed with MSG and stored at 4°C showed the same behavior as those lyophilized with MSG at −20°C and ascorbic acid at 4°C, as shown in Fig. 2.

Second set of experiments. In samples in which RSM was used as a lyoprotector, a progressive decrease in specific activity was detected. The protective effect of this excipient was lower than the control in all the experiments. Tween 80 proved to be a better protective agent than RSM. In both cases, the lowest protective effect was observed at 25°C, as shown in Fig. 3.

The results of the ANOVA test of the effect of RSM and Tween 80 indicated that the main effects were statistically significant and that the effects of the protectors were higher than those produced by the storage temperature. Only the interaction between the lyoprotectors and the storage temperatures was significant as well as the third order interaction (temperature, storage time and lyoprotector).

The biplot graph obtained from this last experiment is shown in Fig. 4, where the PCA of the results obtained using RSM and Tween 80 as protective agents is included. This plot shows the relationships between

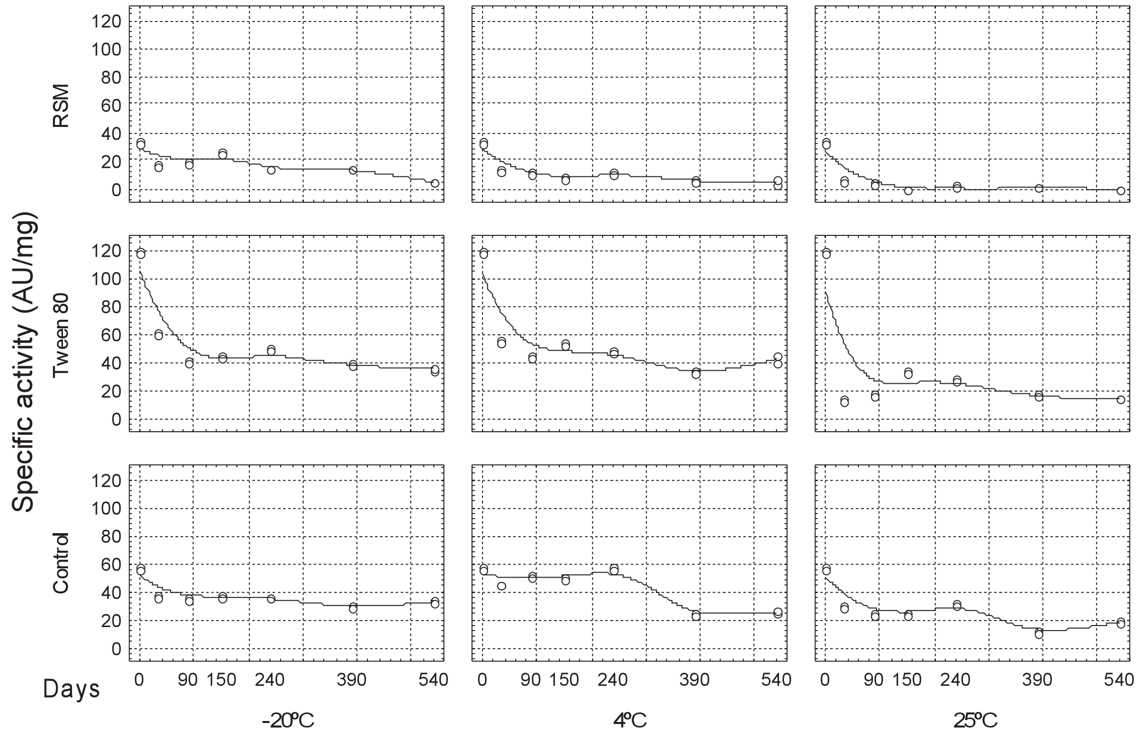

Fig. 3. Plot showing the specific activity of salivaricin (AU mg^{-1}) during eighteen months of storage at 25°C, 4°C and −20°C.

The protectors used were RSM, Tween 80 and control (sample without protector).

