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Survival and stability of *Lactobacillus fermentum* and *Wickerhamomyces anomalus* strains upon lyophilisation with different cryoprotectant agents

Raquel Facco Stefanello[a], Elizabeth Harumi Nabeshima[b], Beatriz Thie Iamanaka[b], Aline Ludwig[c], Leadir Lucy Martins Fries[a], Angélica Olivier Bernardi[a], Marina Venturini Copetti[a,*]

[a] Federal University of Santa Maria, Department of Food Technology and Science, 1000 Roraima Avenue, 97105-900 Santa Maria, RS, Brazil
[b] Institute of Food Technology, ITAL, 2880 Brazil Avenue, 13070-178 Campinas, SP, Brazil
[c] Federal University of Santa Maria, Mycological Research Laboratory, 1000 Roraima Avenue, 97105-900 Santa Maria, RS, Brazil

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ABSTRACT

The stability of microorganisms along the time is important for allowing their industrial use as starter agents, improving fermentation processes. This study aimed to evaluate the survival and maintenance of the cell viability of the lactic acid bacteria *Lactobacillus fermentum* IAL 4541 and the yeast *Wickerhamomyces anomalus* IAL 4533, both isolated from wheat sourdough, after lyophilisation with different cryoprotectant and storage at room temperature along a year. Treatments involved adding control solution (S1 = 0.1% peptone water), and four cryoprotectant solutions S2 (10% sucrose), S3 (5% trehalose), S4 (10% skim milk powder) and S5 (10% skim milk powder plus 5% sodium glutamate) to the microbial cells previously of freeze drying processing. To verify the effect of lyophilisation on the number of microbial cells recovered, microbiological analyses were performed and cell viability was calculated before and after lyophilisation and regularly during a storage period of 365 days at room temperature. Viability after freeze-drying was influenced by the cryoprotectant agent employed, as well the microbial stability conferred along the storage. Differences on the microorganism response to some protectors were observed between the lactic acid bacteria and the yeast evaluated. *W. anomalus* was more affected by absence of cryoprotectant (S1) during freeze drying processing, but this microorganism was more stable than *L. fermentum* along the storage without the presence of protectant agents. For *L. fermentum*, S5 was the best protectant, allowing the recovering of 100% of the bacterial cells after lyophilisation and 87% of cell viability was observed after one year storage, followed by S4 (96 and 74%, respectively). S4 and S5 were the best protectant to *W. anomalus* (viability > 80% after 1 year), but no increase in the yeast cell viability was conferred by addition of glutamate (S5) to skim milk. After 1 year of storage, trehalose was much more effective on protection of the yeast than bacteria (72% and 7% of viability, respectively). S2 was the less protectant agent among the tested, and their effectiveness was higher in *L. fermentum* (allowing 14% of cell recovering up to 120 days of storage) if compared to *W. anomalus* (25% of viability until 90 days of storage). Our results demonstrate that freeze-drying is a realistic technology for the stability and maintenance of the potential sourdough starter *L. fermentum* and *W. anomalus* for long time; however, the choice of cryoprotectant will influence the process effectiveness.

1. Introduction

Sourdough represents a product-mixed fermentation affected by the action of diverse microorganisms naturally present in various cereals (Minervini, De Angelis, Di Cagno, & Gobbetti, 2014). This process is less effective in terms of time but produces breads that are more flavourful and often preferred by some consumers. The large-scale production of fermented foods has become an important branch of the food industry and subsequently has created challenges for producing high quantities of microorganisms (Leroy & De Vuyst, 2004). To guarantee the survival of microorganisms, preservation methods have been tested to ensure their high stability during long-term storage (Sanders, Oomes, Membré, Wegkamp, & Wels, 2015).

Freeze-drying has been the classical method used to produce dry bacterial powders because drying takes place at low temperatures, thus reducing heat degradation (Miyamoto-Shinohara, Sukenobe, Imaizumi, & Nakahara, 2008). However, biological materials can be irreversibly damaged during these treatments. During the freezing process, the

* Corresponding author.
E-mail address: mvc@smail.ufsm.br (M.V. Copetti).

