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Milk Evaluation as Growth and Cold Preservation Medium of a Probiotic Inoculum for Young Calves

[1]L.P. Soto, [1]L.S. Frizzo, [1]E. Bertozzi, [1]A. Diaz, [1]L.E. Marti,
[1]R. Dalla Santina, [1,2]G.J. Sequeira and [1,2]M.R. Rosmini
[1]Departamento de Salud Publica Veterinaria, Facultad de Ciencias Veterinarias,
Universidad Nacional del Litoral, Kreder 2805 (S3080HOF) Esperanza, Santa Fe, Argentina
[2]Facultad de Ciencias Agropecuarias, Universidad Católica de Córdoba,
Camino a Alta Gracia, km 10 (5000) Córdoba, Argentina

Abstract: Production of probiotic products requires preliminary research studies on technological characteristics of strains to verify the growth capacity of microorganisms and their survival rate and stability during storage. On an industrial scale production, it is important to lower the manufacturing cost by using inexpensive media both for propagation and preservation. The objective of the present study was to evaluate milk medium capacity to act as propagation matrix and cold preservation for a potential probiotic bacterial inoculum to be used in young calves bred in intensive systems. Bovine origin strains under study were *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T. Strain propagation and preservation capacities were evaluated in 100 g L^{-1} skim milk powder and de Man, Rogosa and Sharpe medium (MRS) as positive control. Cultures were stored at refrigerated (4°C) and freezing (-20°C) conditions and their viabilities rates were recorded every 21 days for a 3-months period and on months 6, 9 and 12 (180, 270 and 360 days). Biomass production and counts of cell viabilities were done in decimal dilutions in Ringer ¼ solution and spread in MRS agar dishes. The 3-studied Lactic Acid Bacteria (LAB) were able to propagate in milk medium. This inoculum preservation, with bacterial counts higher than the Suggested Minimum Level (SML) (10^6 CFU mL^{-1}) in milk medium, was feasible when it was stored under refrigerated conditions for 84 days and in freezing conditions for 360 days. Manufacturing cost could be substantially reduced if the same medium could be successively used in both, propagation and conservation processes, modifying only the physical conditions.

Key words: Probiotics, calves, preservation, refrigerated, freezing, DSPV

INTRODUCTION

In intensive breeding systems, when calves are weaned from their mothers at an early age, the colonization of the indigenous intestinal microbiota is difficult, specially the one transmitted by mother contact, condition that facilitates the activity of pathogenic microorganisms (Rosmini *et al*., 2004). This situation causes an enteric microorganism misbalance, predisposes to the emergence of digestive disorders and consequently, affects health conditions of animals at an early stage (Nousiainen and Setala, 1998; Timmerman *et al*., 2005), generating, as a consequence, economic loses to the productive system.

In the last decades, the benefits of inoculating the indigenous microbiota with cultures having probiotic capacity in order to inhibit the growth of pathogenic bacteria in calves has been proposed as an alternative to the prophylactic use of antibiotics (Gilliland *et al*., 1980; Vargas *et al*., 2004). Periodical administration of a probiotic inoculum of bovine origin would favor a more stable and well-balanced intestinal microbiota and as a consequence, improve calf health (Abe *et al*., 1995). Moreover, LAB have important implications in modulation of the host's immune response (Matar *et al*., 2001) and is recognized the importance of the host specificity in the selection of probiotic bacteria.

