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Microencapsulation of *Limosilactobacillus reuteri* (DSM 23878) for application in infant formula: Heat resistance and bacterial viability during long-time storage

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ABSTRACT

This study aimed to evaluate the survival capacity of the probiotic culture *Limosilactobacillus reuteri* (DSM 23878) to microencapsulation by spray drying, and its potential as component of an infant formula. Preliminary tests were performed between skim milk (SM) and infant formula (IF) as wall material and two inlet temperatures, evaluating the encapsulation efficiency, moisture content, water activity and stability, to choose the drying parameters. After drying in optimized conditions, the powder of microencapsulated *L. reuteri* was characterized and the viability after dilution in an infant formula at 70 °C was determined. In addition, the survival rate throughout 360 days of storage was assessed. As results, encapsulation efficiency was superior to 90 % in both wall materials. However, the use of IF as for microencapsulation produced microparticles with lower water activity (Aw) and moisture, as compared with the SM. Final microparticles produced with IF as wall material presented values of Aw, moisture content, and particle diameter averaged 0.11 ± 0.02, 2.10 ± 0.35 % and 10.30 ± 0.12 μm, respectively. The viability of microencapsulated *L.reuteri* decreased 1 Log CFU/mL after dilution at 70 °C and the powder maintained a survivor of 73.5 % after 365 days of storage at 4 °C. Thus, the microencapsulation by spray drying under the conditions of this study proved to be an effective technique to protect the probiotic *L. reuteri* for application in infant formulas, obtaining an adequate number of viable cells after reconstitution at 70 °C and during long time the storage.

1. Introduction

Breast milk provides essential nutrients and bioactive compounds that contribute to the growth and immune development during childhood (Lyons et al., 2020). The World Health Organization (WHO) recommends children breastfeeding since the birth and for the first six months of life, followed by continued breastfeeding and other sources of energy nutrients to exceed what is provided by breast milk, (Salminen et al., 2020).

Healthy intestinal development is of great importance during childhood, contributes to the growth and development ensuring the correct digestion and absorption of nutrients (Indrio et al., 2022). The colonization of the gastrointestinal tract of infants is a critical determinant of the gut microbiota, which establishes a critical interaction between food antigens and the external environment.In certain cases, and when breast milk is not available, the use of probiotics can confer health benefits to the host, such as the reduced risk of gastrointestinal infections, reduced antibiotic-associated diarrhea, lower frequency of colic, and infant irritability (Epifanio, 2012; Salminen et al., 2020).

The effectiveness of the addition of probiotics to infant formula is not yet conclusive. However, studies have demonstrated the health benefits to infants in large randomized controlled trials, showing the effectiveness of probiotics in treating acute viral gastroenteritis and preventing antibiotic-associated diarrhea in healthy children (Kumar et al., 2021). In addition, these organisms have been shown to reduce necrotizing

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enterocolitis (NEC) in premature infants (Beghetti et al., 2021). In 2011, the ESPGHAN Nutrition Committee published a systematic review of studies related to infant formulas supplemented with probiotics and prebiotics (Braegger et al., 2011). The study showed an absence of reports about the adverse effects of the administration of infant formulas containing probiotics and prebiotics. The review study showed that clinical effects attributed to a product containing a particular probiotic strain be extrapolated to other products (Rodrigues de Sá et al., 2017). However, if recognized the benefits of specific probiotics, the demand for the use of probiotic-supplemented formulas will increase (Ackerberg et al., 2012; Kent and Doherty, 2014).

The Brazilian National Health Surveillance Agency (ANVISA) has published Resolutions 43, 44, and 45 in 2011, which provides the technical regulations for formulas for newborn babies (0–6 months), follow-on formula for babies six months of age or over, and infant formulas intended for specific dietary needs, respectively. Following WHO (2006) guidelines for reduction of risks associated with *Enterobacter sakazakii* and *Salmonella* these regulations require labeling information about the preparation of the product with water at a temperature of no less than 70 °C, which impairs the addition of probiotics to the formulas since the thermo-resistance of cultures intended for infant use does not reach this temperature. In this context the spray drying technology for microencapsulation of probiotic bactéria, has been explored achieving positive results (Ilha et al., 2015; Etchepare et al., 2019), and can be an alternative to maintain the number of viable microorganisms necessary at high temperatures and long storage periods.

