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STABILITY AND PRESERVATION OF THE PROBIOTIC ADDITIVE WITH *LACTOBACILLUS PENTOSUS* LB-31 FOR ANIMAL PRODUCTION

ESTABILIDAD Y CONSERVACIÓN DEL ADITIVO PROBIÓTICO CON *LACTOBACILLUS PENTOSUS* LB-31 DESTINADO A LA PRODUCCIÓN ANIMAL

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The stability time and storage conditions of the probiotic additive with *Lactobacillus pentosus* LB-31 for animal production were determined. Completely random designs with six repetitions were used to monitor microbial viability for a month (0, 7, 14, 21 and 30 days) when the additive was stored under ambient conditions (24 ±2 ºC) and for six months (0, 30, 60, 90, 120 and 180 days) when stored under refrigeration (4±2 ºC). Cell concentration, pH and purity of the culture were also determined. The results showed that the lactic acid bacteria maintained its viability during the first 14 days of storage at room temperature. After this time, the microbial concentration decreased from 7.64 to 7.02 log cfu/mL ($p=0.0028$) and remained at 91 % viability until 30 days. Under refrigeration conditions, LB-31 was stably up to 60 days of storage with a concentration of $3.74x10^7$ cfu/mL, and subsequently decreased to 10^6 cfu/mL ($p<0.0001$) with a viability of 78 % at the end of the study. Under both conditions, the pH decreased and the colonies maintain their morphological and cultural characteristics. It is concluded that the probiotic additive with *Lactobacillus pentosus* LB-31 without preservatives is stable for 14 days at room temperature (24±2 ºC), and for 60 days under refrigerated conditions (4±2 ºC).

Key words: *animal feeding, bacteria, preservation*

Se determinó el tiempo de estabilidad y condiciones de almacenamiento del aditivo probiótico con *Lactobacillus pentosus* LB-31 destinado a la producción animal. Se utilizaron diseños completamente aleatorizados con seis repeticiones para monitorear la viabilidad microbiana durante un mes (0, 7, 14, 21 y 30 días) al conservar el aditivo en condiciones ambientales (24 ±2 ºC) y durante seis meses (0, 30, 60, 90, 120 y 180 días) cuando se almacenó en refrigeración (4±2 ºC). Se determinó, además, la concentración celular, el pH y la pureza del cultivo. Los resultados mostraron que la bacteria ácido láctica mantuvo su viabilidad durante los primeros 14 días de conservación a temperatura ambiente. Después de este tiempo, la concentración microbiana disminuyó de 7.64 a 7.02 log ufc/mL ($p=0.0028$) y se mantuvo en 91 % de viabilidad hasta los 30 días. En condiciones de refrigeración, LB-31 se comportó estable hasta los 60 días de conservación con una concentración de $3.74x10^7$ ufc/mL, y posteriormente disminuyó a 10^6 ufc/mL ($p<0.0001$) con una viabilidad de 78 % al finalizar el estudio. En ambas condiciones, el pH disminuyó y se comprobó que las colonias mantenían sus características morfológicas y culturales. Se concluye que el aditivo probiótico con *Lactobacillus pentosus* LB-31 sin conservantes es estable durante 14 días a temperatura ambiente (24±2 ºC), y por 60 días en condiciones refrigeradas (4±2 ºC).

Palabras clave: *alimentación animal, bacteria, preservación*

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Introduction

The use of microbial additives, such as probiotics, in animal feeding contributes to improved health and greater use of foods (Rondón *et al*. 2020 and Milián *et al.* 2022), which allows increasing production yields and, consequently, the availability and quality of milk, meat and eggs for the population (García *et al.* 2016). Animal studies use laboratory-grown microorganisms or commercial probiotics. The most important aspects for its production are the adequate selection of the strain or strains, the culture medium and the fermentation conditions that allow obtaining a high level of microbial viability during the process (FAO 2016 and Fenster *et al.* 2019). The stability of the probiotic additive during formulation and storage is an indispensable requirement for a successful commercial production (Ramlucken *et al.* 2021).

The selection of the microbial strain or strains is the first step in the design of a probiotic product. These should be generally recognized as safe (GRAS) microorganisms, able of surviving in the gastrointestinal tract and tolerating low pH and high concentrations of bile salts (Vinderola *et al.* 2017). Other desired characteristics are the ability of probiotic strains to adhere to the intestinal epithelium for subsequent colonization (Endo and Gueimonde 2016), being genetically stable microorganisms and having high growth rates. Furthermore, the chosen strain must maintain its viability and probiotic activity during the manufacturing, transportation and storage processes (Molina 2019 and Kieps and Dembczynski 2022).

