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In-vitro Evaluation of Three Conservation Methods of Probiotics from 42-day-old Tropical Calves

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Authors' contributions

This work was carried out in collaboration among all authors. Author SRG manages the analyses of the study, designed the study, performed the statistical analysis, wrote the first draft of the manuscript, and managed the literature searches. Author LGD helped with the experiments and reviewed the manuscript. Author AS reviewed the manuscript. Author OMI designed the study, reviewed the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The conservation of probiotics is of great importance due to the microorganism's viability; therefore, their properties and benefits depend on it. In this work, it was studied three methods of conservation; refrigeration, cryopreservation, and lyophilization of two probiotics isolated from the rumen of 42 day-old calves, previously evaluated: Animal 1-day 42 (A1D42) and Animal 3-day 42 A3D42.
Place and Duration of Study: Laboratorio de Rumiología y Metabolismo Nutricional, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, between August 2020 and September 2021.
Methodology: It was evaluated the viability of the probiotics after of 0, 30, 60 and 90 days of refrigeration, cryopreservation, and lyophilization as preservation methods. The effect of the lyophilized probiotic A1D42 on the digestibility *in vitro,* such as its effect on the volatile fatty acids (VFAs) production was also determined.

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Results: Cryopreserved and lyophilization methods had better performance about the viability in both probiotics. A1D42 lyophilized maintained its viability (67%) until 60 days and after 90 days it was affected significantly. Despite the fact that VFA production *in vitro* did not increase with probiotic addiction, A1D42 significantly increased *in vitro* digestibility by up to 66%. The results demonstrated that lyophilization is the best method of conservation of probiotic production due to it preserves vial cells, as well as the properties and effects of the probiotic.
Conclusion: Therefore, lyophilized A1D42 probiotic can be used as an additive in calf diets, which could improve their digestibility, and, therefore, their weight gain, which could have a positive impact on animal production, in addition to the health benefits.

Keywords: Probiotics; calves; lyophilization; refrigeration; cryopreservation.

1. INTRODUCTION

Probiotics are live bacteria that provide health benefits such as immunological, nutritional, bacteriostatic, and bactericide effects when given in suitable concentrations [1,2]. Therefore, they can improve animal productivity [3].

The European Union has made it illegal to use antibiotics as growth enhancers in animals (Regulation CEE 1831/2003), this increased the use of probiotics in animals. Therefore, nowadays the need and interest to optimize probiotic production, researching and applying new strategies for production improvement [4], mainly due to the low viability of probiotics during commercial process and storage, for this reason it is necessary to study new methods of preservation to maintain their viability, properties, and effects.

Cryopreservation and refrigeration are easy and cheap preservation methods [5]. Nonetheless, it is reported that there may be other effective preservation methods that maintain the viability of the bacteria better [6].

Some studies have reported that freezing-thawing may damage bacterial cells, and reduce their growth and metabolic activity, which may affect its viability [7], however, this may depend on the type of freezing, whether slow or fast freezing.

Slow freezing causes severe cellular damage and can inactivate the cell, whereas fast freezing can decrease this cellular damage [8]. Moreover, the commercial production of frozen probiotics is complicated to manage, transport and to storage. Then, in recent days, had been studied and used other preservation methods more efficiently, which allows for easier handling of the probiotic. One of these methods is lyophilization (freeze-drying) which was reported to be a great preservation method for probiotics [6,9-11].

The use of probiotics on calves has a positive effect on weight gain, digestively, volatile fatty acids (VFA) production, then, animal production. High feed digestibility and VFA production will provide more energy and allow it to utilize it as a source of fuel for body and animal production. When lactic bacteria (LAB) is added to rumen fluid, it has been demonstrated to boost digestibility [12-14].

Hence, the purpose of this study was to evaluate the effect of three preservation methods; refrigeration, cryopreservation and lyophilization on the viability of two calves' bacterial consortia that previously demonstrated their potential as probiotics. In addition, the effect of a freeze-dried probiotic on digestibility and VFA production in vitro was studied.

2. MATERIALS AND METHODS

2.1 Sample Preparation

The bacteria consortiums (BC) were inoculated in MRS (Man, Rogosa y Sharpe) broth, and were incubated at 37ºC for 18 hours when the bacterial culture was approaching the end of its development cycle (10^9 CFU/mL). After, they were refrigerated (4°C), cryopreserved (-70°C), and lyophilized to conserve them.

2.2 Refrigeration

The BC were conserved in their liquid state (in MRS broth) and were stored at 4 ºC until their use.