In particular, the probiotic culture *Limosilactobacillus reuteri* LRE 02 (DSM 23878), which is recognized as safe for infants, and is commercially available for infant supplementation in the form of a probiotic strain. Some health benefits associated with *L. reuteri* LRE 02 (DSM 23878) include modulation in the gastrointestinal tract of infants, suppression of diarrhea and intestinal inflammation, and protection against pathogenic microorganisms (Mu et al., 2018). In this context, this study aimed to evaluate the *L. reuteri* LRE 02 (DSM 23878) survival to microencapsulation by spray drying using skim milk and an infant formula as wall material, and determine the stability for long time store and viability after reconstitution at 70 °C.

2. Materials and methods

2.1. Preparation of the probiotic culture

The culture *L. reuteri* LRE 02 (DSM 23878) was obtained from Probiotical (Novara, Italy) and maintained at −20 °C prior use. For reconstitution, the culture was grown in 0.1 % v/v MRS broth (Merck, Darmstrat, Germany) for 18 h at 37 °C. After this time, the medium was centrifuged at 4677 g and 4 °C for 12 min, and the pellet was washed with saline solution (0.85 %) and centrifuged. This procedure was performed twice. The cell concentrate was dispersed in the encapsulating solutions to achieve a final concentration of around 10.5 Log CFU/mL.

2.2. Selection of encapsulating materials and process temperatures for spray drying

Two types of encapsulating materials were evaluated, skim milk (SM) powder (Piracanjuba - Brazil) and infant formula (IF) Aptamil 1® (IF, Danone - Argentina), both at 20 % (w/v) concentration, as well as two inlet temperatures (130 and 170 °C) in the spray dryer. According with suppliers the SM contains 50.2% of carbohydrates (mainly lactose), proteins (35.2%) and no lipids; for the IF the main components reported were a 53.1% of carbohydrates (only lactose), 9.8% of proteins (casein and whey proteins 40/60), 25.9% of lipids (vegetal fat), 5.8% of prebiotic fibers and 3.5% of linoleic acid. Before their use in the drying process, the wall agents were diluted in distilled water and autoclaved at 112 °C for 10 min. For the microencapsulation process, the cell concentrate was added to the SM or IF solutions, and the mixtures were subjected to drying in an spray dryer (B290, Büchi, Switzerland) with a 1.5 mm diameter atomizing nozzle, and inlet temperatures of 130 and 170 °C. The outlet temperature was controlled below 75 °C via the aspirator and pump parameters. After the production of the microparticles, they were packed in hermetic glass vials to avoid humidification, sealed and stored in the dark for the stability test, carried out in incubator chambers at 4 °C and 25 °C.

2.3. Encapsulation efficiency

The encapsulation efficiency (EE %) was defined as the percentage of viable cells in the microparticles after the microencapsulation process, compared to the viability of the culture in the liquid encapsulating materials before spray drying, according with the Equation (1) (Etchepare et al., 2019).

$$EE\% = \left(\frac{logN}{logN_0}\right)x100 \quad (1)$$

EE = encapsulation efficiency in %.
N_0 = number of viable cells in the encapsulating matrix before spray drying.
N = number of viable cells in the powder.

2.4. Enumeration of L. reuteri and survival rate

For viable counts determination of *L. reuteri* (DSM 23878) in the microparticles, 1 g of microparticles were rehydrated with 9 mL of 0.1 % peptone water at room temperature for 30 min, as described by Picot et al. (2004), followed by serial dilution and surface plating in MRS agar (Kasvi - Spain) with 0.05 % cysteine (Inlab - Brazil) and incubation in anaerobiosis at 37 °C/72 h. The bacterial count was expressed in Log of CFU/g. The survival rate was calculated by the number of viable cells during storage according to Equation (2) (BAO et al., 2010). Each experiment was conducted in triplicate.

$$SR\% = \left(\frac{logN}{logN_0}\right).100 \quad (2)$$

SR% = survival rate %.
N_0 = number of viable cells before the drying.
N = number of viable cells in the powder.

2.5. Moisture content and water activity

The moisture content in the powders was determined by oven drying at 105 °C, according to the methodology of the Adolfo Lutz Institute (IAL, 2008). The water activity (aw) was determined using a digital water activity meter AquaLab® (São José dos Campos, Brazil) soon after the samples were collected from the spray dryer.

