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Effect of Storage Temperature and Substrate on the Survival of Encapsulated *Lactobacillus acidophilus*

Efecto de la temperatura de almacenamiento y el tipo de sustrato en la sobrevivencia de *Lactobacillus acidophilus* encapsulado

Stalin Gustavo Santacruz Terán[1*]

[1]Universidad Laica Eloy Alfaro de Manabí, Manta, Ecuador.

* Corresponding Author: Stalin Gustavo Santacruz Terán. Universidad Laica Eloy Alfaro de Manabí, Facultad de Ciencias de la Vida y Tecnologías, Av. Circunvalacion, Manta, Ecuador.
stalin.santacruz@uleam.edu.ec

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Abstract: Lactic acid bacteria (LAB) are widely used in fermentation in the food industry and silage for animal feed. Environmental factors such as temperature affect the survival of microorganisms in food and silage. Encapsulation technologies preserve the integrity of encapsulated microorganisms, protecting them from adverse environmental conditions. The present work examined the effect of three storage temperatures, room (25 °C), refrigeration (4 °C), and freezing (-18 °C), in the presence of three substrates (glucose, whey, or distilled water) on the survival of alginate-encapsulated *Lactobacillus acidophilus* through CFU counting. The results showed that encapsulated *L. acidophilus* stored at 4 °C exhibited CFU values between 0.61 and 0.99 for 80 days, these being the highest, while room temperature obtained the lowest CFU, with values between 0.312 and 0.93. Encapsulated *L. acidophilus* in the presence of whey and glucose showed a higher number of CFU, with values between 0.53 and 1, throughout the storage time, compared to those in distilled water, whose values were between 0.3 and 0.99. Glucose and serum are suitable for growing encapsulated *L. acidophilus* at room, refrigeration, and freezing temperatures for 90 days of storage. Regardless of the culture medium, freezing temperature is appropriate for storing *L. acidophilus* for long periods.

Keywords: alginate, normalized CFU, storage time, encapsulation efficiency, glucose, whey.

Resumen: Las bacterias lácticas (BAL) son de amplio uso en fermentaciones de la industria alimenticia y en ensilajes para alimentación animal. Sin embargo, la supervivencia de los microorganismos en los alimentos y en ensilajes se ve afectada por factores ambientales como la temperatura. Por consiguiente, la exploración de tecnologías de encapsulación permitiría preservar la integridad de los microorganismos encapsulados al protegerlos de las condiciones adversas del entorno. En el presente trabajo se examinó el efecto de tres temperaturas de almacenamiento: ambiente (25 °C), refrigeración (4 °C) y congelación (-18 °C), en presencia de tres sustratos: glucosa, suero de leche o agua destilada en la supervivencia de *Lactobacillus acidophilus* encapsulado en alginato a través del conteo de las UFC. Los resultados mostraron que *L. acidophilus* encapsulado y almacenado a 4 °C presentó valores de UFC entre 0,61 y 0,99 durante 80 días, siendo estos los más altos, mientras que la temperatura ambiente presentó los menores números de UFC, con valores entre 0,312 y 0,93, siendo estos los más bajos entre las tres temperaturas analizadas. *L. acidophilus* encapsulado en presencia de suero de leche y glucosa mostró mayor número de UFC, con valores entre 0,53 y 1, a lo largo del tiempo de almacenamiento, en comparación con aquellos en presencia de agua destilada, cuyos valores estuvieron entre 0,3 y 0,99. La glucosa y el suero son los medios adecuados para el cultivo de *L. acidophilus* encapsulado a temperaturas ambiente, de refrigeración y de congelación durante 90 días de almacenamiento. Independiente del medio de cultivo, la temperatura de congelación es la adecuada para el almacenamiento de *L. acidophilus* durante largos períodos.

Palabras clave: alginato, UFC normalizadas, tiempo de almacenamiento, eficiencia de encapsulación, glucosa, suero de leche.

Introduction

Lactic acid bacteria (LAB) are widely used in fermentations in the food industry, in the preparation of probiotic foods (Vega, 2018), and in silages for animal feed (Okoye et al., 2023). Many of these products are preserved by freeze-drying and stored at refrigeration temperatures, requiring a high survival rate of microorganisms during storage. Probiotic food must contain enough live microorganisms to benefit the consumer's health (De Melo et al., 2018). The survival of probiotic microorganisms in food is affected by environmental factors such as temperature, pH, and oxygen (Kaur et al., 2021).