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formation of ice crystals and an increase in the intracellular concentration of salt cause water to leak out of cells and form extracellular ice crystals. This osmotic shock is the main reason for cell viability loss (Nakamura, Takagi, & Shima, 2009). So, cryoprotectants are used to prevent this phenomenon (Morgan, Herman, White, & Vesey, 2006) because they immobilize microorganisms in a material with very high viscosity and low mobility reaction (Semyonov et al., 2010).

Some studies describe the use of cryoprotectants including skim milk and different sugars to decrease the damage caused in microorganisms by low temperatures (Basholli-Salihu, Mueller, Salar-Behzadi, Unger, & Viernstein, 2014; Foerst, Kulozik, Schmitt, Bauer, & Santivarangkna, 2012; Navarta et al., 2014). The positive effect demonstrated by skim milk can be due its ability to provide a coating layer that protects microorganism cells during freeze-drying (Abadias, Benabarre, Teixidó, Usall, & Vias, 2001). Additionally, calcium ions from the skimmed milk may contribute to the protective effect (King & Su, 1993). On the other hand, the protective effect of sugars is attributed to its efficiency in stabilizing cell membranes during freezing (Hubalek, 2003), because they reduce the freezing point and minimize the formation of intracellular ice (Barbas and Mascarenhas, 2009). Besides, the hydroxyl components of sugar molecules have the ability to protect cells from injury caused by freezing (Barbas and Mascarenhas, 2009). Also, protection by trehalose can be due to a protein-stabilizing capacity, replacing water around polar molecules (Morgan et al., 2006). However, microorganisms (i.e. yeasts and bacteria) can respond differently to this preservative processing (Stefanello et al., 2018a). Therefore, it is essential previously to define the best protective agents able to preserve protein activity and cell survivability for each group microorganism intended to be maintained viable.

The production of concentrated starter cultures for the food industry by freeze-drying has been studied (Fonseca, Béal, & Corrieu, 2000; Zhao & Zhang, 2005). However, few studies have focused on effect of liophilisation in microorganism of interest for bread sourdough production (Lattanzi, Minervini, & Gobbetti, 2014; Stefanello et al., 2018a).

The lactic acid bacteria *Lactobacillus fermentum* showed potential for controlling fungal spoilage and improving the shelf-life of breads (Fazeli, Shahverdi, Sedaghat, Jamalifar, & Samadi, 2004). As well the yeast *Wickerhamomyces anomalus* (formerly *Hansenula anomala, Pichia anomala*) is exploited as potential agent for biocontrol of postharvest moulds (Melin, Schnürer, & Håkansson, 2011). When used on sourdough, both species have also demonstrated their potential to produce sensory well accepted panettones with an extended shelf-life (Stefanello et al., 2018b).

Thus, this study aimed to evaluate the freeze-drying method to improve the cell survivability of *L. fermentum* and *W. anomalus* using four cryoprotectant solutions as well as the efficiency of the proposed solutions during a 12-month period of preservation at room temperature, to obtain a simple and fast ready-to-use inoculum for the sourdough process.

2. Materials and methods

2.1. Microorganisms

Lactobacillus fermentum IAL 4541 and *Wickerhamomyces anomalus* IAL 4533 strains were isolated from dried sourdough (Stefanello et al., 2018a) and deposited at Núcleo de Coleção de Micro-organismos (NCMO) of Instituto Adolfo Lutz, São Paulo, Brazil.

The activation of *L. fermentum* IAL 4541 was performed by the resuspension of the lyophilized strain in 3 mL of de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany), followed by vigorous shaking for 30 s in a vortex mixer and incubation at 36 °C for 24 h under microaerophilic conditions (candle jar, about 3–5% CO_2 and 8–10% O_2). An aliquot of 100 µL of *L. fermentum* was transferred to Petri dishes containing solidified MRS agar (spread-plate method). The plates were incubated at 36 °C for 48 h under microaerophilic conditions (candle jar, about 3–5% CO_2 and 8–10% O_2). The inoculum was prepared by scraping the colonies from each plate with an inoculation loop and suspending it in a sterile flask with 3 mL sterile 0.1% peptone solution (Merck, Germany), obtaining a suspension of approximately 4×10^{11} CFU/mL.