2.6. Particle size distribution and morphology of the microparticles

The mean diameter and size distributions of the dried microparticles were determined by laser diffraction in the LV950-V2 particle size analyzer (Horiba, Kyoto, Japan). The samples were dispersed in absolute ethanol and placed in the chamber of the equipment until suitable transmittance indices were reached. The average particle size was expressed as the average diameter (D50) and the polydispersity index (span) was calculated using Equation (3).

$$Span = \left(\frac{D90 - D10}{D50}\right) \quad (3)$$

where D10, D50, and D90 are the diameters corresponding to 10 %, 50 %, and 90 % of the cumulative distribution.

The morphology and microstructure of the samples were evaluated by light microscopy.

2.7. Viability of L. reuteri after thermal reconstitution

Thermal resistance was assessed by mixing 2.5 g of the microparticles into 25 g of infant formula and homogenized in a Schott bottle. Then, 150 mL of water at 70 °C was added and shaken by 1 min. This mixture was finally cooled under running water until reaching 43 °C (temperature of the feeding bottle). Then, serial dilutions and plating were performed using the pour plate technique on MRS agar and incubation in anaerobiosis at 37 °C/72 h for quantification of viable *L. reuteri* (DSM 23878), a sample prior heating was taken in order to calculate the SR%, as described above. This method was used for simulating the standard recommendation of infant formula reconstitution.

2.8. Stability to storage

The stability of the microparticles during storage was evaluated in the preliminary tests for 140 days at 4 °C. In the second step of the study, after selection of wall material for the microparticles production, the stability of the microparticles was evaluated for 360 days under two different conditions: refrigeration temperature (4 °C) and room temperature (25 °C), maintaining the samples in packet in sealed glass flacks and absence of light during the storage test. The survival rate was calculated to the Equation (2), using N_0 as the number of viable cells in the powder before storage and N as the number of viable cells in the powder at the evaluation period. Each experiment was conducted in triplicate.

2.9. Statistical analysis

The results of Aw, M %, EE %, and SR % were analyzed by analysis of variance (ANOVA) and Tukey's test, considering 95 % confidence level (p less than 0.05) using the software Statistica V.12.

3. Results and discussion

3.1. Encapsulation efficiency, water activity, moisture contents of microencapsulated L. reuteri (DSM 23878)

Microencapsulation of L. *reuteri (DSM 23878)* using SM and IF as a wall material resulted in a high yield of microencapsulation (Table 1) of around 85 % for skim milk subjected to 130 °C and 170 °C, and EE of around 83 % in the infant formula at the same temperatures, however, no significate difference ($p \geq 0.05$) was observed between the samples. These results were in agreement to previous studies using skim milk alone or in combination with other wall materials for probiotic bacteria encapsulation. Maciel et al., (2014) reported an encapsulation yield of 77.7 % in the microencapsulation of *Lactobacillus acidophilus* La-5 using SM (30 % total solids). In another study using *L. acidophilus* La-5, Corti et al., (2017) reported a maximum encapsulation yield of 87.6 % using the mixture SM and maltodextrin. On the other hand, different to our results, Gul (2017) reported an EE of 94.07 % using SM (30 % total solids) in the encapsulation of *L. casei* Shirota by spray drying, and in the study conducted by Ilha and co-workers (2015) was found an encapsulation yield of 93.12 % for *L. paracasei* encapsulated in SM reconstituted and cheese whey (40:60 vol ratio). Although the diverse reports of probiotic encapsulation using SM, in our knowledge, this is the first report probiotic bacteria encapsulation using IF as a wall material, and due to its similar composition of protein and carbohydrates as compared with de bovine SM, was expected similar results of EE. Despite to the lack of knowledge in relation of the mechanism of protection, was suggest that the effectiveness of dairy wall materials in protecting cell viability during drying is close related to the presence of lactose and milk proteins, which interact with the bacterial membrane, preventing the leakage during water removal (Garcia, 2011).

Table 1
L. reuteri (DSM 23878) encapsulation efficiency (EE %), water activity Aw and moisture (M %) using two types of encapsulating materials at two inlet temperatures.