2.3 Cryopreservation

The BC were centrifuged at 16,000 x g for 10 min and were washed twice with 10 mL of Ringer solution. The pellet was suspended in 20 mL of MRS broth with glycerol (20 %, v/v, ratio), was vortexed and immediately frozen (fast freezing)

in liquid nitrogen (-196ºC) so as not to affect the cells. Afterwards, it was stored at -70ºC [15,16].

2.4 Lyophilization

2.4.1 Medium for cryopreservation

Skim milk powder was used as cryoprotectant in this investigation (Svelty, Nestlé, México) in 24%, which showed high capacity as a cryoprotectant [9,10,15,17-18]. Moreover, it is cheap and safe to use in the food industry. The cryoprotectant medium was prepared by suspending the cryoprotectant 24 g in 100 mL of distilled water and was sterilized at 121ºC for 20 min [17].

2.4.2 Sample preparation for lyophilization

Cells were centrifuged at 16,000 x g for 5 min, the pellet was separated from the liquid. The pellet was washed twice with 40 mL of sterile distilled water and centrifuged again. In 50 mL of skim milk 24%, the pellet was resuspended in special glass containers to be subjected to lyophilization [17,19].

2.4.3 Lyophilization procedure

The pellet mixed with the skim milk (24%) was frozen with dry ice (CO_2) and acetone, and it was stored at -20ºC. The samples were freeze-dried at -20 ºC for 8 h in a freeze-dryer (LABCONCO) to 5 x10^2 mbar [17-18].

2.5 Viability of Bacteria

The number of viable cells (CFU/mL) were determined in the BC before and after refrigeration, cryopreservation, and lyophilization. 100 mL of suitable dilutions of each BC before refrigeration, freezing, and lyophilization was seeded on MRS agar plates and was incubated at 37 ºC for 24 h for subsequent counting. After 30, 60, and 90 days of refrigeration, freezing [15], and lyophilization, the CFU/mL was determined. The freeze-dried samples were resuspended in skim milk by shaking, and incubated for 15 min at room temperature, afterwards, 100 mL of appropriated dilutions were seeded in MRS agar plates at 37 ºC for 24 h, and the number of bacterial colonies was determined.

The viability of cell was determined using the equation bellow [18]:

$$Viability\ (\%) = \frac{Viable\ cells\ after\ treatment\ (\frac{CFU}{mL})}{Viable\ cells\ before\ treatment\ (\frac{CFU}{mL})} X100$$

2.6 Digestibility of the Lyophilized Probiotic

The diet used for the digestibility test was grass dried in an oven for 24 h at 60ºC and ground.

Ankom F57 bags (ANKOM Technology Corp., Fairport, NY) were used to heat seal the diet. Ruminal fluid was collected (2 L) through a rumen cannula and was brought to the laboratory in a prewarmed container.

The reagents used to produce the buffer solution were: solution A (10 g of KH2PO4, 0.5 g of MgSO4·7H2O, 0.5 g of NaCl, 0.1 g of CaCl2·H2O, and 0.5 g of urea in 1 L of deionized water) and solution B (15 g of Na2CO3 and 1 g of Na2S9H2O in 100 mL of deionized water). Warming solutions A and B to 39ºC and adding 20 mL of solution B to 1 L of solution A made a buffer solution right before each digestion run. The pH of the buffer solution was raised to 6.8.

Each digestion vessel received ten bags, 1600 mL (5:1) of the buffer solution, 400 mL of ruminal fluid and the probiotic at doses 4X10^{11}, 4X10^{12} and 4X10^{13} UFC/g were added; the vessels were injected with CO_2, and were positioned in the DAISY^{II} digestor at 39 ºC for 48 h rotating. After that, the bags were washed, dried and were weighted to calculate the digestibility.

2.7 Determination of the Total Production of Volatile Fatty Acids (VFA's)

2.7.1 Quantification of VFA's

For the determination of VFA concentration, the sample was acquired from the rumen liquid of the *in vitro* digestibility of each treatment. 1.5 mL of the ruminal fluid was taken and centrifuged at 3,500 x g for 10 min at 4°C for pellet sedimentation, then, 1200 µL of supernatant was recovered in a new Eppendorf tube and 240 µL of 25% metaphosphoric acid was added to obtain a 5:1 ratio. The tubes were incubated on ice for 30 min to promote protein sedimentation and immediately centrifuged at 12,000 x g for 15 min at 4°C. The supernatants were filtered through glass fiber membranes. For measurement of VFA, a Varian 3300 gas chromatograph with a Zebron ZB-FFAP capillary column and a flame ionization detector with characteristics of length 15 cm, internal diameter of 0.53 mm (Phenomenex, CA, USA) were used. The operating conditions of the gas chromatograph were as follows: column temperature 70°C for

1.5 min and increased to 130°C at a rate of 10°C per min, reaching the final temperature at 7.5 min; injector temperature 190°C; detector temperature 210°C; mobile phase: nitrogen at a flow rate of 9.5 mL/min; pressure: air 60 psi, N_2 70 psi, and H_2 40 psi.