Microscopy studies show cell death during freeze-thaw results from cell membrane damage (Conrad et al., 2000). Therefore, membrane stabilization is vital for cell recovery after freezing and thawing. Typical compounds that minimize these effects (cryoprotectants) include dimethyl sulfoxide and methanol or larger molecules such as polyvinylpyrrolidone and hydroxyethyl starch (Meryman, 2007). The literature has shown that sugars stabilize cell membranes during freezing (Gómez-Fernández et al., 2012). The best cryoprotective effects are obtained using glucose among monosaccharides and the disaccharides with glucose in their composition.

Encapsulation technologies preserve the integrity of encapsulated materials or microorganisms, which are protected by a physical barrier that prevents their exposure to adverse environmental conditions. During the application of these techniques, liquids, solids, or microorganisms are trapped within a thin film of a food-grade microencapsulating agent (Chew et al., 2019). Silage storage is done under ambient conditions, at temperatures below or above 0 °C, depending on the geographical location and the season. It is, therefore, of interest to know the effect of storage temperature on the viability of LAB.

Lactobacillus acidophilus is widely recognized to have probiotic effects and is one of the most suggested organisms for dietary use (Altamirano-Ríos et al., 2022). The most common probiotics reported in yogurt and other fermented foodstuffs are *Lactobacillus, Escherichia, Bifidobacterium, Bacillus, Enterococcus, Streptococcus,* and some fungal *Saccharomyces* strains (Masoumi et al., 2021). Additionally, the extensive characterization of this microorganism makes this LAB a model system for many studies. These bacteria grow optimally between 37 and 42 °C (Midik et al., 2020); therefore, encapsulation appears as an alternative to maintaining viability at temperatures far from the optimum range. Studies of the viability of *L. acidophilus* have been performed using whey supplemented with protein extracts as a substrate during storage in freezing conditions (Burns et al., 2008); whey and storage at 10 °C for 28 days (Pescuma et al., 2010); glucose together with protein and ethanol in a digestion model (Matouskova et al., 2021). The effect of refrigerated storage temperature was studied at 4, 25 and 35 °C on the viability of *L. acidophilus* during 90 days (Soukoulis et al., 2013); temperatures of 4 and 20 °C for 180 days of storage (Laličić-Petronijević et al., 2015); and addition of *Lactobacillus rhamnosus* B 442 to ice cream stored in a freezer for 90 days (Kozlowicz et al., 2019). No studies have been conducted to compare glucose, whey, and water as substrates for *L. acidophilus* stored at refrigeration, freezing, and room temperature of 25 °C. Therefore, and based on previous information, the present work examined the CFU counting of encapsulated *L. acidophilus* stored under room (25 °C), refrigeration (4 °C), and freezing (-18 °C) conditions in the presence of three substrates: glucose, whey or distilled

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water. The present study may guide the selection of a proper storage temperature or substrate that could maintain a high survival of *L. acidophilus* during storage.

Materials and Methods

Frozen cultures of *L. acidophilus* were obtained from Chr. Hansen A/S (Denmark). LAB were thawed and activated in glucose solution (0.02 g/mL) for 24 h. Encapsulation was done with Shandong Jeijing Group Corporation (China) sodium alginate. Whey was obtained from an artisanal cheese production in Chone (Manabí province, Ecuador). The substrates for the encapsulation of LAB were 10 % (v/v) whey solution, 0.5 % glucose solution, and distilled water. Whey solution was prepared by diluting the whey 1:10 with distilled water.

Encapsulation of *Lactobacillus acidophilus*

The encapsulation of *L. acidophilus* was done according to Santacruz and Castro (2018). An experimental device of two cylindrical containers connected with a valve was used. The capsules were formed in the upper container by adding drop by drop 6 mL of a 1.8 % m/v sodium alginate solution to a mixture of palm oil (La Fabril, Ecuador) and 0.1 M calcium chloride solution (100:2 volumetric ratio of palm oil/0.1 M $CaCl_2$) which was kept stirred at 500 r.p.m. (overhead stirrer, Fisher Scientific BDC2002, Canada). The 1.8 % m/v sodium alginate solution contained 7×10^7 CFU/mL of *L. acidophilus* and the respective substrate (0.5 % glucose, 10 % (v/v) whey solution, or distilled water). Once the previously described mixture was shaken, the valve between the two containers was opened, allowing the capsules to pass into a lower container containing the $CaCl_2$ solution. The sedimented capsules were collected and stored at 4 °C (refrigeration), -18 °C (freezing), and 25 °C (room temperature) for 90 days. The viable cell was determined every ten days of storage, according to Zhao et al. (2015). Capsule size was determined by light microscopy (LaboMed, U.S.A.) with the aid of a grid slide, according to Colville and Bassert (2009).