Treatment	SM130[1]	SM170[1]	IF130[2]	IF170[2]
EE (%)	85.86 ± 1.55[a]	85.04 ± 1.36[a]	83.76 ± 1.43[a]	83.28 ± 0.99[a]
Aw	0.172 ± 0.064[b]	0.279 ± 0.084[a]	0.115 ± 0.027[b]	0.104 ± 0.033[b]
M %	4.20 ± 0.78[a]	5.80 ± 1.26[a]	1.82 ± 0.27[b]	1.73 ± 0.39[b]

1 SM130: reconstituted skim milk microparticles subjected to 130 °C; SM170: reconstituted skim milk microparticles subjected to 170 °C.
2 IF130: infant formula microparticles subjected to 130 °C; IF 170: infant formula microparticles subjected to 170 °C.
Values are the mean ± standard deviation of 3 (three) independent experiments. Different superscript letters denote significant differences ($p < 0.05$) between all samples.

Table 1 also presents the results of water activity (Aw) and moisture contents (M %) of the microparticles in the preliminary tests. The Aw values were below 0.175 for the IF in both inlet temperatures and for SM at 130 °C with no statistic difference; the sample using SM and dried at 170 °C (SM170) presented a higher Aw value (0.279). Similar values of Aw were found by Dianawati et al. (2016b) and Fritzen-Freire et al. (2012), who reported water activity values from 0.073 to 0.406 for spray drying of probiotic bacteria using fixed inlet temperatures of 120 °C and 150 °C, respectively, with the same outlet temperature of 55 °C; both works reported the best bacterial viability in the powders with lower values of Aw. Samples prepared with IF presented water activity below 0.2 at both temperatures, indicating good stability of the dried product, that can help to prolong the shelf life of encapsulated probiotic bacteria due to the limitation of water available for microbial multiplication In addition, was reported that, values between 0.11 and 0.23 prevent cell death during storage (Chávez and Ledeboer, 2007; Dianawati et al., 2016a). In this regard, Weinbreck et al. 2010 reported that water activity values > 0.7 impaired the stability of encapsulated *L. rhamnosus* GG, reducing the number of viable cells (>10 log10) within two weeks, confirming that water activity has a strong correlation with the viability of spray-dried probiotic bacteria.

Concerning the moisture content (M %), significant differences were observed between different formulations (SM and IF), with no difference in the different inlet temperatures with the same wall material. Higher moisture values were found in the SM microcapsules (Table 1), moisture of 5.80 % and 4.20 % for inlet drying temperature of 130 and 170, respectively. On the other hand, for IF the moisture was around 1.8 % for the two temperatures. Guergoletto et al. (2017) reported moisture contents between 2.7 and 4 % in spray-dried *L. reuteri* LR92 using different encapsulating materials and Ilha et al., (2015) also reported values close to 4 % in dried *L. paracasei* using SM as wall material, both works obtaining satisfactory viable cell stability after drying. The reason for the lower values of moisture and Aw in the IF powders could be related to the higher amount of carbohydrates and the presence of prebiotic fibers (around 9% more than SM). Low molecular weight carbohydrates provide stabilization by closely interacting with the lipid bilayer of the cell membranes (Perdana et al., 2014); in addition, Fritzen-Freire and coworkers (2012) reported that SM containing prebiotics, used as wall material for encapsulation of *Bifidobacterium* BB-12, resulted in lower values of Aw and moisture as compare with the single SM. The moisture content of dried probiotic cultures must be properly controlled in order to achieve long-term storage stability and is recommended to set below 5 %, due to less water will be available for degradative reactions and the solubilisation/mobility of components in the formulation (Peighambardoust, Tafti & Hesari, 2011). In this context, moisture content values close to 2% were reported for spry drying encapsulation of probiotic bacteria *Lactobacillus reuteri* and *Lactobacillus rhamnosus*, LGG, with no degradation of the bacterial viability, on the contrary, the survivor rates and storage stability were superior in the samples with lower moisture content (Schell & Beermann, 2014; Sohail et al., 2013). In addition, based in the data, we also

conclude that the inlet temperature (130 °C or 170 °C) not affect the physico-chemical parameters of the IF powders. In the case of SM powders, the inlet temperature of 170 °C result in a slightly higher Aw as compare with the process at 130 °C. Thus, the differences on the physico-chemical parameters of the powders are mainly related to the composition of the wall materials.