2.8 Statistical Analysis

Data were tested in triplicate (viability and digestibility) and expressed as means ± standard deviation (SD); differences between samples were examined using Tukey's Kramer testing, and the differences with *P* = .05 were considered significant. Data were analyzed using the JMP version 8.0 software (SAS Institute, Cary, NC).

3. RESULTS AND DISCUSSION

3.1 Viability of Bacteria

The viability of A1D42 was conserved with the lyophilization method, nevertheless, at 90 days of lyophilized was reduced significantly (Fig. 1). The viability of refrigerated was affected significantly (*P* = .05) after 30 days (least of 18%), the viability of cryopreserved also was affected after 30 days, although it was maintained until the 90 days (65%).

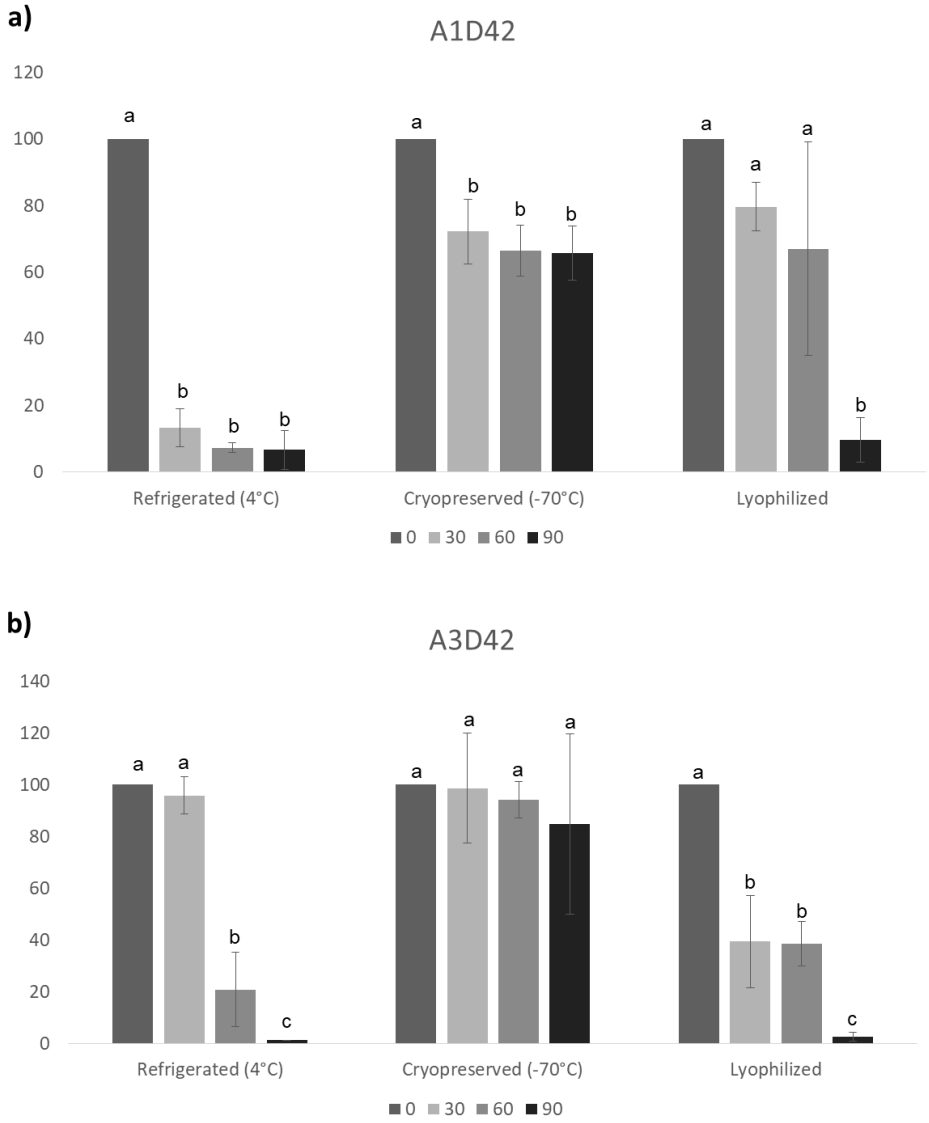

Fig. 1. Viability of BC A1D42 (a) and A3D42 (b) at 30, 60, and 90 days of different methods of preservation (% of the CFU/mL)

The data collected are the mean ± S.D. Different letters indicate differences (*P* = .05)

Unlike the A1D42, the viability of A3D42 was better cryopreserved than lyophilized and liquid (Fig. 1). Despite 95% of the liquid being maintained up to 30 days, at 60 days it was significantly (*P* = .05) affected (20%). Lyophilization was affected significantly after 30 days (39%).

