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Preservation of frozen strawberries enriched with Saccharomyces boulardii using gelatin-based coating

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ABSTRACT. Although freezing is considered one of the easiest ways to preserve the quality of food, it is harmful to strawberries. Thus, the objective of this study is to evaluate the conservation of frozen strawberries using gelatin as a coating and of the probiotic yeast, *Saccharomyces boulardii*. Strawberries were selected, sanitized and subjected to the following treatments: Treatment 1 - control 1 (strawberries stored at -18ºC); Treatment 2 - control 2 (strawberries stored at -80ºC); Treatment 3 - strawberries added with citric acid, calcium chloride and *S. boulardii* stored at -18ºC; Treatment 4 - strawberries with gelatin, citric acid, calcium chloride, glycerol and *S. boulardii* stored at -18ºC. Drip loss, soluble solids, titratable acidity, color, firmness, ascorbic acid, total phenolic compounds, monomeric anthocyanins, antioxidant activity, polyphenoloxidase activity and viability of *S. boulardii* were analysed. The gelatin coating added with citric acid and calcium chloride, in combination with freezing at -18ºC, reduced the drip loss, the metabolic reactions and exerted a protective effect on bioactive compounds, making it possible to use them instead of deep-freezing, mainly for agribusinesses that do not have the equipment. The use of gelatin coating on strawberries minimized the effects of freezing and promoted the protection of *S. boulardii*, obtaining in up to 60 days of storage the minimum viable concentration of the microorganism to exercise its functional property. On the other hand, in the absence of gelatin, *S. boulardii* had a negative influence on the physical-chemical parameters, in addition to showing less viability. Thus, the study demonstrated that the coating composed of gelatin, citric acid and calcium chloride can represent an interesting alternative for application on frozen strawberries enriched with *S. boulardii*.

Keywords: *Fragaria* x *ananassa* duch; camarosa strawberry; edible coating; functional food.

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Introduction

The strawberry (*Fragaria* x *ananassa* Duch.), belonging to the Rosaceae family, is among the most consumed red fruits in the world for presenting pleasant aroma and taste, as well as a juicy texture. Besides, it is considered source of dietary fiber (1.7 g 100 g^{-1}), potassium (184 mg 100 g^{-1}), vitamin C (63.6 mg 100 g^{-1}), manganese (10 mg 100 g^{-1}) and antioxidant substances such as phytochemicals (Oszmianski & Wojdyło, 2009; *Tabela Brasileira de Composição de Alimentos* [Taco], 2011).

Strawberry is non-climacteric, however, it is highly perishable and can only be stored for short periods, less than 5 days (Han, Zhao, Leonard, & Traber, 2004; Yu & Liao, 2016). Thus, effective preservation techniques are necessary to prolong the shelf life of the strawberry, in order to maintain its physical and nutritional properties. Freezing is one of the most common and well-established food preservation techniques: it decreases the rate of deterioration, reducing molecular mobility, microbial, enzymatic and oxidative activities and respiratory rate (Bilbao-Sainz et al., 2019).

Although freezing is considered one of the easiest ways to preserve the quality of food, it is harmful to strawberries. It causes changes in the color of the fruit, due to the loss of anthocyanins, oxidation of phenolic compounds by polyphenoloxidase and oxidation of vitamin C, in addition to drip and texture losses, due to the damage caused by ice crystals (Reno, Prado, & Resende, 2011; Abd-Elhady, 2014; Bulut, Bayer, Kırtıla, & Bayındırlıa, 2018). Thus, different pretreatments have been evaluated in order to maintain the characteristics of the strawberry subjected to freezing (Han et al., 2004; Reno et al., 2011; Abd-Elhady, 2014; Yu & Liao, 2016).

In general, these studies use a firmness agent (calcium chloride, calcium lactate or calcium gluconate) and an antioxidant agent (citric acid or ascorbic acid), using mainly carbohydrates as a coating, such as sucrose, glucose and pectin; few studies have evaluated the use of other polysaccharides, such as chitosan and alginate (Han et al., 2004; Oszmiański, Wojdyło, & Kolniak, 2009; Reno et al., 2011; Abd-Elhady, 2014; Yu & Liao, 2016). The coatings mainly influence the reduction of drip loss due to the control in the formation of ice crystals and oxidation reactions due to the influence on gas exchange (Oszmiański et al., 2009; Reno et al., 2011).