In Cuba, the Instituto de Ciencia Animal (ICA) has been developing a research group for several years aimed at obtaining and evaluating probiotics with beneficial effects on the health and productive performance of animals. Based on the main results of these researchers, a group of strains was selected as those of greatest interest for the development of microbial additives and were deposited in the Microorganism Collection, belonging to the Bank of Microorganisms for Animal Production (BAMIAP) from ICA (Sosa *et al.* 2017). One of these strains is *Lactobacillus pentosus* LB-31, of avian origin, isolated from fermented excrement of broilers. The LB-31 strain showed the greatest probiotic potential in *in vitro* tests. Its beneficial action was confirmed in broilers (García *et al.* 2016), rainbow trout (García and Pérez 2015), growing pigs (Ayala *et al.* 2014) and pelibuey lambs (Gutiérrez *et al.* 2020).In addition, LB-31 was used as an additive to improve the protein content of mixed silages for ruminant animals (Rodríguez *et al.* 2020).

In recent studies, the process of obtaining the liquid probiotic with L. *pentosus* LB-31 was defined for its future production on an industrial scale (Sosa 2021). For this purpose, an economical culture medium was selected that can replace the traditional medium De Man-Rogosa-Sharpe (MRS, pH 6.2±0.2), designed by De Man *et al.* (1960), which is very expensive for use on an industrial scale. Different operating conditions were also evaluated in laboratory bioreactors and fermentation was scaled up to 30 L. Also, it was found that the new conditions for obtaining the additive did not affect the activity of the probiotic strain in broilers (Sosa *et al.* 2021). However, it is necessary to carry out stability and conservation studies of the additive to ensure high viability of the microorganisms during the storage process and, consequently, that they can preserve their probiotic effect on the host's gastrointestinal tract. For these reasons, the objective of this researcher was to determine the stability time and storage conditions of the probiotic additive with *Lactobacillus pentosus* LB-31 for animal production.

Materials and Methods

The experiment was conducted at the Food Production Laboratory of Instituto de Ciencia Animal. This center is located at km 47 ½ of the Central Highway, at 22º 53' north latitude, 82º 02' west longitude and 92 m o. s. l, in San José de las Lajas municipality, Mayabeque province, Cuba.

Design and experimental treatments: Completely random designs with six repetitions were used to evaluate the stability and conservation of the probiotic additive under ambient conditions (24±2 ºC) for a month (0, 7, 14, 21 and 30 d) and under refrigeration (4±2 ºC) for six months (0, 30, 60, 90, 120 and 180 d). A total of six samples were taken at each time for the analysis that subsequently was eliminated.

Microorganism and preparation of the probiotic additive:

The *Lactobacillus pentosus* LB-31 strain was used, belonging to the Bank of Microorganisms for Animal Production (BAMIAP) from Instituto de Ciencia Animal (Mayabeque, Cuba). The LB-31 was identified by sequencing of the 16S ribosomal RNA gene and its sequence is deposited in the GenBank with accession number: FR717464 (García *et al.* 2016). The probiotic additive was obtained from three fermentation batches in an 11 L bioreactor (BIONET, Spain) with an effective volume of 8 L of a culture medium designed with sugar cane molasses, urea, sodium acetate and ammonium citrate. The operating conditions of the equipment were established according to the methodology proposed by Sosa (2021).

Stability and preservation of the probiotic additive: A liquid culture from the *Lactobacillus pentosus* LB-31 strain with a concentration of $4.33x10^7$ cfu/mL was used. The additive was packaged in sterile 100 mL glass bottles with plastic tops and placed at room temperature and in refrigeration. Cell viability, purity and pH were determined. Samples were taken and serial dilutions were made in saline solution (0.85 %, w/v) and the culture was seeded on plates with Rogosa agar. These were incubated for 24-48 h to determine the concentration of viable cells

(cfu/mL) by visual counting of colonies. Gram stains were performed to check the purity of the culture and the pH was measured with a digital pH meter (Sartorius, Germany) with a precision of ±0.01 units.

Statistical analysis: The experimental data were processed with the Infostat statistical package (Di Rienzo *et al.* 2012). All variables fulfill the theoretical assumptions and followed a lognormal distribution. When necessary, Duncan (1955) multiple comparison test was used to discriminate differences between means at $p<0.05$.