The viability can be preserved over time by the lower temperature (freezing) or by the reduction of the available water (freeze-drying) [20] as can be seen in the Fig. 1. According to the results, it was evident that the refrigeration cannot be a good conservation method as was reported by Serna-Cock et al. [6], the viability of *W. confusa* (LAB) grown in MRS too decreased gradually, up to and including the six-weeks of storage period. Meanwhile, the viability of lyophilized strain persisted unchanged for six weeks, it was preserved throughout time.

Nevertheless, it is reported that slowly freezing produce outside ice that causes severe cellular damage, whereas fast freezing can decrease this cellular damage [8]. During the lyophilization process, the viability of the probiotic bacteria may decrease because the freezing step can inactivate the cell [21], although, 60-70 percent of cells that made it through this step will be able to withstand the dehydration procedure [22]. On the other hand, the drying process reduces bacterial cell bound water, causing damage to surface proteins, cell walls, and the cell membrane. The loss of water can cause the structural integrity of cellular components to be compromised, leading in function loss or deterioration [8].

On the other hand, the skim milk used as a cryoprotectant in this study has demonstrated a great capacity as a cryoprotectant in *Lactobacillus*, *L. lactis* ssp. Lactis CECT 5180 remained 44.3% cell viable [9], *L. salivarius* had 22.4% of cell viable [10], *Lactobacillus delbrueckii* subsp. *Bulgaricus* 86.53% mixed with glycerol, sorbitol, and sucrose [18] and *L. plantarun* mixed with sucrose, and trehalose [17]. The cryoprotect have the capacity to maintain the cell membrane components and form a porous structure in freeze-dried products that enables rehydration simpler [11]. Furthermore, proteins in skim milk form a protective layer around the cells during the process, preventing har [23].

Several factors influence the survival of probiotic bacteria in dry powders during the storage, including storage temperature, relative humidity, oxygen content, light exposure, powder moisture content and storage materials [20]. The viability of powder bifidobacterial probiotics during storage was greater at low temperatures (-18 ºC) than at 15 ºC, room temperature (20 ºC), and 25 ºC it was reported that the viability of probiotic storage at these temperatures decreased significantly the bacteria viable [24,25]. The above may explain that although freezing and freeze-drying can better preserve the probiotic over time, the viability of the probiotic decreases slightly after these processes. Moreover, the probiotic cellular oxidation of membrane fatty acids during storage is associated also with the decrease in viability [26]. Therefore, combining antioxidants with vacuum storage and regulating water activity should be a better and more effective way to preserve probiotics [27].

As a result, despite the minor damage and loss bacterial viability caused by challenges such cold, oxygen, and osmotic stress during drying and storage, freeze- drying can be employed for large-scale manufacture of probiotics powders [20].

In addition, due to better probiotic management, it is easier and feasible to manipulate dry powder than frozen, A1D42 was conserved appropriately lyophilized until 60 days maintaining its viability (67%), therefore, this lyophilized probiotic was selected to continue with the next analysis.

3.2 Digestibility of the Lyophilized Probiotic

All the concentrations of probiotics had higher percentage of digestibility than the control (*P* = .05). The digestibility of A1D42 at $4x10^{13}$ UFC/g concentrations had the greatest digestibility (66%), but it was no different with the A1D42 4x10 12 (Fig. 2).

Our results were similar to those obtained by Ridwan et al. [13], where the addition of LAB strain 32 *L. plantarum* in rumen fermentation resulted in digestibility of 65-75%.

Feed digestibility is an essential criterion to measure rumen fermentation, high feed digestibility indicates that the animal will receive more energy from the feed and metabolize it as a source of energy for the body and animal production. According to several studies, adding some LAB straight to the rumen fluid improves digestibility [12-14].