Having in mind the benefits that strains produce on the host, their selection is done by means of *in vitro* and *in vivo* studies of the probiotic properties. Likewise, technological characteristics are assessed to verify microorganism growth capacity, their survival rates and stability throughout storage (Dunne *et al*., 2001). Viability and amount of microorganisms at the moment of being

Corresponding Author: Marcelo Raul Rosmini, R.P. Kreder 2805, CP S3080HOF, Esperanza, Santa Fe, Argentina

Table 1: Pharmaceutical shape of probiotic products in market

Product name	Company	Country	Host	Microorganism	Pharmaceutical shape	Microbial count	Product application
[a]Provita protect	Provita	UK	Calves	*Enterococcus faecium* SF 101; *Lactobacillus acidophilus* LA 101; *L. acidophilus* LA 107	Oil based liquid	NAI*	2.5-5 mL day^{-1}
[b]Lactobac premier B soluble	Park tonks	UK	Calves	*E. faecium* (NC1B11181)	Powder	1×10^{9} CFU g^{-1}	2 kg t^{-1} calf milk replacer
[c]Rumen aider®	Bio vet	EEUU	Adult cattle	*Propionibacterium freudenreichii; L. acidophilus; L. casei; L. lactis; E. faecium; Pediococcus cereviciae; Saccharomyces cereviciae*	Capsules	2×10^{10} CFU capsule^{-1}	1-2 capsules day^{-1}
[d]Gener™ microbial paste	Bio vet	EEUU	Livestock	*Bacillus licheniformis; B. subtilis; L. acidophilus; L. casei; L. lactis; E. faecium; P. cereviciae; S. cereviciae*	Paste	$1,6\times10^{9}$ cfu mL^{-1}	5-15 mL $dose^{-1}$; 1-2 doses day^{-1}
[e]Equi-bac oral gel	Kaeco group	EEUU	Horses and foals	Bacteria; yeast	Gel	1×10^{10} CFU $dose^{-1}$	NAI
[f]Biofresh™ Bolus	Biofresh	EEUU	Dairy cattle	*Lactobacillus* sp.	Bolus	NAI	NAI
[g]Tech-o-lyte®	Kaeco group	EEUU	Livestock	*S. cereviciae*; *L. acidophilus; L. casei; L. fermentum; L. plantarum; Streptococcus faecium*	Soluble granules	NAI	NAI
[h]Bio-premium	Protexin®	UK	Horses	*S. cereviciae* 1026 (CBS 493.94)	Pellets	2×10^{8} CFU g^{-1}	10-20 g day^{-1}

*NAI: Not Available Information; [a]http://www.provita.co.uk/; [b]http://www.parktonks.co.uk/?/animal/supplements/probiotic/lactobac_b_soluble.htm; [c]http://www.bio-vet.com/AdultCattleCapsulesRA.htm; [d]http://www.bio-vet.com/sheepgoatsothers.htm; [e]http://kaeco.com/equine.htm#EquiBac; [f]http://www.selectsires.com/biofresh_bolus.html; [g]http://kaeco.com/livestock.htm; [h]http://www.protexin.com/vet/product.php?id=33

inoculated is of vital importance since to produce the probiotic effects (Perdigon *et al*., 1991), the SML of bacteria is 10^{6} CFU mL^{-1}, achieved by a 300-400 g consumption/week of the probiotic product (Vinderola *et al*., 2000).

At an industrial level, in the probiotic manufacturing process, it is very important to appropriately select the growth medium. When doing so, different factors should be considered, like cost, high production capacity of active cells as well as an easy recuperation of microorganisms (Mayra-Makinen and Bigret, 1998). Bacterial proliferation requires appropriate growth medium that supply nurturing substances microorganisms need to grow. There are specific commercial media for each specie or bacterial group; such is the case of de Man, Rogosa and Sharpe medium (MRS) for *Lactobacillus* (Gaudreau *et al*., 2005). In general, these growth media are so highly priced that their use is very limited at an industrial level (Rodrigues *et al*., 2006). On the other hand, in the market, there exist many alternatives of raw material available that could be used as nutrients for large-scale fermentation, such is the case of milk that lets to grow different *Lactobacillus* species (Elli *et al*., 1999) and consequently, milk could be used in the industrialization of bovine probiotic inocula.