Encapsulation Efficiency

The encapsulation yield (EY) of microcapsules was calculated according to Caceres de Menezes et al. (2019). Capsules were added to tempered (37 °C) peptone saline (1 g/L peptone, 8.5 g/l NaCl) and shaken for 30 min. EY was calculated through Equation 1.

$$EY = \frac{N}{N0} * 100\,\% \quad (1)$$

Where N and N0 are the number of viable cells released from the microspheres and the initial cell concentration used in the microencapsulation process, respectively. Viable cells were counted according to Zhao et al. (2015).

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Statistical Analyses

All measurements were performed in triplicate. ANOVA and Tukey's test, with a significance level of 5 %, were run using InfoStat statistics software (v. 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

Results and Discussion

The present work showed the encapsulation of *L. acidophilus* in capsules between 60 and 500 µm (Figure 1). Two groups of microcapsules (<100 µm diameter) and macrocapsules (>100 µm diameter) were obtained (John et al., 2011). Vemmer and Patel (2013) established that a high size distribution is expected for encapsulation based on emulsification. Encapsulation efficiency of cells was 49.6 %, which is similar to 54.5 % reported by Poletto et al. (2019) using Hi-maize starch as encapsulation material, whereas was lower than 72.5 % using pectin + whey protein concentrate (Caceres de Menezes et al., 2019) and 92 % using alginate + high methoxyl pectin (Motalebi et al., 2021). Different results may be due to different encapsulating materials or a different pH within the capsule, which may cause biological damage.

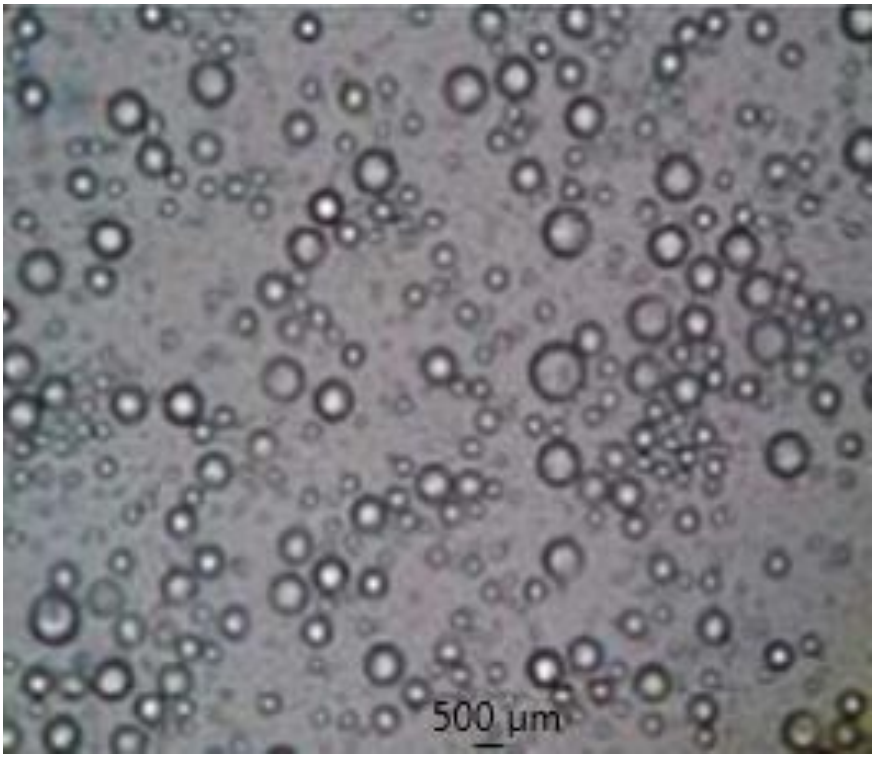

Figure 1. Microcapsules seen with an optical microscope, 10x.
Source: Author's archives.