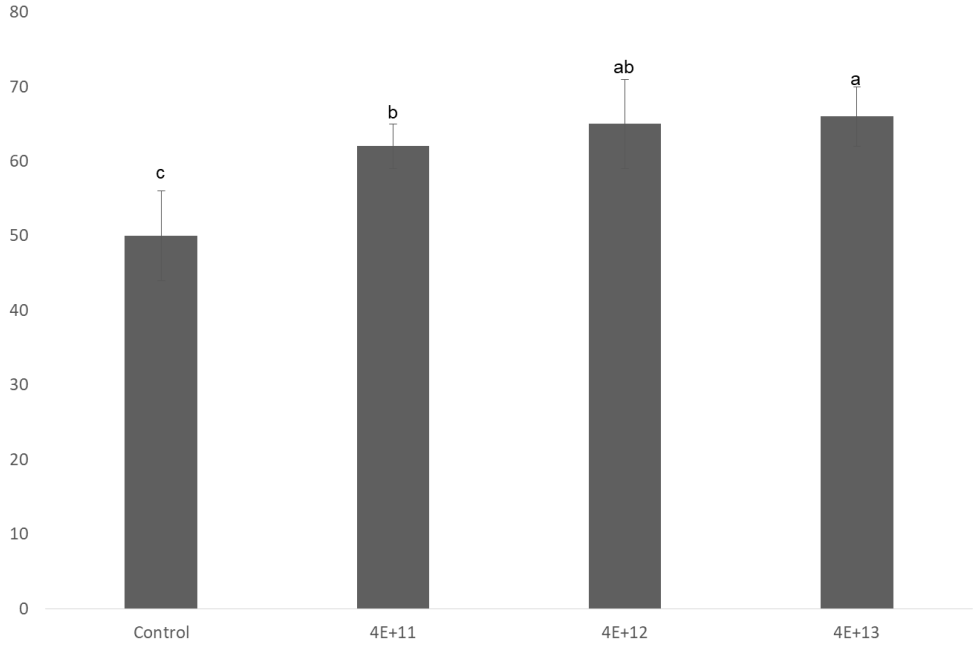

Fig. 2. Digestibility of A1D42 lyophilized probiotic at different doses
The data collected are the mean ± S.D. Different letters indicate differences (P = .05)

A study report that *L. plantarum* increased significantly the digestibility *in vitro* in rumen compared to the control. The fact that LAB can operate as a probiotic by promoting rumen bacteria activity demonstrates that it can act as a probiotic [14].

The effect of the probiotic on the increase of the digestibility is because the probiotic improves cellulolytic bacteria in the rumen [28] and increases the pH ruminal, consequently, increasing the fiber degradation [29]. Some LAB break carbohydrates and produce simple carbohydrates (monosaccharides) like glucose, which is essential to produce energy, then, the animal can make better use of the food, since improved the carbohydrates bioavailability [30].

Other LAB are reported to produce enzymes that help to digest the fiber carbohydrates, all of this improve animal performance, therefore, increasing the animal productivity [30,31].

3.3 Production of Volatile Fatty Acids (VFA's)

The acetic, propionic, and butyric acids are produced in higher concentrations during food fermentation in the rumen by the action of rumen bacteria. Therefore, it was thought that rumen probiotics could improve VFA production. However VFA concentrations remain similar to the control and were no observed increase with the addition of the probiotic or with the increase of the doses. Despite an increase in digestibility, the probiotic inoculation did not benefit the production of VFA (*P*=0.05, data not showed). Even decreased one VFA, acetate with $4X10^{11}$ and $4X10^{12}$ doses of probiotic.

Our results are similar to other studies that report no significant changes in VFA concentrations by adding probiotics in the rumen fluid. A study report that LAB has had no substantial effects on VFA concentration after 72 h of incubation at 37ºC in rumen compared to the control [14]. Another study also reported no significant increase of VFA concentration by LAB in comparison to the control [32]. The ruminal VFA concentrations were unaffected by a probiotic that contains *E. faecium* strain 26, *L. plantarum* strain 220, and *Clostridium butyricum* strain Miyari [33]. Nor was an increase in VFA production observed using *Prevotella bryantii* 25A as a probiotic in cows [34].

4. CONCLUSIONS

In summary, the viability was greater in cryopreserved and lyophilized than refrigerated in both probiotics. Nevertheless, lyophilized is easier to handle due to it being a dry powder, A1D42 lyophilized had better viability than A3D42 lyophilized, and this was maintained until

60 days. Moreover, A1D42 was shown to increase significantly the digestibility, which improved with increasing probiotic dosage up to 66%. The VFAs production did not augment the addiction to the probiotic.

Based on the results, it was concluded that lyophilization is a great conservation method for the production of rumen probiotics, which proved not to affect the properties and beneficial effects of the probiotic *in vitro*. Nevertheless, its preservation could be improved with the addition of antioxidants, with storage under vacuum at low temperature and controlling water activity.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s) (UNAM CICUAE.DC-2019/4-2).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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