3.2. Survivor rate during storage

The survival rates (SR) of *L. reuteri* (DSM 23878) encapsulated with SM and IF at inlet temperatures of 130 °C (SM130) and 170 °C (SM170) were evaluated during 140-day storage. As shown in Table 2, the *L. reuteri* maintained high values of SR (around 90 %) after 75 and 140 days of storage at 4 °C. The SR mean results were analyzed for the effect of the same temperature for different formulations, and different temperatures for the same formulation, with no significant difference ($p \geq 0.05$) between the treatments. High levels of SR during storage were reported by Gul (2017), according with the author, encapsulated *L. casei* Shirota (with SM as wall material), and loss less than 5 % of SR after 60 days under refrigerated conditions and at 25 °C. Maciel et al, (2014) also reported SR superior to 90 % using SM for *L. acidophilus* La-5 encapsulation, after 90 days of storage at 4 °C. Considering the stability of dried *L. reuteri* free cells is expected a rapid decrease of viability without encapsulation, in fact, a recent work has reported survival losses of around 87% after 60 days of storage at 4 °C, the authors used an inlet temperature of 120 °C and outlet temperature of 75 °C as spray drying main parameters for *L. reuteri* TF-7 (Puttarat et al., 2021).The viability of microencapsulated cells during storage depends on diverse factors such as number of irreversible damaged cells during drying, presence of oxygen, storage temperature and humidity (Morgan et al., 2006). Therefore, the high SR obtained in this work could be attributed to several factors such as the protective mechanisms of SM and IF during storage, reducing the rate of water loss from the intermediate environment and some intrinsic resistance characteristics of the strain (Abe et al., 2009).

Based on the data of EE, SR%, Aw and M%, both encapsulating materials were able to protect the microorganism from heat during drying and storage. However, the microencapsulation processes using infant formula as a wall material exhibited lower moisture content and water activity, that could be an advantage in relation to the SM. Based in these results and due to the IF encapsulation do not present significant differences in during the drying at 130 and 170 °C, this wall material was chosen for additional analysis of stability.

3.3. Additional analysis

After evaluating all results and considering the possibility of better stability, the infant formula was considered for an additional set de analysis. Therefore, a novel batch of spray dried *L. reuteri* was carried out with inlet temperature of 170 °C (this temperature was chosen due to is closer to the values used in the industry) and flow rate adjusted to keep the outlet temperature at 75 °C. This new probiotic powder production was characterized and their resistance to hydration at 70 °C and long-time storage stability in different temperatures was assessed.

Table 2
Survival rate (SR %) of different formulations of encapsulated *L. reuteri* (DSM 23878) at 75 and 140 days of storage at 4 °C.

L. reuteri counts (CFU/g)[1]								
	SM130[2]		SM170[2]		IF130[2]		IF170[2]	
Time (days)	75	140	75	140	75	140	75	140
SR(%)	95.3 ± 5.47[a]	95.0 ± 2.87[a]	95.0 ± 5.36[a]	93.9 ± 5.58[a]	92.9 ± 3.65[a]	89.1 ± 3.13[a]	92.8 ± 5.37[a]	90.7 ± 6.33[a]

1 CFU/g: colony-forming units per gram of sample.
2 SM130: reconstituted skim milk microparticles subjected to 130 °C; SM170: reconstituted skim milk microparticles subjected to 170 °C; IF130: infant formula microparticles subjected to 130 °C; IF 170: infant formula microparticles subjected to 170 °C.
Values are the mean ± standard deviation of 3 (three) independent experiments. Different superscript letters denote significant differences ($p < 0.05$) between all samples.

3.4. Physical properties of the microcapsules

The results of encapsulation efficiency, Aw and moisture in the second preparation are shown in Table 3. Small variations in the values were observed as compare with the first preparation, indicating well reproducibility in the drying processes, showing higher value of EE (90.41 %), and low values of Aw (0.112) and Moisture (2.10 %), which were close to the values obtained in the preliminary test within the range predicted in the preliminary tests, 83.28%, 0.104 and 1.73%, respectively.

3.5. Particle size distribution and morphology

The infant formula microparticles showed a mean diameter (D50) of 10.30 μm and polydispersity of 1.42 (Table 3). Fritzen-Freire et al., (2012) reported mean diameter ranging from 14.45 to 18.78 μm for spray-dried microparticles containing reconstituted skim milk and prebiotics such as inulin and oligofructose. Ilha et al. (2015) reported mean diameter value 10.96 μm when using SM as encapsulating material for *L paracasei* at 160 °C inlet temperature. Thus, our results of size and polydispersity are typical of spray-dried probiotic bacteria and are within the size range observed in the scientific literature for spray-dried microparticles.