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Figures 2a, 2b, and 2c show that the CFU of encapsulated *L. acidophilus* in the presence of any of the three substrates decreased along the storage time at the three storage temperatures. Encapsulated LAB stored at 25 °C and refrigerated (4 °C) in the presence of whey and glucose had higher CFUs along the storage time than those in distilled water (Figures 2a, 2b). Figure 2b also shows that the CFU of encapsulated LAB in the presence of whey in refrigeration conditions was higher than in glucose from day 50 to the end of storage. Refrigeration had the highest normalized CFU during 80 days of storage, with values between 0.61 and 0.99. In contrast, freezing had the highest normalized CFU after 90 days of storage for any of the three substrates (Table 1). Room temperature led to the lowest normalized CFU throughout the storage time, with values between 0.312 and 0.93. Storage of *L. acidophilus* at freezing temperatures (-18 °C) may be the best choice to keep a high CFU during 80 days of storage. This fact is supported by Marques Da Silva et al. (2018), who demonstrated that the stability of microencapsulated probiotics was superior at low temperatures.

Table 1. Normalized CFU of encapsulated *L. acidophilus* using either water, whey, or glucose as substrate stored for 90 days at room (25 °C), refrigeration (4 °C), and freezing (-18 °C) temperatures

Substrate	Temperature (°C)	Time (days)								
		0	10	20	30	40	50	60	70	80	90
Water	25	1	0.77 ± 0.035^{A}	0.73 ± 0.025^{A}	0.71 ± 0.000^{A}	0.61 ± 0.006^{A}	0.53 ± 0.000^{A}	0.48 ± 0.040^{A}	0.38 ± 0.006^{A}	0.30 ± 0.125^{A}	0.24 ± 0.050^{A}
Glucose	25	1	0.92 ± 0.000^{B}	0.89 ± 0.030^{B}	0.79 ± 0.025^{B}	0.72 ± 0.016^{B}	0.68 ± 0.100^{B}	0.66 ± 0.016^{D}	0.59 ± 0.050^{C}	0.53 ± 0.200^{C}	0.44 ± 0.195^{C}
Whey	25	1	0.93 ± 0.010^{C}	0.89 ± 0.015^{B}	0.83 ± 0.095^{C}	0.75 ± 0.010^{C}	0.71 ± 0.035^{C}	0.63 ± 0.025^{C}	0.61 ± 0.105^{D}	0.58 ± 0.000^{D}	0.53 ± 0.195^{D}
Water	4	1	0.99 ± 0.002^{E}	0.96 ± 0.002^{D}	0.93 ± 0.003^{E}	0.90 ± 0.000^{E}	0.78 ± 0.001^{D}	0.60 ± 0.001^{B}	0.55 ± 0.002^{B}	0.49 ± 0.014^{B}	0.39 ± 0.001^{B}
Glucose	4	1	1.02 ± 0.003^{G}	1.01 ± 0.002^{F}	1.00 ± 0.012^{H}	0.96 ± 0.002^{F}	0.91 ± 0.001^{G}	0.88 ± 0.005^{H}	0.82 ± 0.001^{G}	0.78 ± 0.000^{F}	0.64 ± 0.006^{E}
Whey	4	1	1.01 ± 0.005^{F}	1.00 ± 0.007^{E}	0.98 ± 0.002^{G}	0.98 ± 0.003^{G}	0.96 ± 0.004^{H}	0.93 ± 0.005^{I}	0.92 ± 0.003^{H}	0.87 ± 0.004^{G}	0.75 ± 0.004^{G}
Water	-18	1	0.98 ± 0.001^{D}	0.94 ± 0.001^{C}	0.92 ± 0.001^{D,E}	0.84 ± 0.014^{D}	0.82 ± 0.012^{E}	0.79 ± 0.000^{G}	0.71 ± 0.001^{F}	0.68 ± 0.005^{E}	0.68 ± 0.002^{F}
Glucose	-18	1	0.98 ± 0.003^{D}	0.95 ± 0.007^{C,D}	0.94 ± 0.005^{F}	0.90 ± 0.001^{E}	0.81 ± 0.001^{E}	0.68 ± 0.006^{E}	0.64 ± 0.003^{E}	0.61 ± 0.001^{D}	0.86 ± 0.001^{I}
Whey	-18	1	0.99 ± 0.002^{E}	0.95 ± 0.001^{C}	0.91 ± 0.002^{D}	0.89 ± 0.002^{E}	0.85 ± 0.002^{F}	0.75 ± 0.001^{F}	0.69 ± 0.002^{F}	0.66 ± 0.001^{E}	0.83 ± 0.012^{H}

Note. Different capital letter superscripts in a column indicate a statistically significant difference ($p < 0.05$). Normalized CFU values shadowed in darker colors are higher than those in lighter shades.
Source: Prepared by the authors.