The activation of the *W. anomalus* IAL 4533 strain was by the subculture of cells from a GYP (glucose 20 g/L; yeast extract 5 g/L; peptone 10 g/L; agar 20 g/L) plate, and incubation for 48 h at 30 °C under aerobic conditions. The inoculum was prepared by scraping 1/3 of the culture growth on the plate with an inoculation loop and suspending it in a sterile flask with 3 mL of sterile 0.1% peptone solution (Merck, Germany), giving a suspension of approximately 4×10^{10} CFU/mL.

2.2. Cryoprotectant agents (CPAs)

Five treatments were evaluated, namely a control solution (S1) and four cryoprotectant solutions (S2, S3, S4 and S5), having the following components: S1–0.1% peptone water solution; S2–10% sucrose solution (Merck, Germany); S3–5% trehalose solution (Tovani; Treha™); S4–10% skim milk solution; and S5 - stabilizer solution containing 5% sodium L-glutamate monohydrate p.a. and 10% Molico® skim milk powder. The control solution (S1) was autoclaved (121 °C/1 atm) for 15 min. The solutions containing the 5% trehalose (S2) and 10% sucrose (S3) were sterilized by filtration using a sterile syringe and filter. For S3, a 0.22 µm filter (BIOFILM® Syringe Filter) was used. For S3 filtration, a 0.46 µm filter (KASVI-k18–430) was used, since this solution was not able to cross the 0.22 µm filter. Cryoprotective solutions S4 and S5 were sterilized by tyndallisation (100 °C/30 min). Tyndallisation was repeated after 24 h, and the vials containing solutions S4 and S5 were sealed with Parafilm® after closing (Bemis NA, USA).

The inoculum prepared in Section 2.1 was aseptically resuspended in sterile glass vials containing 3 mL of the solutions of each treatment (S1 to S5) for further lyophilisation, reaching a final concentration of 1×10^{11} CFU/mL *L. fermentum* and 1×10^{10} CFU/mL *W. anomalus*.

2.3. Freeze-drying

Initially, the vials were held at −20 °C for 24 h. Lyophilisation caps were positioned on the flasks containing the *L. fermentum* IAL 4541 and *W. anomalus* IAL 4533 inocula. The flasks were immediately frozen (at −80 °C) using an ultralow temperature freezer (Thermo Scientific, USA). After 12 h of freezing, cultures were transferred to a freeze-dryer (K105, Liotop, Brazil). Freeze-drying was carried out for 24 h at −50 °C and at a pressure of 0.15 mbar (Keivani Nahr et al., 2015). After closing, the vials containing lyophilized strains were sealed with Parafilm® and stored protected from light at room temperature for 1 year.

2.4. Microbiological counts and cell viability

The quantification of the viable cell number of lyophilized strains (IAL-4541 and IAL-4533) was performed eight times during storage: before (−1) and after (0) lyophilisation, and after 30, 60, 90, 120, 210 and 365 days of storage. Freeze-dried samples were rehydrated adding autoclaved MRS broth (*L. fermentum* IAL 4541) and GYP broth (*W. anomalus* IAL 4533) to match the sample weight before dehydration, and vortex-mixed intermittently for 2 min. After waiting up to 15 min in room temperature for resuscitation, 100 µL samples were surface inoculated in Petri dishes. The viability of microorganisms before and after freeze-drying was determined by 10-fold serial dilutions and plate assays on MRS and GYP agar for bacteria and yeasts, respectively. Plates were incubated at 35 °C for 72 h for bacteria and 30 °C for 48 h for yeasts. Colonies with 30–300 per plate were selected for counting, and the mean values of the three plates were reported.