Based on the optical microscopy (Fig. 1), the probiotic suspension was evenly distributed within the entire volume of the particle and exhibited a typical matrix structure. The microparticles produced were dense and spherical. The spherical shape is interesting because this format could facilitate the flow of material. The particle surface is very similar to other spray-dried samples reported in other studies (Fávaro-Trindade and Grosso, 2002; Fritzen-Freire et al., 2012; Alvim et al., 2016; Fadini et al., 2018), indicating well defined particle structure.

3.6. Thermal resistance of the microencapsulated probiotic culture

Besides of most of the thermal resistance test in microencapsulated bacteria are carried out by exposing the dry powders to different temperatures (generally 65 °C), during 30 to 60 min; in this work, the IF and the dried probiotic (using the same IF as wall material) was resuspended in water preheated at 70 °C, and cooled to 43 °C, as a simulated infant formula preparation (SIFP) before child consume, because it represent more closely the conditions of temperature, exposition time and Aw, to

Table 3
Physical properties of the microcapsules of *L. reuteri* (DSM 23878) produced with IF as wall material in the second batch production at 170 °C and 75 °C of inlet and outlet temperatures, respectively.

Infant formula microparticles	
Parameter	**Value**
EE (%)	90.41 ± 0.12
Aw	0.112 ± 0.016
M %	2.10 ± 0.35
SPAM	1.42 ± 0.25
D90	21.43 ± 2.77
D50	10.30 ± 0.12
D10	6.80 ± 0.05
SR% (SIFP)	92.75 ± 0.25

The values represent the statistical mean and standard deviations of at least three independent experiments. SIEP: Simulated infant formula preparation.

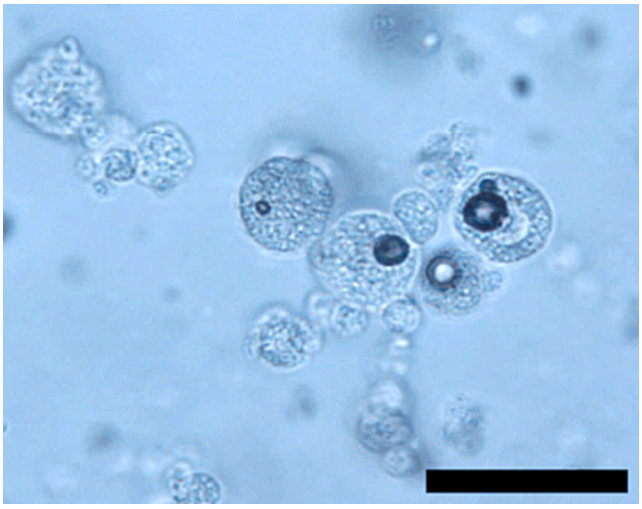

Fig. 1. Morphology by light microscopy of infant formula microparticles. Bar = 20 μm.

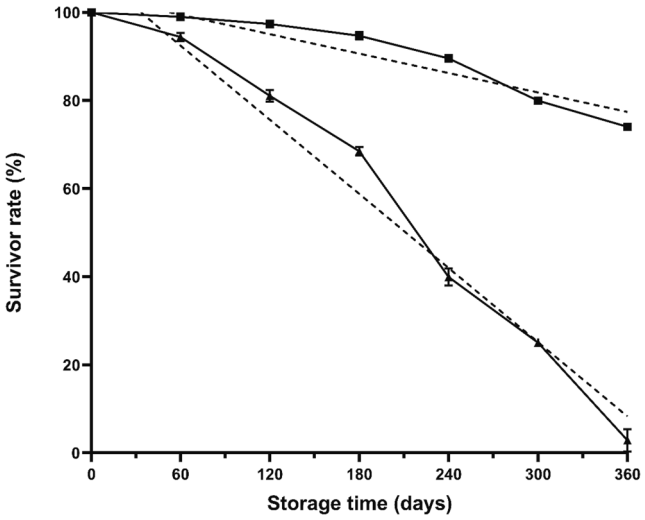

Fig. 2. Survival rate of *L. reuteri* (DSM 23878) using infant formula as wall material, during storage at 4 and 25 °C. Continued line wih a square symbol represents the SR% at 4 °C and the continued line with a triangle symbol is the SR(%) at 25 °C of storage temperature. Dotted lines represent the linear regression of each survival curve.