Gelatin is a protein of animal origin and widely used in the food industry. As a coating, it has shown good results in the conservation of fresh and minimally processed vegetables (Licodiedoff et al., 2016; Mannucci et al., 2017; Aitboulahsen et al., 2018). However, studies with the application of gelatine in frozen strawberries were not found in the literature.

Functional foods contain ingredients that bring specific health benefits (Annunziata & Vecchio, 2013), which is the case of foods added with probiotics. Probiotic microorganisms have been added to minimally processed vegetables (Shigematsu et al., 2018; Speranza et al., 2018), but on frozen vegetables, no reports were found in the literature. *Saccharomyces boulardii* is a probiotic microorganism that acts in the regulation of pathogenic bacteria. It interferes in the ability of pathogens to colonize and infect the mucosa, by modulating immune responses, stabilizing the gastrointestinal barrier, inhibiting pro-carcinogenic enzymes and inducing enzymatic activity, thus promoting the absorption of nutrients (Vandenplas, Brunser, & Szajewska, 2009). However, the viability of *S. boulardii* is dependent on environmental conditions (Khodaei, Hamidi-Esfahani, & Lacroix, 2020). The ice crystals formed during freezing can also affect the microbial viability (Silva, 2000).

Based on what has been reported, it is believed that the preservation of strawberries by freezing with the use of gelatin and probiotics can be advantageous to extend the shelf life of fruits and provide them with functional characteristics for fresh consumption.

Thus, the objective of this study was to evaluate the conservation of frozen Camarosa strawberries added with a coating based on commercial gelatin and of the probiotic yeast, *S. boulardii*.

Material and methods

Material

Strawberries (*Fragaria* x *ananassa* Duch.) cultivar Camarosa were used as raw material for the study. They were purchased in October 2019, from a producer in the district of Cerrito Alegre, in the city of Pelotas/state of Rio Grande do Sul (latitude: -31.776, longitude: -52.3594, 31° 46' 34" S, 52° 21' 34" W).

Methods

Strawberries were selected for the absence of physiological defects, for size and color (> 75% of red-colored surface). Afterwards, they were washed with water and sanitized in 200 mg L^{-1} sodium hypochlorite solution, with pH between 6.5 and 7.0, for 15 min. Entire strawberries were used, with their sepals and pedicels.

The following treatments were evaluated:

Treatment 1 (control 1) - the strawberries were stored in a freezer at -18ºC (Consul, Brazil),

Treatment 2 (control 2) - the strawberries were stored in an ultrafreezer at -80ºC (Coldlab, Brazil).

Treatments 3 - a freeze-dried culture of the yeast *S. boulardii* CNCM I-745 was used. It contained the aroma of tutti-frutti, magnesium stearate, fructose and lactose (Merck, France), in a concentration of 2.2 x 10^8 UFC mL^{-1}, added to the coating solution. In addition, citric acid (1.0% w v^{-1}) and calcium chloride (1.0% w v^{-1}) were added, under stirring for 10 min at room temperature.

Treatment 4 - unflavored gelatin (Royal, Brazil) was used, which was prepared by dissolving 5% (w v^{-1}) in distilled water. The solution was homogenized on a magnetic stirrer at 60ºC for 30 min. After dissolution, the lyophilized microorganism *S. boulardii* CNCM I-745 (Merck, France), in a concentration of 2.2 x 10^8 UFC mL^{-1}, calcium chloride (1.0% w v^{-1}), citric acid (1.0% w v^{-1}) and glycerol (1.0% w v^{-1}) were added, under stirring for 10 min. at room temperature.

For treatments 3 and 4, the fruits were completely submerged in the solutions for 30 s and dried under forced ventilation at room temperature (15ºC), and stored at -18ºC.

The strawberries were packed in high density polyethylene bags, with a standardized amount of fruit per package (200 g), totaling 120 strawberries per treatment. They were stored at -18 or -80ºC according to treatment, for 90 days.