There are different pharmaceutical formulations of probiotic inocula for veterinary use (Table 1), which allow preserving the product at room temperature. Though, liquid cultures have a shorter average life in general, they are interesting to use since their action is faster in the gastrointestinal tract. They are commercially available in oil, water or milk probiotic suspensions (Table 1). Cold preservation is one of the most used methods to keep the viability of bacteria in liquid cultures. Refrigeration is generally used to preserve cells for only a short period of time since long time-storage could be a stress factor for the bacteria (Gilliland and Lara, 1988). For this reason, freezing is the recommended method to use in long term preservation (Fonseca *et al*., 2001). Though, freezing causes ice crystal formation that could damage the cells, usually, cryoprotector agents like glycerol, sugar and/or milk are added to the bacterial culture before exposing it to low temperatures (Champagne *et al*., 2000; Fonseca *et al*., 2003).

To take maximum advantage of the benefits of microorganisms with probiotic capacity in livestock farming it is necessary to develop growth media that is economic, ensure inoculum viability during the industrialization and storage stages and permit their administration to animals in farm conditions.

The objective of this study was to evaluate milk medium capacity to act as propagation matrix and cold preservation of a bacterial inoculum potentially probiotic for young calves raised in an intensive cattle raising system.

MATERIALS AND METHODS

Microorganisms: *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T, 3 strains of a potential probiotic inoculum of bovine origin belonging to the Departamento

de Salud Publica Veterinaria (DSPV), Facultad de Ciencias Veterinarias (FCV), Universidad Nacional del Litoral (UNL), were studied. Their Genbank accession numbers are: FJ787305, FJ787306 and FJ787307, respectively. Strains were isolated from healthy calves and kept at -80°C in MRS broth (Britania®) with 350 mL L^{-1} sterilized glycerol. Strains were identified by 16S rDNA gene sequence (Schneider *et al*., 2004) and their probiotic properties were evaluated *in vitro* (Frizzo *et al*., 2006), *in vivo* in mice (Frizzo *et al*., 2005, 2007) and in calves (Frizzo *et al*., 2008).

Propagation and preservation media: Strains were subcultured twice in MRS broth at 37°C before a final inoculation (1% v v^{-1}) in the media detailed next: MRS broth as positive control for LAB propagation and 100 g L^{-1} sterile rehydrated skim milk powder (Milkaut®). Strains were stored during the preservation period in the same medium where they had been propagated.

Growth conditions: Each strain was inoculated in 30 mL growth media before mentioned and were incubated during 24 h at 37°C. After decimal dilutions in Ringer ¼ solution had been done, viable cell counts were determined by pour-plating method in MRS agar and then incubating the Petri dishes at 37°C for 48 h. All determinations were done in triplicate.

Storage conditions: An aliquot of 1.2 mL of each one of the homogenized cultures was dispersed in sterile vials. They were stored at 4°C and -20°C so as to evaluate the preservation capacity in refrigerated and freezing storage, respectively. Viable cell counts were done every 21 days during 3 months and on the 6, 9 and 12 months (180, 270 and 360 days). To do so, decimal dilutions in Ringer ¼ solutions were spread in MRS agar plates. They were incubated at 37°C for 48 h. All determinations were done in triplicate. Viability rate was calculated by relating the number of live microorganism in the different times that had been studied with respect the initial count.

Statistical analysis: Bacterial growth and viability data were analyzed by way of ANOVA and Tukey test. Both were done using Statgraphics Plus for Windows, ver. 3.0 and taking $p<0.05$ as the significant difference between means.

RESULTS

Bacterial growth: The 3-studied strains showed less growth in milk medium ($p<0.05$) than in MRS. Statistical differences were found ($p<0.05$) among the different strains when they were propagated in the same culture medium, except for *L. casei* DSPV 318T and *L. salivarius* DSPV 315T that presented a quite similar growth in MRS broth (Table 2).