The CFU of LAB encapsulated in water were the lowest compared to LAB encapsulated in whey and glucose throughout the entire storage. Pescuma et al. (2010) found a reduction of *L.*

acidophilus CFU along 60 days of storage at 25 °C and 105 days at both 4 and -18 °C, using a mixture of whey protein and pectin as substrate. Adding pectin to the whey protein concentrate led to a slower reduction of the CFU. Pectin stimulates the growth of *Lactobacilli*, causing a lower decrease in CFU (Islamova et al., 2017). Adding a solute such as whey to pure water resulted in lower water activity (a_w) of the solution (Di Giacomo et al., 2017).
Lower a_w during storage must have limited biological reactions, thus increasing bacterial stability during storage (Bodzen et al., 2021). Figure 1c shows no differences in the CFU of LAB encapsulated at freezing temperatures for any of the three substrates along the storage time. The cytoplasm of a cell remains unfrozen down to −15°C. Extracellular ice crystals usually form first during freezing, creating a gradient of osmotic pressure (Santivarangkna et al., 2011). This difference in osmotic pressure causes water outflow of the cell and cell dehydration (Bodzen et al., 2021). None of the three substrates may reduce cell freezing so that cell survival may be reduced. Microencapsulation of probiotic cells has been shown to preserve them from detrimental environmental factors such as cold shocks induced by process conditions such as deep freezing (Shah & Rarvula, 2000). No differences in survival in the presence of whey, glucose, or water in frozen conditions up to 80 days of storage were found, possibly due to the large size of the capsules. Microencapsulation of probiotic bacteria in beads with a diameter of about 20 μm can increase the viability of probiotics (Homayouni et al., 2008). Sheu and Marshall (1993) reported that a mean diameter of 30 μm for calcium alginate beads was desirable for frozen dairy desserts. Present results showed capsules above 60 μm, which may promote viability; however, a comparison of the survival of encapsulated and no encapsulated cells under the same storage conditions is needed.

Figure 3a shows that CFUs of encapsulated *L. acidophilus* in the presence of water decreased more rapidly than glucose and whey at the three storage temperatures (room, refrigeration, and freezing). There was no difference in the CFUs of encapsulated LAB under refrigeration and freezing conditions until day 40. The CFU of LAB stored in freezing conditions was higher than in refrigeration from day 50 to the end of storage. Figures 3b and 3c show that CFUs of encapsulated *L. acidophilus* in the presence of glucose and whey were lower in freezing conditions than in refrigeration. The CFU of LAB stored at room temperature was the lowest among the three temperatures and for the three substrates. There were smaller differences in CFU among the three storage temperatures for whey and glucose compared to water.

Bacteria need a minimum of nutrients: water, a carbon source, a nitrogen source, and some mineral salts (Maier & Pepper, 2015). However, the present work suggests that glucose may be a proper medium for growing encapsulated *L. acidophilus* along 90 days of storage at room—, refrigeration, and freezing temperatures. Nutrients in whey and glucose may lead to those minor differences. Arepally et al. (2020) reported that encapsulated *L. acidophilus* stored at 4 °C had higher viability than 25 °C. The reduction in cell viability when increasing the storage temperature is mainly attributed to the oxidation of membrane lipids and protein denaturation, leading to the denaturation of macromolecules in bacterial cells (Arepally et al., 2020). Storage temperature is the critical parameter for cell survival, and the encapsulated bacteria stored above refrigeration temperature may increase the rate of metabolism and, hence, cause survival loss (Ranadheera et al., 2010).