The experimental design used was entirely random, in a 4 x 4 factorial scheme, with 4 treatments (1, 2, 3 and 4) and 4 evaluation periods (0, 30, 60 and 90 days of storage). The evaluations were performed on strawberries thawed at 4ºC for 16 hours, performed at least in triplicate.

Drip loss

Drip loss (DL) was determined by the difference in weight before and after thawing the strawberries (Han et al., 2004), being calculated as a percentage, according to Equation 1:

$$DL(\%) = \frac{Weight of fresh strawberry - Weight of strawberry after thawing}{Weight of fresh strawberry} x100 \quad (1)$$

Titratable acidity

The titratable acidity was determined by potentiometric titration of 10 g of crushed sample and homogenized with 100 mL of distilled water. The sample was titrated using 0.1 mol L^{-1} NaOH solution to a pH range (8.2-8.4). The results were expressed as a percentage of citric acid (*Instituto Adolfo Lutz* [IAL], 2008).

Vitamin C

Twenty grams of crushed strawberries were mixed with 50 mL of water, 10 mL of 20% (v v^{-1}) sulfuric acid solution, 1 mL of the 10% (v v^{-1}) potassium iodide solution and 1 mL of the solution 1% (w v^{-1}) starch. The titration was performed with 0.002 mol L^{-1} potassium iodate solution until reaching a pinkish color. The results were expressed in mg 100 g^{-1} of sample (IAL, 2008).

Soluble solids

Soluble solids were determined in an Abbé bench-top refractometer, at 20ºC (IAL, 2008).

Firmness

The measures of firmness of the strawberries were determined using a texturometer (Stable Micro Systems TA.XTplus, United Kingdom) and the P-2N probe. The compression test was performed to measure firmness, or the strength that would cause the fruit to rupture. The operational parameters used were: pre-test speed of 1.0 mm s^{-1}, test speed of 1.00 mm s^{-1}, post-test speed of 10.00 mm s^{-1} and driving force of 0.245 N. The firmness obtained was automatically registered using the Texture Exponent 32 software. The reading was performed in the equatorial central region of the strawberry, and the results were expressed in Newton (N).

Color

The color was determined using a colorimeter (Minolta CR 400, Japan). The standard used was CIE L*a*b*, where the L* coordinate expresses the degree of luminosity of the measured color (L* = 100 = white; L* = 0 = black), the a* coordinate expresses the degree of variation between red (+60) and green (-60) and the b* coordinate expresses the degree of variation between blue (-60) and yellow (+60).

Preparation of hydroalcoholic extract

To prepare the hydroalcoholic extract, strawberries were crushed and 5 g of them were added with 50 mL of methanolic solution (70% methanol/ 30% water). The extract was stirred for 3 hours at room temperature, after which it was subjected to filtration on qualitative paper.

Total phenolic compounds

The determination of total phenolic compounds followed the methodology proposed by Singleton, Orthofer, and Lamuela-Raventós (1999) with some modifications. Aliquots of 1 mL of the hydroalcoholic extract (70% methanol/ 30% water) were added with 1 mL of Folin-Ciocalteu solution and, subsequently, 8 mL of distilled water. After 3 min of reaction, 1 mL of Na_2CO_3 1 mol L^{-1} was added and the mixture was incubated at 37ºC for 30 min. The absorbance of the resulting solution was measured on a spectrophotometer (AAKER, Brazil) at 750 nm. Quantification was performed using the calibration curve performed with gallic acid at concentrations from 0 to 0.5 mg mL^{-1}. The results were expressed in mg GAE 100 g^{-1} of strawberries.