Table 2: Bacterial growth of the studied strains in MRS and milk media

	Population (log_{10}CFUs)[1]	
Strain	MRS	Milk
L. casei DSPV 318T	9.47±0.09^{a,A}	8.51±0.11^{b,A}
L. salivarius DSPV 315T	9.54±0.07^{a,A}	8.04±0.16^{b,B}
P. acidilactici DSPV 006T	9.12±0.02^{a,B}	8.13±0.01^{b,B}

[1]The values correspond to average±SD. ^{a,b} Values with the same letter are not significantly different ($p<0.05$) inside the same row. ^{A,B} Values with the same letter are not significantly different ($p<0.05$) inside the same column

Preservation in refrigerated and freezing conditions of microorganisms in MRS medium: Cell viability in MRS medium was different ($p<0.05$) for the 2 evaluated temperature (Fig. 1). In refrigerated conditions, strains were viable at least for over 84 days. In freezing conditions it was possible to find live cells of all studied strains throughout the entire study (360 days). Likewise, it was observed a large variability among the 3 strains.

The 2 *Lactobacillus* strains were the ones presenting less survival time (84 days) and no differences ($p>0.05$) in their behaviors when kept in refrigerated conditions.

L. casei DSPV 318T kept in refrigeration produced a lesser decrease in viability rates than in freezing storage ($p<0.05$). Up to 84 days of the experiment, a higher viability rate was found ($p<0.05$), in refrigerated than in freezing storage conditions, however, in the latter it was possible to find viable cells up to the very end of the study. Counts obtained were higher than the SML up to 63 and 21 days of the study in refrigerated and freezing conditions, correspondingly (Fig. 1a).

In refrigerated condition, *L. salivarius* DSPV 315T presented a similar behavior to the one described for *L. casei* DSPV 318T. However, in freezing conditions, cells kept the SML up to the end of the study (Fig. 1b).

Unlike, the other studied strains, *P. acidilactici* DSPV 006T remained viable during 360 days in both temperatures presenting no significant differences ($p>0.05$) between the studied storage temperatures up to 42 days inclusive. However, starting on 63 days, viability loss was significantly lower in freezing conditions ($p<0.05$), reaching a high live cell rate and keeping the SML up to the end of the study. In refrigerated conditions, SML was only kept up to 84 days (Fig. 1c).

Preservation in refrigerated and freezing conditions of microorganisms in milk medium: The same as in MRS, the preservation in milk medium showed significant