The survival rate (x, expressed as a %) was calculated using Eq. (1), considering the microorganism population before the process, i.e. the result obtained in the analyses before freezing (−1), and the

microorganism population after the process, both for freezing followed by lyophilisation and for the other storage stages at room temperature (for 365 days).

The viability (x) of the cell suspension for each protective medium was calculated using the following equation (Huang et al., 2006):

$$x\ (\%) = \frac{\text{Viable cells after freeze} - \text{drying}}{\text{Viable cells before freeze} - \text{drying}} x\ 100 \quad (1)$$

2.5. Experimental design

The experimental design was completely randomized with measures repeated in time, in a 2 × 5 × 8 factorial scheme, totalling 80 treatments, with three replicates of each treatment: two microorganisms (*L. fermentum* IAL 4541 and *W. anomalus* IAL 4533), five different cryoprotectants (S1: 0.1% peptone water, S2: 10% sucrose, S3: 5% trehalose, S4: 10% reconstituted skim milk, S5: 10% reconstituted skim milk with 5% monosodium glutamate) and eight times (−1, 0, 30, 60, 90, 120, 210, 365). The mathematical model used is described in the formula below:

$$Y_{ijkl} = \mu + M_i + C_j + (M*C)_{ij} + T_k + (M*T)_{ik} + (C*T)_{jk} + (M*C*T)_{ijk} + \varepsilon_a + \varepsilon_{ijkl} \quad (2)$$

Data were submitted to residual analysis (normality test, test for independence and test for homogeneity), analysis of variance, F-test and, when significant at 5%, means were analysed by the Tukey test (5%) and contrast studies. For the time factor and its interactions, when significant (5%), correlation analysis and polynomial regression were used. Repeated measurements of viable counts after freeze-drying were carried out to compare the protective effect of different agents. All experiments were repeated three times independently. The statistical analyses were performed using SAS University Edition, version 9.4 M4 (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

This study is the first investigation of the resistance of *L. fermentum* and *W. anomalus* to freeze drying processes. Figs. 1 and 2 show the microbial cells count and viability, respectively, of *L. fermentum* IAL 4541 and *W. anomalus* IAL 4533 after freeze-drying with different cryoprotectants over time. Based on the presented results, is possible to see that the lyophilisation process with cryoprotectant agents improved, in different degrees, the cell viability after processing (exception was sucrose for *L. fermentum*). *W. anomalus* was more affected by absence of cryoprotectant (S1), but, under this condition, this microorganism was more stable than *L. fermentum* along the storage. All the cryoprotectant agents studied were also adequate for improving the recovering of viable cells of both the *L. fermentum* IAL 4541 and the *W. anomalus* IAL 4533 isolates along the storage, when compared to the control.

Freeze-drying provides higher cell viability and is used for the long-term preservation of bacteria and yeasts (Jarque, Bittner, & Hilscherová, 2016). Different factors affect cell viability during freeze-drying (Hubálek, 2003). Freezing conditions (rate and duration of freezing), temperature of drying and the presence of protectants used to suspend cells before freezing are important parameters because they usually affect cell survival (Amine et al., 2014; Stephan, Da Silva, & Bisutti, 2016)). This can be explained because during the drying process, more specifically lyophilisation, cells undergo thermal stress that can cause membrane injuries, protein denaturation and damage to cellular DNA (Basholli-Salihu et al., 2014).

A marked difference was observed between the efficacy of the different agents in maintaining viability of both yeast and bacteria throughout the storage. For *L. fermentum*, skim milk supplemented with glutamate (S5) was the best protectant, maintaining 87% of cell viability after one year storage, followed by the skim milk alone (S4) (74%). Regarding the sugars, trehalose only maintained the bacterial cell viability ≥ 50% until 4 months of storage, and sucrose for 2 months. Although differing from the control (S1), sucrose (S2) was only adequate for the maintenance of the yeast *W. anomalus* for one month, showing a marked drop in the next 30 days. On the other hand, the other agents (S3, S4 and S5) allowed recoveries higher than 70% after 1 year of storage at room temperature. The best results were obtained by the skim milk (S4) and, unlike that observed for the bacteria *L. fermentum* IAL 4541, the supplementation of the skim milk with glutamate (S5) had no additional effect on the cell viability of the yeast *W. anomalus* IAL 4533 ($p > .05$).