Monomeric anthocyanins

The content of monomeric anthocyanins was determined by the differential pH method (Lee, Durst, & Wrolstad, 2005). For this, 1 mL of the extract was added with 4 mL of potassium chloride buffer pH 1.0 (0.025

mol L^{-1}) and in another portion of the sample added with 4 mL of sodium acetate buffer pH 4.5 (0.4 mol L^{-1}). After 20 min with the sample in the dark, reading was performed using spectrophotometer (AAKER, Brazil) at 520 nm and 700 nm wavelengths. The results were calculated according to Equation 2 and 3, and the results expressed in mg eq. pelargonidin-3-glycoside per 100 g (wet basis).

$$A = (A520nm - A700nm)_{pH1} - (A520nm - A700nm)_{pH4,5} \quad (2)$$

$$Monomericanthocyanins(mg.100g^{-1}) = \frac{(AxPMxFDx1000)}{(\varepsilon x1)} \quad (3)$$

where:
A = absorbance; PM = molecular weight of the predominant anthocyanin in the sample, in this case pelargonidin-3-glycoside (PM = 433 g.mol^{-1}); FD = dilution factor; 1000 = conversion factor from g to mg; ε = 22,400 molar absorptivity for pelargonidin-3-glucoside; 1 = optical path traveled (cell width).

Antioxidant activity

The determination of antioxidant activity was performed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, according to Brand-Williams, Cuvelier, and Berset (1995). For this, 750 µL of the hydroalcoholic extract (70% methanol/ 30% water) were added in 3750 µL of DPPH (0.05 mM). The reading was performed after 20 min in a spectrophotometer (AAKER, Brazil), at 515 nm, and the results were expressed as a percentage of inhibition.

Polyphenoloxidase activity

For the extraction of the enzyme, 1 g of the frozen tissue was homogenized with 10 mL of 0.05 mol L^{-1} phosphate buffer, pH 7, containing 1% polyvinylpyrrolidone, and then immediately filtered. The homogenate obtained was centrifuged for 15 min. at 7,000 g and a temperature of 3ºC (Matsuno & Uritani, 1972). The resulting supernatant was used to determine the activity of the polyphenoloxidase enzyme.

In determining the activity of the polyphenoloxidase enzyme, 1 mL of enzyme extract was added to 3.6 mL of 0.05 mol L^{-1} phosphate buffer pH 6 and 0.1 mL of 0.1 mol L^{-1} catechol. The obtained solution was incubated for 30 min. at 30ºC, being then immediately cooled in an ice bath, and the absorbance reading was performed on a spectrophotometer (AAKER, Brazil), at a wavelength of 395 nm. The enzymatic activity of polyphenoloxidase was expressed in units (enzymatic activity capable of altering 0.001 absorbance at 395 nm) per gram of fresh pulp per minute (UAE g^{-1} $min.^{-1}$) (Campos & Silveira, 2003).

Counting of *Saccharomyces boulardii*

The yeast count was performed according to the procedures proposed by Downes and Ito (2001). For all samples, serial dilutions were made in sterile saline solution (0.85%) up to the 10^{-9} dilution for the quantification of yeasts in the coating solution and up to 10^{-7} for counting in strawberries submitted to treatments 1, 2, 3 and 4. The analyses were performed in triplicate.

For the counting of *S. boulardii*, expressed through the counting of fungi, the method of plating in Potato Dextrose Agar was used, being the plates incubated at 25ºC. Counts were performed at three and five days of incubation. The result was expressed in log CFU g^{-1}.

Statistical analysis

The results obtained were subjected to analysis of variance and the comparison of means between treatments was performed by the Tukey test with a significance level of 5%, using the Statistix 10 program. The 95% confidence interval was calculated for the evaluation of the storage time.

Results and discussion

In Figure 1A, it can be seen that there was maintenance ($p > 0.05$) of the values of drip loss from strawberries submitted to treatments 1 and 2; however, in treatments 3 and 4 there was a significant increase ($p \leq 0.05$) in values during storage.

When evaluating the influence of treatment, it is observed that in 30 days of storage, strawberries submitted to treatments 2 and 4 showed significantly the lowest values of drip loss in relation to the other treatments, without significant distinction between them (data not shown). In 60 days, the drip loss values of strawberries from treatments 2 and 4 were still the lowest in relation to the others ($p \leq 0.05$), whereas in treatment

3 the highest values were significantly obtained ($p \leq 0.05$). However, in 90 days of storage, the drip loss in treatment 2 did not differ from treatment 1, in which the lowest values were obtained. In treatment 3, significantly greater losses of exudate from strawberries were observed, in relation to the other treatments ($p \leq 0.05$).