which the microparticles will be subjected. As results, the survival rate was up than 90 % (Table 3) and the number of viable cells was around 10^8 CFU/ml, which is the desired value for probiotic products for different authors (Brandão, 2014; Salminen et al., 2020; Tabbers et al., 2011), and within the recommendations of the Brazilian Health Regulatory Agency (Anvisa) in Resolutions 43 and 44 of 2011, which determined that the viable bacteria counts in infant formulas should be between 10^6 and 10^8 CFU/g of the product ready for consumption. The high SR% obtained could be the result of different factors such as composition of the dissolution media, low residence time at high temperature, and slow solubilization of the wall material, resulting in low exposition of the probiotic to harmful temperatures.

3.7. Stability of the microparticles under different storage conditions

The survival rates of microencapsulated *L. reuteri* (DSM 23878) in IF decreased from 10^{11} CFU/g to 10^8 CFU/g of viable cells (26 %) during 360 days of storage at 4 °C (Fig. 2). On the other hand, as showed in the same figure, at 25 °C the rate decreased around 97 %, during the same time of storage (360 days) indicating that refrigerated storage is the most suitable preservation of the spray-dried probiotic culture of this study, as it exhibited greater stability during storage. Using the data, was make a linear regression on the survival rate (SR%) along storage to determine the decay constant (k), as results were obtained values of $-0.280\ ^{day\text{-}1}$ ($R^2 = 0.969$) for the storage temperature of 25 °C and values of $-0.073\ ^{day\text{-}1}$ ($R^2 = 0.896$) for the storage at 4 °C. However, is important to note that, the microcapsules stored a 25 °C maintained a SR of around 70% (10^8 CFU/g) after 180 days of store. Similar decrease of probiotic survivor at 25 °C of storage were reported by others researchers (Ranadheera et al., 2015; Gul, 2017). This effect could be the result of higher reactivity and diffusivity of intracellular oxygen species and by accumulation of toxic waste resulting from increase of cell metabolic activity (Ranadheera et al., 2015); whereas at 4 °C, the cell viability decrease was probably due to nutrient limitation and changes in the Aw, which resulted from cell metabolic activity (Munoz-Celaya et al., 2012).

The recommendation of probiotic-supplemented infant formulas intake depends of well-designed and carefully conducted randomized controlled trials in infants, and also depends on the strain (Braegger et al., 2011). For example, for preterm infants, the Committee on Nutrition of the European Society for Pediatric Gastroenterology and Nutrition (ESPGHAN) Working Group for Probiotics and Prebiotics conditionally recommended the use of *L. rhamnosus GG* ATCC 53103 at a daily dose of 10^9 CFU and for *B. infantis* Bb-02 and *Str. thermophilus* TH-4, a daily dose of 3.0 to 3.5 × 10^8 CFU of each strain, as treatment for necrotizing enterocolitis (NEC) stage 2 or 3 in preterm infants (Akker et al., 2020). However, no recommendation was be made regarding the use of *L. reuteri* strains. Overall, in this work the particles during the storage at 4 °C losses less than 10% of viability until 180 days and present suitable counts for application in infant formulas until 300 days or 180 days, when stored at 25 °C, based in a criteria of the viable bacteria counts in infant formula between 10^6 and 10^8 CFU/g of the product ready for consumption.

4. Conclusions

The microencapsulation technique by spray drying proved to be effective to produce microparticles with high viability of *Limosilactobacillus reuteri* LRE 02 (DSM 23878). The 20 % reconstituted infant formula used as an encapsulating material, the inlet air temperature of 170 °C and outlet air temperature around 75 °C were adequate to protect the culture during spray drying, and the results of viability, water activity, and moisture contents were within the expected according to the literature. The microencapsulation using IF as wall material provided and longtime stability at 4 °C of storage and a thermo protective effect, enabling the protection of the probiotic after powder dispersion at 70 °C, thus being suitable for application in infant formulas, and application in other food products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Author's Contribution

MAGV and **IDA** conducted the analysis of characterization and stability in the microparticles formulations and wrote the original draft. **CMBP** was responsible by the data curation, manuscript revision and

edition. **GV, PBZRS, MIB and ATSeA,** were responsible for the project supervision, obtaining financial resources, revision, and edition of the final manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Ethics approval and consent to participate

This article does not contain any studies performed with humans and/or animal models.

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