Even though the primary objective of using cryoprotectants is enhancing cell survival during freeze-drying, the results demonstrate that the presence of these substances concentrated after processing can affect the amount of cells surviving along the storage. Also, the agent efficacy can vary according to the microorganism (i.e. the bacteria *L. fermentum* and the yeast *W. anomalus*). Therefore, it is important to optimize the freeze-drying process and choose a medium or solution capable of protecting the cell both in freezing and in drying (Berk, 2013), as well experimenting treatments to ensure higher survival rates of the interest microorganism over time.

Abadias et al. (2001) also reported that there was no significant difference on viability of yeast cells of *Candida sake* between using Skim milk plus glutamate.

The positive effect observed for skim milk and trehalose on the viability of the two strains after freezing have been reported in a previous studies (Stephan et al., 2016), however the viability and stability reported in *Pseudomonas* sp. were lower than the observed in our study. Also the results of our studies, both regarding processing survival and stability during storage of *L. fermentum* and *W. anomalus,* were much better than the reported for *Lactobacillus salivarius* (Zayed & Roos, 2004) using different protectant agents (sucrose, trehalose and skim milk) and combinations, which demonstrated improvement on the conferred stability. The combination of trehalose, sucrose and skim milk was the most effective protection and gave a survival rate between 83% and 85% after freeze-drying, however the stability along the storage cannot be compered, since in that study samples were maintained at −85 °C (Zayed & Roos, 2004). An average concentration of skim milk and a high concentration of trehalose also improved tolerance to freezing followed by the drying of *L. plantarum* (Keivani Nahr et al., 2015).

Increasing the trehalose concentration from 1% to 10% increased the survival of *C.sake* cells after freeze-drying (Abadias et al., 2001). The addition of 10% and 15% of trehalose before lyophilisation also demonstrated a cryoprotective effect directly proportional to the added concentration of trehalose in microorganism viability in the preparation of a lyophilized sourdough (Stefanello et al., 2018a). The survivability of *L. plantarum* cells after freeze-drying also increased as the concentration of trehalose rose from 0% to 30% (Keivani Nahr et al., 2015) and this could be an option to improve the viability rates when trehalose was the protectant agent of the microrganism evaluated in this study (*L. fermentum* and *W. anomalus)*, however, due to economic reasons higher amounts were not tested in our study. The main objective of our study was to find good and relatively cheap agents allowing for commercially viable production of starters of *W. anomalus* IAL 4533 and *L. fermentum* IAL 4541, and skin milk powder, as well glutamate, are cheaper and more economically advantageous than the use of trehalose. Besides, our results also demonstrated the possibility of the conservation and maintenance of strains at room temperature, reducing refrigeration and transportation costs.

In a study by Brandão et al. (2013), four different cryoprotectants used in the lyophilisation of *Escherichia coli* were evaluated (Black, Zannini, Curtis, & Gänzle, 2013). Four batches were produced using a 10% skim milk solution and the same solution added of glycerol, sucrose and trehalose. Long-term stability was studied at ≤ −70 °C,