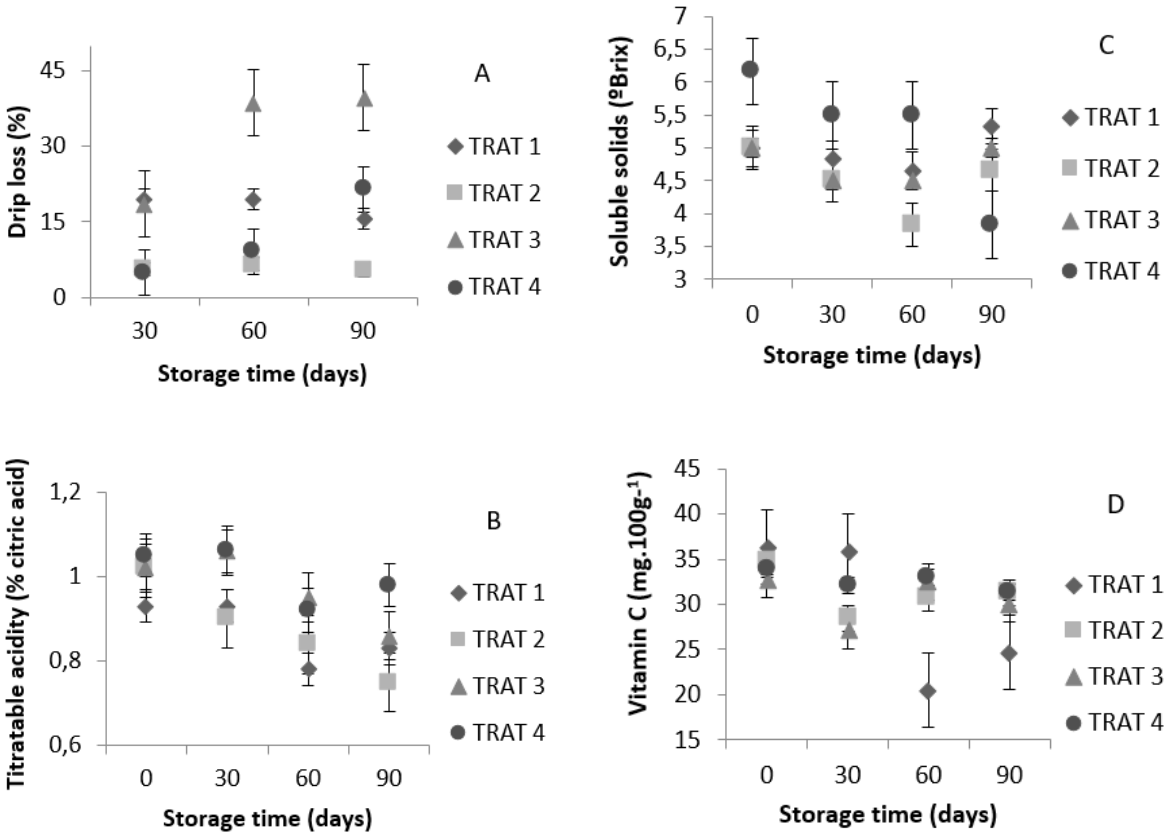

Figure 1. Drip loss (%) - A, titratable acidity (% citric acid) - B, soluble solids (ºBrix) - C and vitamin C (mg 100 g^{-1}) - D of frozen strawberries stored for 90 days. Treatment 1: Control 1 - strawberry stored at -18ºC; Treatment 2: Control 2 - strawberry stored at -80ºC; Treatment 3: strawberries added with citric acid, calcium chloride and *S. boulardii*, stored at -18ºC; Treatment 4: strawberries added with gelatin, citric acid, calcium chloride, glycerol and *S. boulardii*, stored at -18ºC. Vertical bars represent the 95% confidence interval.

The loss of cellular fluid is an important quality parameter of freezing, being related to the degree of rupture of the cellular structure. The greater loss of cellular fluid indicates greater damage to tissues (Reno et al., 2011).