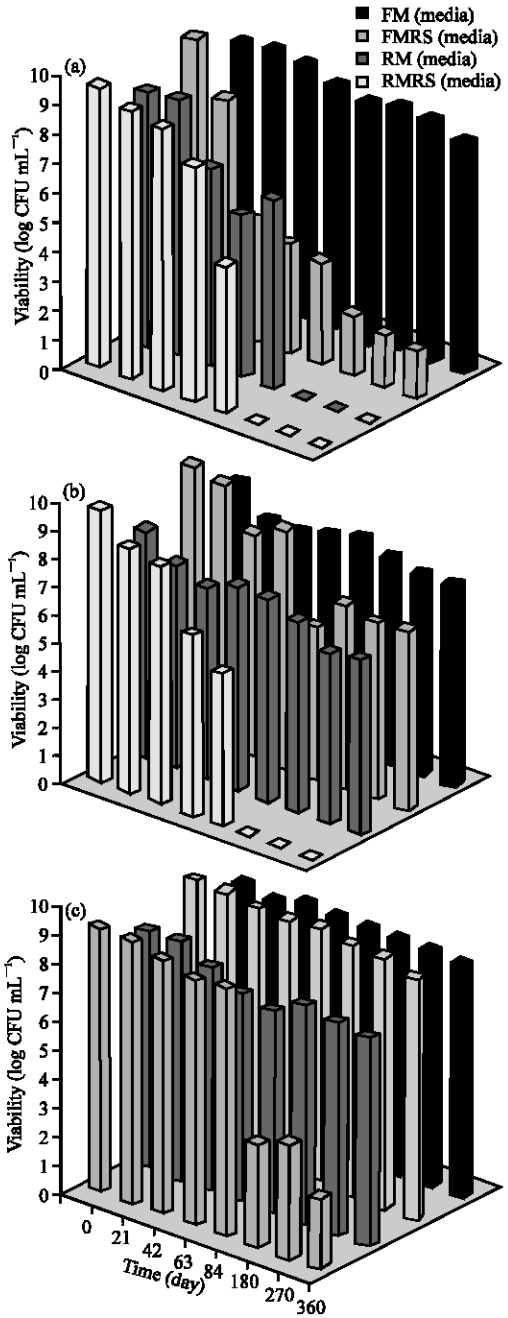

Fig. 1: Cell viability of *L. casei* DSPV 318T (a), *L. salivarius* DSPV 315T (b) y *P. acidilactici* DSPV 006T (c) during Frozen (F) or Refrigerated (R) storage in Milk (M) or MRS medium for 360 days

differences between the 2 studied storage temperatures, being the microorganisms' viability rate higher under freezing storage conditions (Fig. 1).

L. casei DSPV 318T was the only strain that did not survive in refrigerated conditions >84 days. The comparison of the strain viability rates, in both temperatures, did not show significant differences ($p>0.05$) up to 21st days inclusive. From that moment on, the viability rate loss in refrigerated conditions was significantly higher. However, in such conditions, cell concentration values were superior to the SML up to day 84 of the experiment (Fig. 1a). On the other hand, in freezing conditions, a quite high viability rate was present until 360 day, with values that exceeded the SML (Fig. 1a).

Unlike, *L. casei* DSPV 318T, the other 2 analyzed strains, stayed viable in both storage conditions during all the experiment. As regards bacterial counts, *L. salivarius* DSPV 315T did not present significant differences ($p>0.05$) between the 2 studied temperature conditions up to 63 day inclusive. Starting on 84th day, its viability rate loss was higher ($p<0.05$) in refrigerated condition, though, in this condition cell counts exceeded SML up to the end of the study (Fig. 1b).

On the other hand, in freezing conditions, *P. acidilactici* DSPV 006T presented higher cell counts ($p<0.05$) when compared with the ones in refrigerated conditions from day 42 on. Despite this, preservation in refrigerated conditions allowed keeping high viability with values that exceeded the SML up to the end of the experiment (Fig. 1c).

Comparison between media of preservation and storage conditions: In general, in both evaluated storage conditions, it was observed that viability values were higher in milk medium than in MRS.

In both media, *L. casei* DSPV 318T remained viable in refrigerated conditions up to 84th day and in freezing conditions up to the end of the experiment (Fig. 1a). Nevertheless, in refrigeration storage, the viability rate between the two studied media was different ($p<0.05$), finishing the study with a higher bacterial concentration in milk medium. When freezing, *L. casei* DSPV 318T also showed better results in milk medium throughout the experience. This strain kept 22.3% viability in milk medium up to the end of the study, being quite superior ($p<0.05$) to MRS which kept alive <1% of the cells with respect the initial amount.

L. salivarius DSPV 315T presented significant differences ($p<0.05$) in the cell preservation time between both media in refrigerated conditions, while in MRS, cell viability was observed up to 84th day, in milk medium, cells were kept viable up to the end of the study (Fig. 1b). Moreover, in milk medium, cell counts higher than in MRS ($p<0.05$) were found in all the determinations done. On the other hand, in freezing conditions viable cells were found up to the end of the study in both media; however, starting on 42nd day inclusive, viability rate in milk medium were higher ($p<0.05$) than in MRS.

P. acidilactici DSPV 006T presented a different viability ($p<0.05$) between the studied media, both in refrigerated and freezing conditions. There were no differences between media at 4°C, up to day 42 and from that moment on; the viability rate was higher ($p<0.05$) for MRS up to 84th day. Afterwards, the highest viability was found in milk medium ($p<0.05$), keeping 12.1% of live cells up to the end of the study (Fig. 1c). There were some differences ($p>0.05$), from day 42 inclusive, in freezing conditions, ending with a 74.4% of live cells in milk medium up to the end of the study, exceeding the viability percent in MRS which, only reached 18.8%.