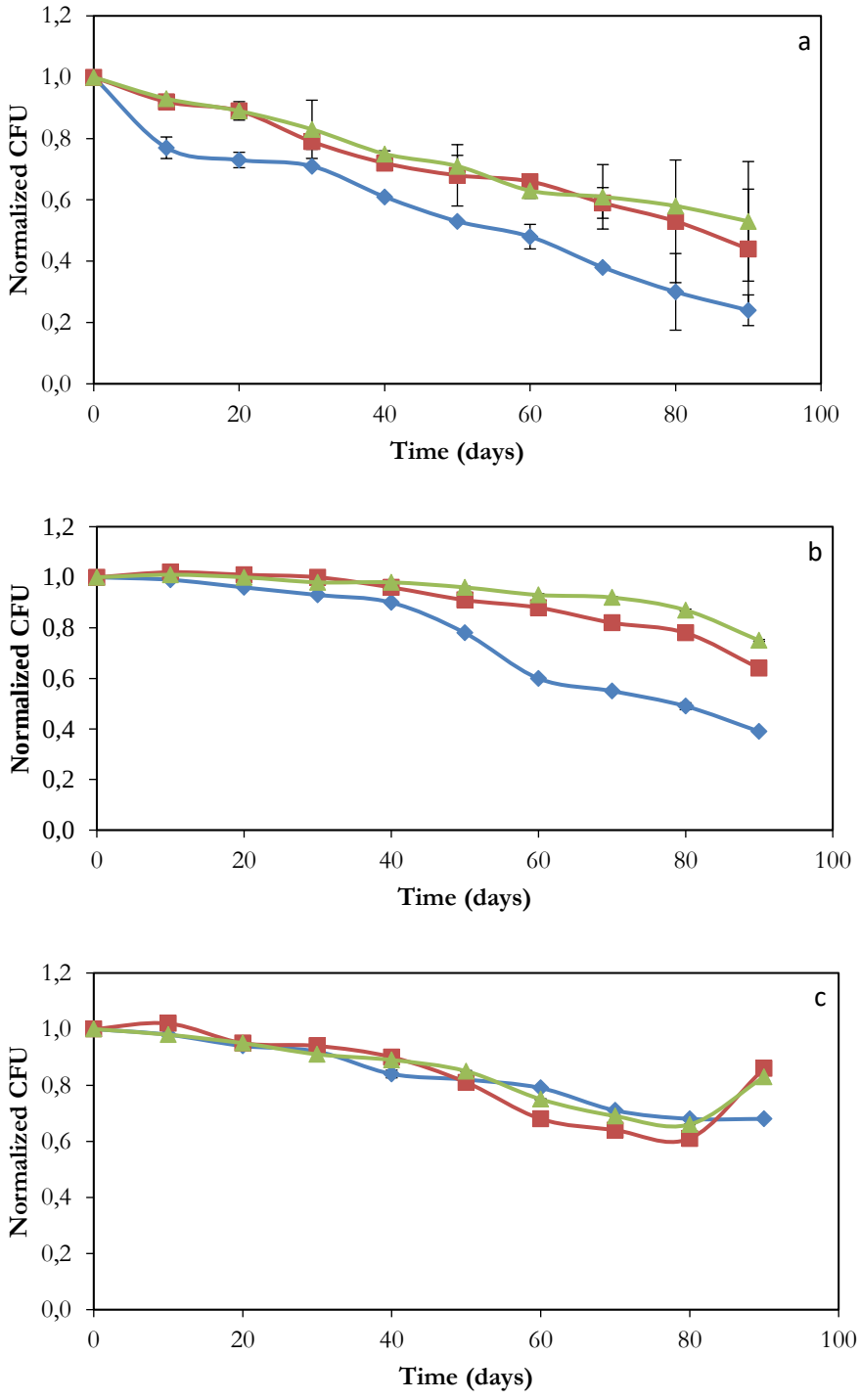

Figure 2. Normalized CFU of encapsulated *L. acidophilus* using either water ♦ whey ▲ or glucose ■ as substrate stored for 90 days at **a)** 25 °C **b)** 4 °C, and **c)** -18 °C.
Source: Prepared by the authors.