Strawberries frozen at -80ºC (treatment 2) showed low values of drip loss during the entire storage period. This is because rapid freezing provides the formation of small intracellular ice crystals, which do not cause the cells to rupture, thus reducing the drip loss. On the other hand, in slow freezing (treatment 1), there is the formation of large ice crystals, which break the cells and cause greater drip loss (Alcarde, D'arce, & Spoto, 2006; Bulut et al., 2018).

Thus, it appears that the gelatin coating (treatment 4) was efficient in reducing the drip loss up to 60 days of frozen storage. Reno et al. (2011) demonstrated that coatings based on pectin, calcium chloride and glucose reduced drip loss due to the control in the formation of ice crystals. According to Han et al. (2004), the coating helps to retain the exuded liquid and prevents the migration of moisture from the fruit to the environment during the freezing and thawing processes.

The reflections of the presence of yeast in the gelatin coating were only observed after 60 days, due to the presence of gelatin that changes the internal atmosphere of the fruit, reducing the enzymatic activity, However, after 60 days, the drip loss in treatment 4 increased significantly. It was higher than treatment 1, possibly due to the degradation of gelatin by proteases produced by yeast. According to Im and Pothoulakis (2010), *Saccharomyses boulardii* produces a series of proteases that are responsible for cleaving toxins produced by pathogenic microorganisms.

The addition of probiotic yeast without the presence of gelatin (treatment 3) had a negative influence on the results, since there was an increase in the drip loss, even in relation to treatment 1. This can be due to the production of carbohydrases and proteases by the yeast *S. boulardii* (Im & Pothoulakis, 2010).

There was no influence of the addition of citric acid and calcium chloride in treatments 3 and 4, since the drip loss values differed significantly.

Different studies have demonstrated the influence of coatings based on solutions of sugars and/or polymers associated with firmness agents in maintaining the integrity of the cell structure. This would occur by reducing crystallization and strengthening the cell wall with the formation of calcium pectate, respectively (Han et al., 2004; Reno et al., 2011).

When analyzing the acidity data of frozen strawberries in relation to time, a significant reduction in values ($p \leqslant 0.05$) can be observed during storage, for treatments 1, 2 and 3. In treatment 4, the reduction of values was not significant ($p > 0.05$) (Figure 1B). There was no influence of the addition of citric acid on the behavior in relation to time for strawberries subjected to treatments 3 and 4, as these presented different behaviors. However, the presence of gelatin in treatment 4 reduced ripening, as values were maintained.

At the end of the 90 day storage period, it can be seen that strawberries submitted to treatment 4 had a percentage of acidity significantly ($p \leqslant 0.05$) higher than strawberries submitted to freezing at -80ºC (treatment 2). This demonstrates that the combination of gelatin coating and freezing at -18ºC was more effective in maintaining the values, when compared to freezing in an ultra-freezer. However, there was no significant difference ($p > 0.05$) in the acidity values of strawberries submitted to treatments 1, 3 and 4, as well as between treatments 1, 2 and 3 (data not shown).

With the ripening of the fruits, there is a reduction in acidity due to the oxidation of organic acids and their conversion into sugars (Mattiuz, Durigan, & Rossi Júnior, 2003). Freezing only reduces the metabolic activity of the fruits, in this sense the addition of gelatine may have altered the internal atmosphere, delaying ripening, as observed by Han et al. (2004) in strawberry coatings with chitosan subjected to freezing.

On the other hand, Yu and Liao (2016) did not observe variation in acidity values during 6 months of storage of control frozen strawberries and those coated with lemon juice, alginate and calcium chloride, using cryomechanical freezing (liquid nitrogen and forced air 5°C) and air blast (forced air -25°C) and storage at -18°C.

It can be observed that the soluble solids of frozen strawberries tended to decrease up to 60 days, regardless of the treatment (Figure 1C). However, after this period, for treatments 1, 2 and 3 there was a significant increase ($p \leqslant 0.05$) in values, whereas in treatment 4 there was a reduction ($p \leqslant 0.05$) in the soluble solids content.