Independent from the studied strain, freezing preservation was the one that showed higher microorganisms' survival due to the fact that live cells were found up to the end of the study (360 days). From the point of view of SML, all frozen strains in milk medium kept such viability levels up to the year, whereas, *L. salivarius* DSPV 315T and *P. acidilactici* DSPV 006T were the only ones that did so in MRS, *L. casei* DSPV 318T kept the SML only up to 21st day. In refrigerated milk, the SML of all evaluated strains was kept at least for 84 days (Fig. 1).

DISCUSSION

It is necessary that the inoculum has a satisfactory amount of microorganisms at the moment of being administered to the animal to facilitate the expression of the probiotic effects. Moreover, this is important, when the inoculum must be conserved using any of the preservation methods due to the need of having an amount of microorganisms sufficient enough to compensate the decrease in cell number caused by the very same technological process and storage stage. It has been mentioned that 10^6 CFU mL^{-1} is the bacteria SML applicable to produce probiotic effects (Vinderola *et al*., 2000). On the other hand, it has been verified that inoculation of 10^9 CFU kg^{-1} live weight was sufficient enough to show colonization and persistence effects represented by the high level of inoculum present in calves' intestinal tracts when bred in intensive systems (Frizzo *et al*., 2008). In some cases of commercial probiotic products marketed for veterinary administration, the strain components of the inoculum are mentioned, but information concerning cell concentration is not specified. Other products specify values that exceed SML, but they have up to 8 different species and each strain amount is not determined. Then again, in some other cases neither is informed the recommended doses of such inoculum (Table 1).

Growth of microorganisms that are part of this research showed differences for each one of the strains and each one of the studied culture medium. This is in direct relation with the requirements peculiar to each microorganism and on the other hand, with the nutrient offer it was administered to. LAB are capable of growing in milk, but it is not an optimum medium for microorganisms' development (Mayra-Makinen and Bigret, 1998). In some cases milk medium must be enriched with energy sources, precursors for cell division and growth stimulant substances (Elli *et al*., 1999), specially to compensate the different capacities the probiotic bacteria have to metabolize lactose and milk proteins (Østlie *et al*., 2003). Some examples of such cases have been shown; for example, *L. rhamnosus* GG was not capable of fermenting lactose and *Bifidobacterium animalis* BB12 and *L. reuteri* SD 2112 did not grow quite well in milk without triptona addition (Østlie *et al*., 2003). In other cases, it was necessary to add undefined chemical substances so *L. johnsonii*, *L. gasseri*, *L. crispatus*, *L. amylovurus* and *L. gallinarum* strains could grow in 100 g L^{-1} low fat milk and in UHT milk (Elli *et al*., 1999). Despite these antecedents and considering that growth was best in the specific culture medium that was studied (MRS), strains used in this investigation were capable of growing in milk medium. Nevertheless, it is necessary to do new studies to evaluate if milk matrix enrichment would permit to increase microbial counts up to reaching the levels of the specific medium. A better nutrient offer from the matrix side will benefit in having more possibilities of a higher microbial load than the SML and in consequence, the preservation period could be longer.

As regards different storage conditions, it was possible to observe that refrigerated storage was adequate only in the short-term preservation, while for long-term it would be necessary to freeze the inoculum. This situation could be confirming that during storage in refrigerated conditions the effect of cell stress directly affects its viability (Gilliland and Lara, 1988).

It can be stated that milk was the medium that best kept the viability of the studied strains, both in refrigerated and freezing conditions. These results show an important advantage of milk medium over the (MRS) specific medium, situation that could be given by the cryoprotector effect attributed to milk (De Antoni *et al*., 1989).