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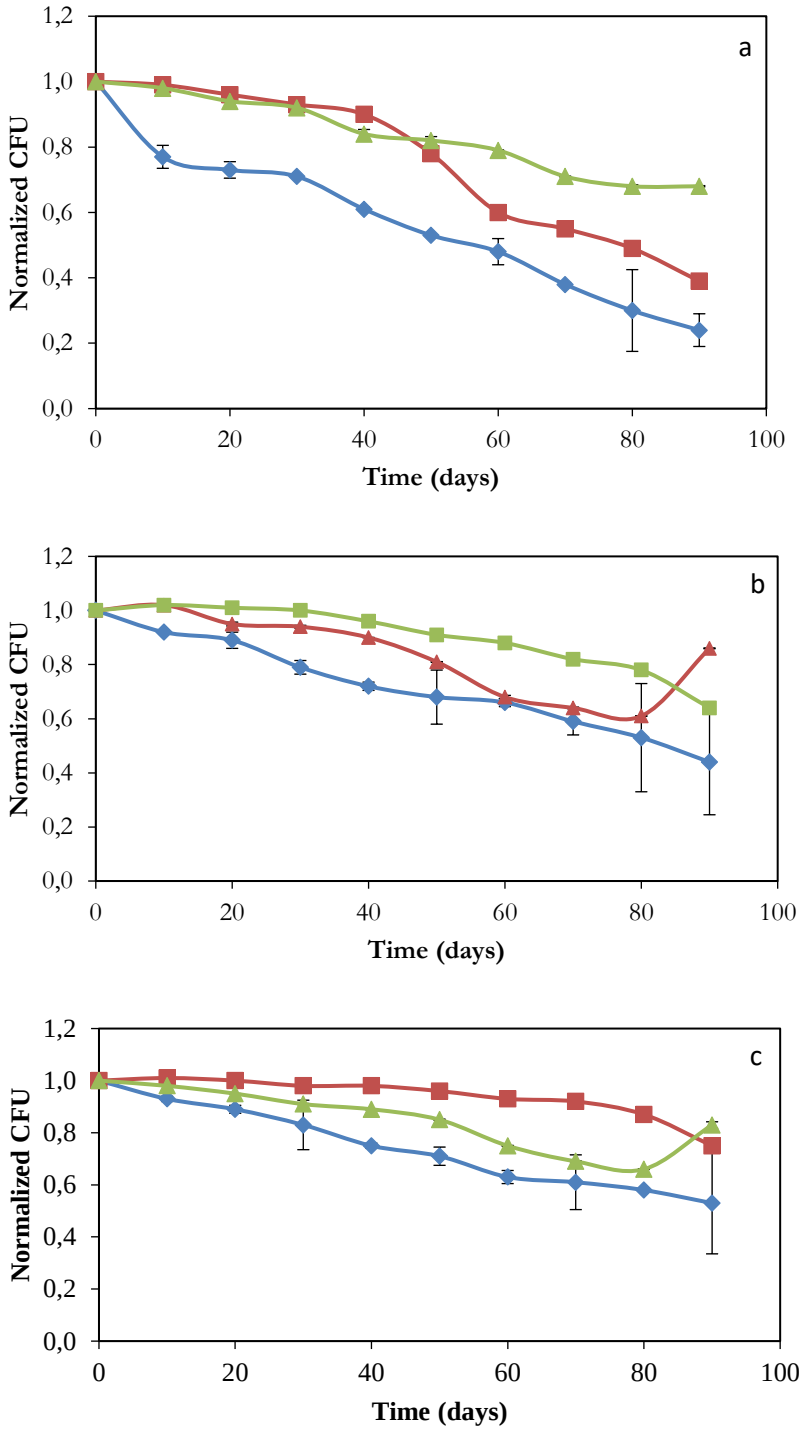

Figure 3. Normalized CFU of encapsulated *L. acidophilus* stored for 90 days at either 25 °C ♦ - 18 °C ▲ or 4 °C ■ using as substrate: **a)** water **b)** glucose, and **c)** whey.
Source: Prepared by the authors.

In the present work, pure substrates served as a food model and guided the development of fermented food. The storage of food containing LAB should be further studied to guarantee the high survival rate of the bacteria. More research is needed, including using different food (complex models), and the survival of encapsulated and non-capsulated bacteria should be studied.

Conclusions

Glucose is a proper medium for growing encapsulated *L. acidophilus* at room, refrigeration, and freezing temperatures for 90 days of storage. CFU counting of encapsulated L. acidophilus stored at -18 °C for 80 days showed the highest number, followed by refrigeration (4 °C) and room temperature (25 °C). L. acidophilus encapsulated in the presence of whey and glucose showed higher CFUs during storage compared to those in distilled water.

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Authors' Contributions

Stalin Santacruz made contributions in design and development of the project, methodology, data collection and analysis, and writing of the manuscript.

Ethical implications

The author declares that this article has no ethical implications.

Conflict of interest

The author declares no conflicts of interest in this study.

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