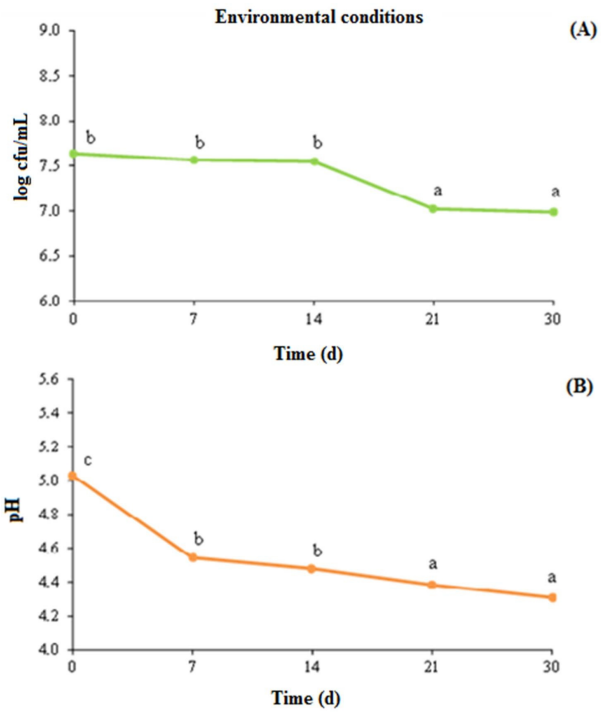
Results and Discussion

Figure 1 shows the performance of the microbial concentration (A) and pH (B) of the probiotic additive with *Lactobacillus pentosus* LB-31 during a month of storage at room temperature. There were no differences in the viability of lactic acid bacteria for the first 14 d of storage (figure 1A). After this time, the microbial concentration decreased from $4.33x10^7$ to $1x10^7$ cfu/mL (7.64 to 7.02 log cfu/mL) and remained at 91 % viability until 30 d. Regarding pH (figure 1B), there was a decrease at 7 d and from this moment on it remained stable.

Figure 2 shows the stability of the additive during six months of refrigerated storage. There were no differences in the concentration of lactic acid bacteria during the first 60 d of storage (figure 2A), since it maintained values of $4.33x10^7$ cfu/mL (7.64 log cfu/mL) and subsequently decreased to 10^6 cfu/mL (6.01 log cfu/mL) and reached 78 % viability at the end of the study. The pH decreased at 30 d and remained stable until 180 d (figure 2B).

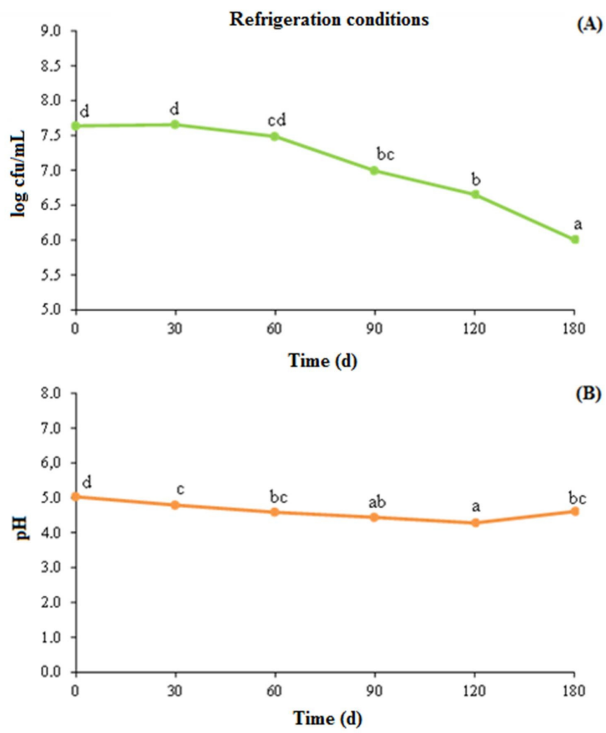
FAO/WHO (2002) suggested that probiotics should have a minimum concentration of 10^6-10^7 cells/mL or g of product to ensure their efficacy. This study shows that the additive with *Lactobacillus pentosus* LB-31 maintains an adequate concentration to be used as a probiotic and that, in addition, it is stable up to 14 d at room temperature ($4.33x10^7$ cfu/mL) and 60 d under refrigerated conditions ($3.74x10^7$ cfu/mL).

In both storage conditions, macroscopic and microscopic observations of the culture showed that the colonies maintained their morphological and culture characteristics. In addition, the presence of contaminants was ruled out (figure 3).