The obtained results allowed observing different behaviors from each strain when facing the studied growth media and storage conditions, being *L. casei* DSPV 318T strain the most sensitive one to cold effect. These results agree with those informed by

Juarez Tomas *et al.* (2004), when reporting that the differential behaviour among strains could be related to the different types and proportions of fatty acid of lipid fraction. In the case of *P. acidilactici* DSPV 006T, a high viability rate was kept during 360 days, result that agrees with Guerra *et al.* (2007), who reported a limited loss of viability (0.22-$\log_{10}$ CFU mL^{-1}) for *P. acidilactici* NRRL B-5627 stored at -20°C in milk medium for 3 months. On the other hand, though its viability rate diminished, *L. casei* DSPV 318T in milk medium and freezing conditions kept the SML up to the end of the study, agreeing with Juarez Tomas *et al.* (2004) report for *L. paracasei* CRL 1251 and *L. paracasei* CRL 1289 that kept high counts for a year.

Storage in freezing conditions is generally used to preserve LAB viability over a long-time period, keeping their technological properties (acidification activity, organoleptic properties, etc) (Fonseca *et al.*, 2003). Results in this research allowed determining that freezing is a method quite effective to keep the inoculum over a year. Saxelin *et al.* (1999) reported that with temperatures below -35°C it is possible to obtain cells counts for 12 months, though in the present research, viability values were high when preserving the cells at -20°C. If we consider that microorganism's metabolism is reduced to a minimum during the freezing period, most of the nutrient contribution offered by that MRS as a specific medium for LAB loses relative importance when facing the benefit of the cryoprotector effect given by milk matrix (Juarez Tomas *et al.*, 2004). This would justify that the best results were obtained with milk medium and in freezing conditions. Adding different cryoprotectors to milk (yeast extract, sucrose, etc.), to improve microorganisms survival has being reported (Østlie *et al.*, 2003; Juarez Tomas *et al.*, 2004). Results mentioned before are more valuable if we consider they were obtained without the addition of the cryoprotectors. This raises the possibility of verifying potential improvements through new studies of supplementation the milk medium with these substances.

The principal industrial interest to preserve probiotics in refrigerated conditions is related with the manufacturing of refrigerated food for human consumption, especially yogurts. In these matrixes, microorganisms present little stability and generally the SML is not maintained within the product shelf-life (Schillinger, 1999), situation different to the one observed in acid milks (Vinderola *et al.*, 2000). In this study, the matrix characteristics were those to the acid milk and the SML was kept up to 84th day minimum period (*L. casei* DSPV 318T), in the case of *L. salivarius* DSPV 315T and *P. acidilactici* DSPV 006T and up to a year.

Survival capacity of the probiotic strain to the passing through the gastrointestinal tract may be attributed principally to its acid and bile tolerance. This is one characteristic of the strains which could be improved by the use of feed as delivery vehicle (Charalampopoulos *et al.*, 2003). In this form, milk would act as a microbial developing medium, preservation matrix and protective medium that prevents viability loss *in vivo* during the oral administration.

The inoculum preparation in milk medium and its storage in refrigerated or freezing conditions would avoid added cost to the microorganisms' separation process out of the growth medium (centrifugation, filtration, etc.) and new medium addition to store it. One disadvantage liquid concentrate has is that when kept by using cold, have high cost in transporting and storing (Mattila-Sandholm *et al.*, 2002), reason that would justify the need to further studies that would allow evaluating new preservation procedures (microcapsules, lyophilisation), or pharmaceutical formulas for veterinary products (Table 1) and comparing costs when using different technologies. In all these cases and for each one of the evaluated techniques, the microbiologic stability study must be done and after that, the real possibilities of administering the inoculum to animals in farm conditions should be controlled.

CONCLUSION

To conclude, it is possible to affirm that the studied inoculum may be growth and stored in milk, keeping the values higher than SML for 84 days in refrigerated conditions and up to 360 days in freezing conditions. Cultures preserved in such way could be administered to calves in direct form.

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