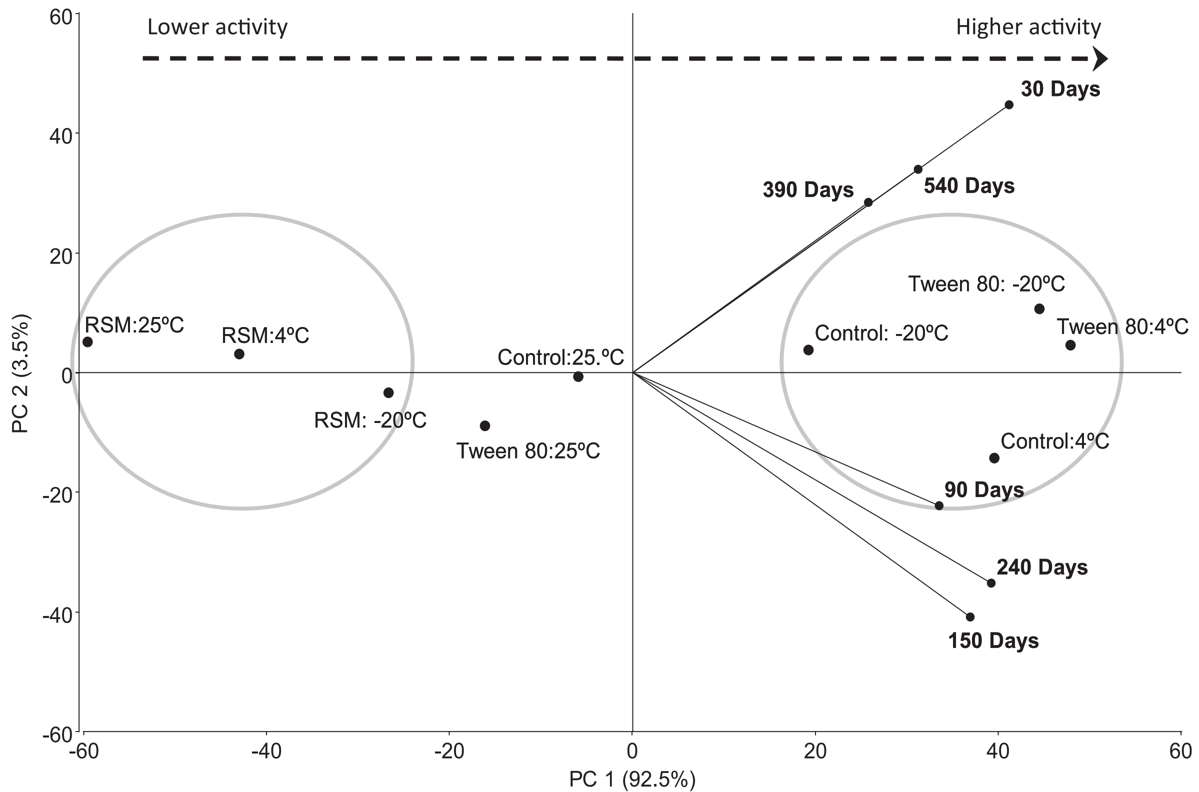

Fig. 4. Representation of the first two components of PCA.

The plot shows the specific activity (AU mg^{-1}) of salivaricin CRL 1328 during eighteen months of storage at 25°C, 4°C and −20°C. The protectors used were RSM, Tween 80 and control (sample without protector).

activity values after 30, 90, 150, 240, 390, and 540 days of the samples stored with RSM, Tween 80 and their control at the three temperatures assayed. The first component of the biplot graph accounts for 92.5% of the variability of the data along the horizontal axis and the second only for 3.5%. PC 1 is associated with all the specific activities determined between 30 and 540 days. Consequently, because of their relative position in the graph, Tween 80 at 4°C and −20°C are the conditions in which the best protection of salivaricin was obtained after 540 days of storage. The control samples stored at −20°C also showed an acceptable average behavior during the storage time, although at 4°C protection was higher during the first 240 days of storage.

The use of RSM as a protective agent was not effective during storage at any of the temperatures assayed. This effect decreased with higher temperatures, as shown on the left in Fig. 4.

Discussion

In the design of pharmaceutical bioactive formulas, storage of the final product should be given great consideration. The freeze-drying of bioactive compounds prior to formulation and storage is a strategy frequently used since the stability of different substances in a solid state has some advantages over storage in liquid media, mainly in the case of those bioactive compounds included in pharmaceutical products. However, there are different types of physical and chemical degradation of peptides in the solid state. Some of the main factors affecting the stability of powdered peptides are the storage temperature and formulation excipients included in such products (Connors et al., 1986; Mi and Wood, 2004). In the present work salivaricin was freeze-dried by using different lyoprotectors and stored at three different temperatures in order to select the optimal freeze-drying and storage conditions.

Salivaricin could be included in a pharmaceutical product for the prevention of urogenital infections because it is heat-stable and active against different urogenital pathogens (Ocaña et al., 1999; Vera Pingitore et al., 2009a). The incorporation of this bacteriocin into probiotic products could be highly beneficial, mainly because the biological activity of salivaricin could act in a synergistic way with the biological effect of the different strains of lactobacilli with beneficial properties, also previously evaluated by our research group (Juárez Tomás et al., 2004; Zárate et al., 2005). Therefore, the optimal excipients for the design of this type of combined product would be those that are able to protect salivaricin and that are also compatible with the probiotic microorganisms included in the product. The excipients evaluated in this study were selected trying to obtain stability of the activity of salivaricin during the lyophilization process, maintaining also its biological effect during storage. There were no available publications on the storage of bacteriocins by using the type of excipients assayed in this work to allow the comparison of our results. However, due to the peptide nature of bacteriocins, our findings are compared with some results published on the stability of proteins and peptides.