[a, b, c]Different letters per graph differ at $p<0.05$ (Duncan 1955)

Figure 1. Stability performance of the probiotic additive with *L. pentosus* LB-31 for 30 d at room temperature (24±2 ºC): (A) Microbial concentration (SE±0.13, p=0.0028) and (B) pH (SE±0.03, p<0.0001)



[a, b, c, d]Different letters per graph differ at $p<0.05$ (Duncan 1955)

Figure 2. Stability performance of the probiotic additive with *L. pentosus* LB-31 for 180 d at 4±2 ºC: (A) Microbial concentration (SE±0.44, p<0.0001) and (B) pH (SE±0.08, p<0.0001)

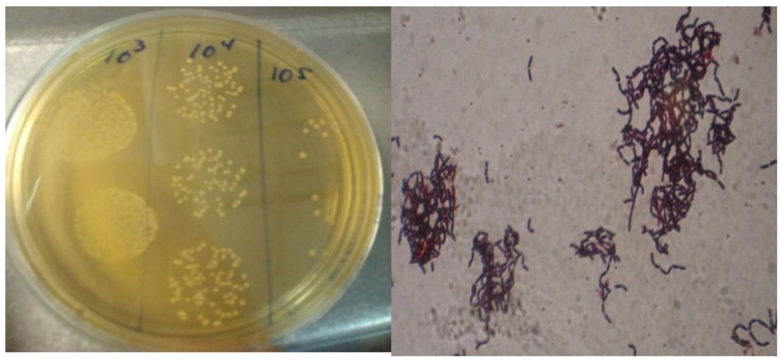


Figure 3. Macroscopic and microscopic observation of the purity of the probiotic culture with *L. pentosus* LB-31

Brizuela (2003) and Rondón (2009) evaluated the stability of probiotic additives with different strains of lactobacilli in glass bottles at room temperature and under refrigeration conditions for 180 d. Both researchers reported that the additives were stable up to 30 days, and that from this moment on the viability of the microorganism decreased under environment and refrigerate conditions.

The international scientific literature reports several stability studies of probiotic microorganisms using different types of substrates and stability times shorter than those of this study. Among them are those of dos Santos *et al.* (2019), who studied the stability of *Lactobacillus casei*, cultured in cocoa juice for the same time and under the same conditions, and reported that the microbial concentration decreased from 10^8 to 10^7 cfu/mL. Fernandes *et al.* (2019) evaluated the stability of *Lactobacillus rhamnosus* DTA 79 and *Lactobacillus paracasei* DTA 83 in skimmed milk at 20 and 40 d of storage under refrigerated conditions (7 °C). The mentioned authors highlighted that, although one of the strains decreased its viability at 40 d, both remained at a concentration higher than 10^8 cfu/mL. Likewise, Tavares *et al.* (2018) showed that a probiotic drink based on fermented corn with the commercial probiotic *Lactobacillus paracasei* LBC-81, individually and in co-culture with several yeast strains, maintained its viability for 28 d under refrigeration conditions (4 °C) and that the concentration was within the recommended range for its use as a probiotic.

International patent databases also protect stability studies of some liquid probiotic products. Patent ES2674353 T3 (2018), for example, deal with a method for preparing liquid starter cultures with high stability and fermentative activity. These cultures reach concentrations higher than 10^9 cfu/mL and are only stable for 6 d between 3 and 5 °C.

The results of this study are encouraging and comparable with other probiotic products preserved under refrigeration, which have shorter stability times than the additive with LB-31. It should also highlight that no preservatives were added to the probiotic additive that could improve the survival of LB-31. However, despite these advantages and the container-closure system used (glass bottles with plastic tops) is the most commonly used for laboratory studies (Rondón 2009, García *et al.* 2013 and Freire *et al.* 2017), it is necessary to evaluate other types of packaging that facilitate the transport and storage processes when this type of product is industrially produced. Another aspect to take into account is the storage temperature, since the average ambient value in Cuba is 30±2 °C and this study was carried out at lower temperatures, mainly due to the season of year and the climatic conditions where it took place. It should also be considered that several researchers show that fermentation conditions can affect the viability, stability and functionality of the strain (Farnworth and Champagne 2016 and Aragón *et al.* 2018), so all these parameters must be checked at each stage of the scaling process.

Conclusions

The probiotic with *Lactobacillus pentosus* LB-31, without preservatives, is stable in glass bottles for 14 d at room temperature and for 60 d under refrigerated conditions.

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