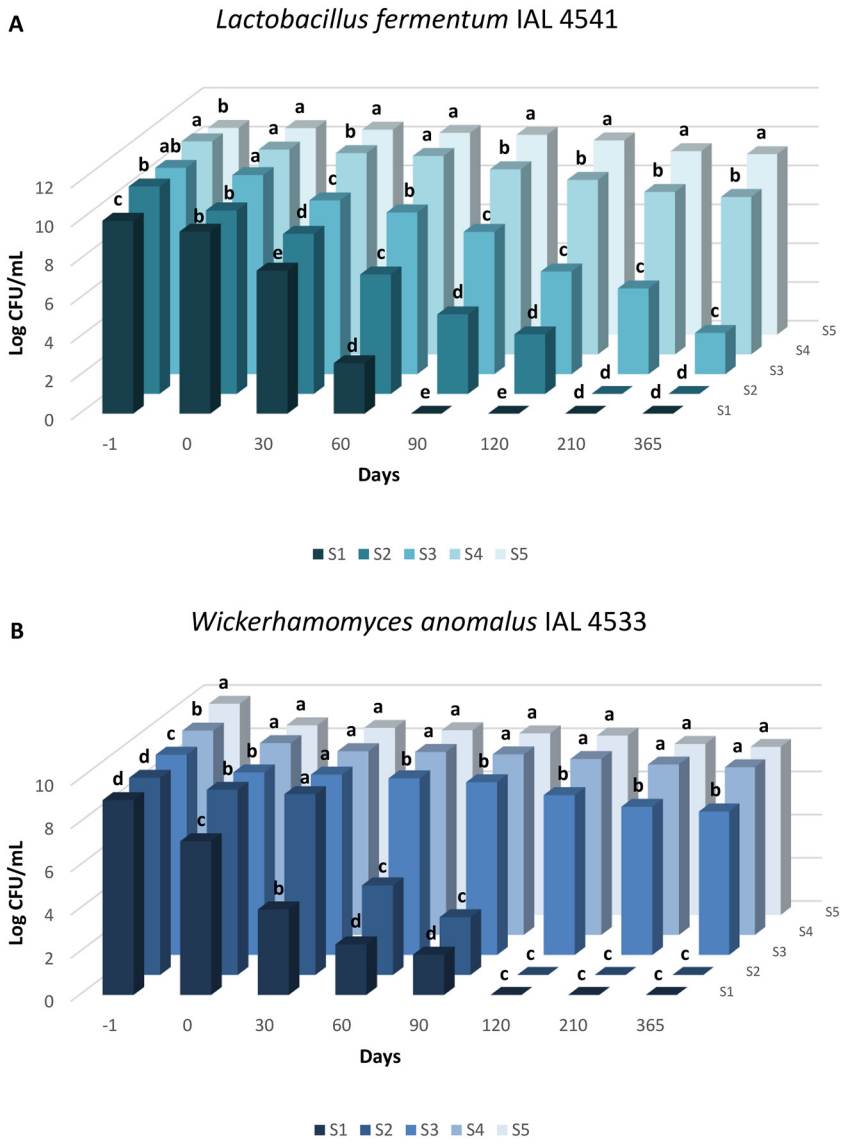

Fig. 1. Viability (Log CFU/mL) of *Lactobacillus fermentum* IAL 4541 (A) and *Wickerhamomyces anomalus* IAL 4533 (B) lyophilized with different cryoprotectant agents and stored at room temperature for 365 days. Different superscript letters on columns within the same day group indicate significant differences ($p < .05$) among the samples. S1 = Control: peptone water; S2 = Sucrose 10%; S3 = Trehalose 5%; S4 = Skim milk powder 10%; S5 = Skim milk powder 10% plus sodium glutamate 5%.

−20 °C, 4 °C, 25 °C and 35 °C for five days. Batches remained stable at ≤ −70 °C for four months. The sample containing glycerol presented unsatisfactory stability results at the end of five days. Sucrose and trehalose cryoprotectants were stable at the studied temperatures and were considered to be suitable for lyophilizing the microorganism in question. An increase in the survival of freeze-dried species of *Geotrichum candidum* was reported by Hamoudi et al. (2007) using disaccharides such as trehalose, sucrose and maltose at a concentration of 23%.