Ascorbic acid has been shown to increase the viability of lactic acid bacteria during storage and can also help to maintain a low pH in the vagina (Polatti et al., 2006; Zárate et al., 2005; Zárate and Nader-Macias, 2006). MSG is an excipient used in the pharmaceutical industry for the storage of lactic acid bacteria (Carvalho et al., 2003; Martos et al., 2007) and also, in the form of glutamic acid, as an acidifier for vaginal formulations (Garg et al., 2001). However, in the case of salivaricin, it showed a slight effect, and should be considered only if its incorporation into the design of a novel product is highly beneficial.

Sucrose is one of the excipients of choice for the stabilization of proteins due to the inhibition of the unfolding of protein and also because it provides a glassy matrix, as explained by Carpenter et al. (2002). The higher stability of salivaricin during storage was obtained by using this excipient combined with PEG, but the specific effect of sucrose in salivaricin is not known yet. PEG was previously used for optimal stability of the model protein lactate dehydrogenase with promising results (Corveleyn and Remon, 1996; Mi and Wood, 2004). In the present study, when sucrose was combined with mannitol, a commonly used bulking agent, a lower activity of salivaricin was obtained, even lower than with sucrose + PEG. Mannitol was used by Passot et al. (2005) to stabilize proteins. RSM is a protective agent frequently used in the storage of lactic acid bacteria that has been shown to stimulate the growth of lactobacilli against the detriment of uropathogens in in vitro assays (Reid et al., 1998; Schoug et al., 2008). However, RSM would be difficult to include in a formula containing salivaricin because it proved to be

ineffective during the storage stage. These results could be explained by the presence of reducing sugars such as lactose included in RSM that could degrade proteins via the Maillard reaction between carbonyls of the sugar and free amino groups in the protein (Li et al., 1996). Tween 80 is a surfactant that disperses hydrophobic substances; therefore, it may promote the insertion of bacteriocins into cell membranes, inhibit the aggregation of bacteriocins, stabilize a favorable configuration of the bacteriocin molecules, and/or produce a sensitization of the target cell, perhaps through destabilization of the cell membrane (Joosten and Nuñez, 1995; Nissen-Meyer et al., 1992). The addition of Tween 80 significantly increased salivaricin activity prior to the freeze-drying process. This effect was previously reported for different bacteriocins, such as lactacin F, lactococcin G and bovicin HC5, but the reason for this increase has not been yet studied (Houlihan and Russel, 2006; Muriana and Klaenhammer, 1991; Nissen-Meyer et al., 1992).

The activity of divercin, a bacteriocin produced by *Carnobacterium divergens* AS7 was increased after Tween 80 addition. The authors reported that Tween 80 inhibited the aggregation of this bacteriocin and proposed a possible mechanism of action (Sip and Grajek, 2001). Other bacteriocins, such as nisin and enterocin 4, are adsorbed on polypropylene disposables and on glassware and the adsorption is avoided by using Tween 80, thus increasing apparent bacteriocin titers (Joosten and Nuñez, 1995). However, Tween 80 did not increase the stability of salivaricin during storage, probably because of some type of oxidative damage or aggregation that has been described for IL-2 mutein (Wang et al., 2008).

All the results obtained from experimental assays were subjected to statistical evaluation. The Analysis of Principal Components biplot allowed us to obtain additional information of the data collected, since it enabled the easier interpretation of the effects of the main interaction of the evaluated factors by using the relationships between them and the relative positions of the points in a biplot graphic. This type of plot has been extensively used in different research fields (Li et al., 2008; Maisuradze et al., 2009; Mendoza et al., 2009). However, this is the first time that this type of analysis has been applied for the evaluation of the storage of a bacteriocin. The results obtained showed that the stability of the freeze-dried salivaricin was dependent on the protectors assayed and on the storage temperature. But, the mechanistic interpretation must be strengthened with experimental assays, which are not included in this work. The best conditions for the storage of salivaricin were obtained at 4°C and −20°C. However, it is important to emphasize that the freeze-dried bacteriocin, in spite of reducing the antimicrobial activity, remained active at least during the 18-month period assayed at a temperature of 25°C, a relevant characteristic for its potential application. These results are underlined by the fact that in the design of a pharmaceutical products, one of the most desirable properties is the stability of the bioactive compounds and the maintenance of the biological activity during long times of storage, and if possible, at room temperature.

Acknowledgments

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT). The *L. salivarius* CRL 1328 strain was licensed to Probiotical-Anidral for industrial applications.

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