4. Conclusion

Using lyophilized strains as starter to obtain natural ferments is a practical alternative for the production of fermented products. However, this study showed that the cryoprotectant agent employed influences the recovering rates after the processing, as well as the microbial cell viability along the storage time, and so, can affect the process effectiveness. Differences on the response of the lactic acid bacteria *L. actobacillus fermentum* IAL 4541 and the yeast *Wickerhamomyces anomalus* IAL 4533 to some protectant were observed. Sucrose was the less effective protectant agent among the tested, and their effectiveness was higher in *L. fermentum* if compared to *W. anomalus*. On the opposite, trehalose was shown to be much more efficient in maintaining the yeast cells than the bacterial cells. Skim milk was the best protectant to the yeast *W. anomalus* IAL 4533, while the addition of glutamate to it improved their efficacy in the protection of bacterial cells, making it the best choice for the preservation of *L. fermentum* IAL 4541. These two matrixes are relatively cheap and are more economically advantageous than the use of trehalose. Our results also demonstrated the possibility of the conservation and maintenance of strains at room temperature, reducing refrigeration and transportation costs.

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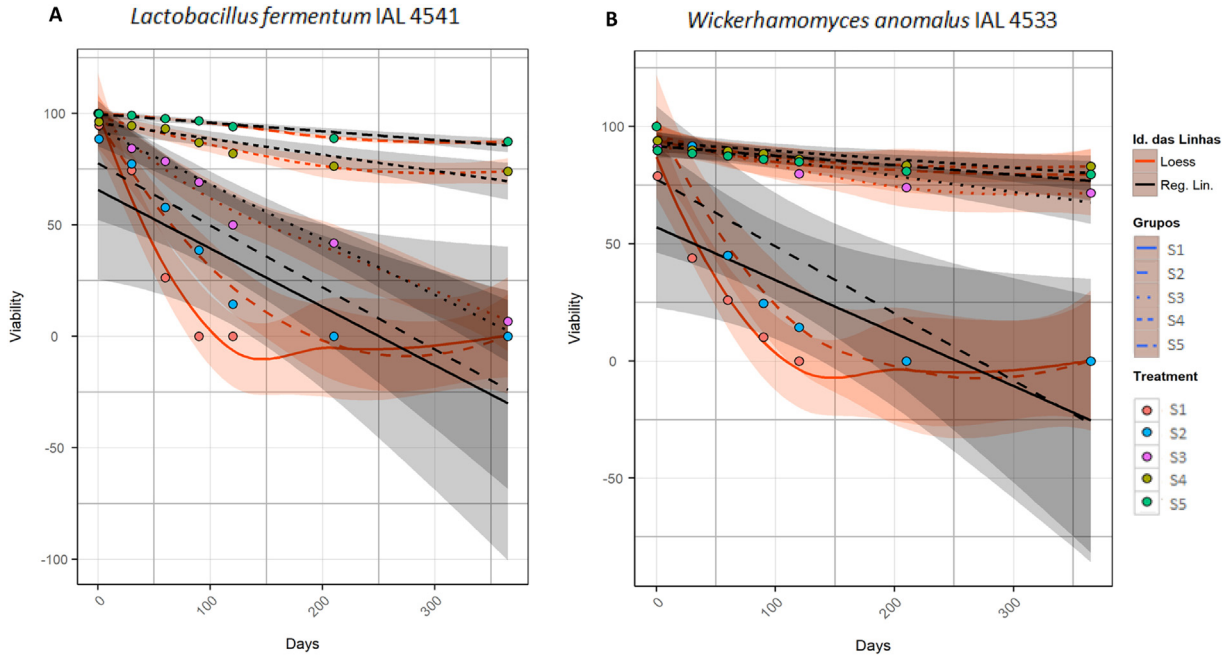

Fig. 2. Viability (%) of *Lactobacillus fermentum* IAL 4541 (A) and *Wickerhamomyces anomalus* IAL 4533 (B) before and after lyophilisation with different cryoprotectant agents along the storage at room temperature for 365 days. S1 = Control: peptone water; S2 = Sucrose 10%; S3 = Trehalose 5%; S4 = Skim milk powder 10%; S5 = Skim milk powder 10% plus sodium glutamate 5%.

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