Regarding the influence of treatment on the different storage times (data not shown), it can be seen that the values of soluble solids of strawberries submitted to treatment 4 were significantly higher than the others ($p \leqslant 0.05$) at point 0. In 30 and 60 days of storage, the values of the soluble solids of the strawberries submitted to treatment 4 were higher than those obtained in treatments 2 and 3 ($p \leqslant 0.05$), but they did not differ from treatment 1 ($p > 0.05$). In 90 days, the lowest values of soluble solids in the strawberries of treatment 4 were obtained, with no distinction from those submitted to treatment 2.

The reduction in the content of soluble solids during storage may be related to the process of respiration of the fruit, on the other hand the increase in such content may have occurred due to the ripening process, in which there is conversion of starch and acids into glucose (Alharaty & Ramaswamy, 2020), even though the freezing temperatures reduce the pace of these processes (Alcarde et al., 2006). Thus, it is suggested that the gelatin coating in treatment 4 has reduced the maturation process of strawberries, even in relation to the use of deep-freezing in treatment 2.

The higher content of soluble solids initially observed in treatment 4 may be due to the presence of gelatin. In treatment 3, however, values did not change. Different behavior has been observed in the literature depending on the coating. Reno et al. (2011) observed that the impregnation of pectin, glucose and calcium chloride in strawberries subjected to freezing at -18ºC, caused an increase in the soluble solids content. Yu and Liao (2016), on the other hand, did not observe a significant change in the soluble solids content during the storage of frozen strawberries for 6 months, coated with lemon juice, alginate and calcium chloride, using cryomechanical freezing (liquid nitrogen and forced air -25ºC) and air blast (forced air -25ºC) and storage at -18ºC.

When analyzing the vitamin C data of frozen strawberries in relation to time, it can be observed that there was a significant decrease ($p \leqslant 0.05$) in values during storage for treatments 1 and 2, as shown in Figure 1D. In treatment 3 there was a significant reduction in 30 days with a subsequent increase. In treatment 4, values were maintained ($p > 0.05$) in 90 days of storage.

At the end of storage (data not shown), strawberries submitted to treatment 1 had the lowest vitamin C content ($p \leqslant 0.05$). There was no difference in values among treatments 2, 3 and 4.

Vitamin C is very sensitive to degradation due to its oxidation (Turmanidze, Jgenti, Gulua, & Shaiashvili, 2017). The low temperatures of treatment 2 reduced vitamin C degradation when compared to treatment 1,

possibly by inhibiting the enzyme ascorbate oxidase. According to Alcarde et al. (2006), fast freezing is better for food quality in relation to enzymatic activity, when compared to slow freezing. Already, the presence of citric acid in treatments 3 and 4 has influenced the achievement of higher values of vitamin C, compared to treatment 1, due to its antioxidant activity (Koblitz, 2008). Gelatin coating does not seem to have influenced the values.

There was no significant variation ($p > 0.05$) in the content of total phenolic compounds of frozen strawberries until 60 days of storage, regardless of treatment (Figure 2A). However, after 60 days there was an increase in the content of total phenolic compounds ($p \leq 0.05$), in all treatments.

When evaluating the influence of the treatment on the different storage times (data not shown), it is noticed that strawberries submitted to treatment 4 tended to present the highest values, although they were significantly distinct ($p \leq 0.05$) from the other treatments only by 30 and 90 days. In relation to the other treatments, similar values were observed without significant distinction between them, at times 0, 60 and 90.

The tendency of increasing the content of phenolic compounds during storage has been justified in the literature due to the damage caused to the cell wall and membrane by freezing, resulting in the greater extraction of phenolic compounds (Oliveira, Coelho, Alexandre, Almeida, & Pintado, 2015; Bulut et al., 2018).

In this study, the highest concentration of phenolic compounds was obtained at the end of storage in treatment 4, but it was not the treatment with the greatest drip loss. Therefore, it is not possible to relate the results to cell damage. However, it is suggested that the presence of the edible coating, which seals the fruit, has created a protective layer against oxygen absorption, which reduces the enzymatic oxidation of phenolic compounds (Oszmiański et al., 2009).

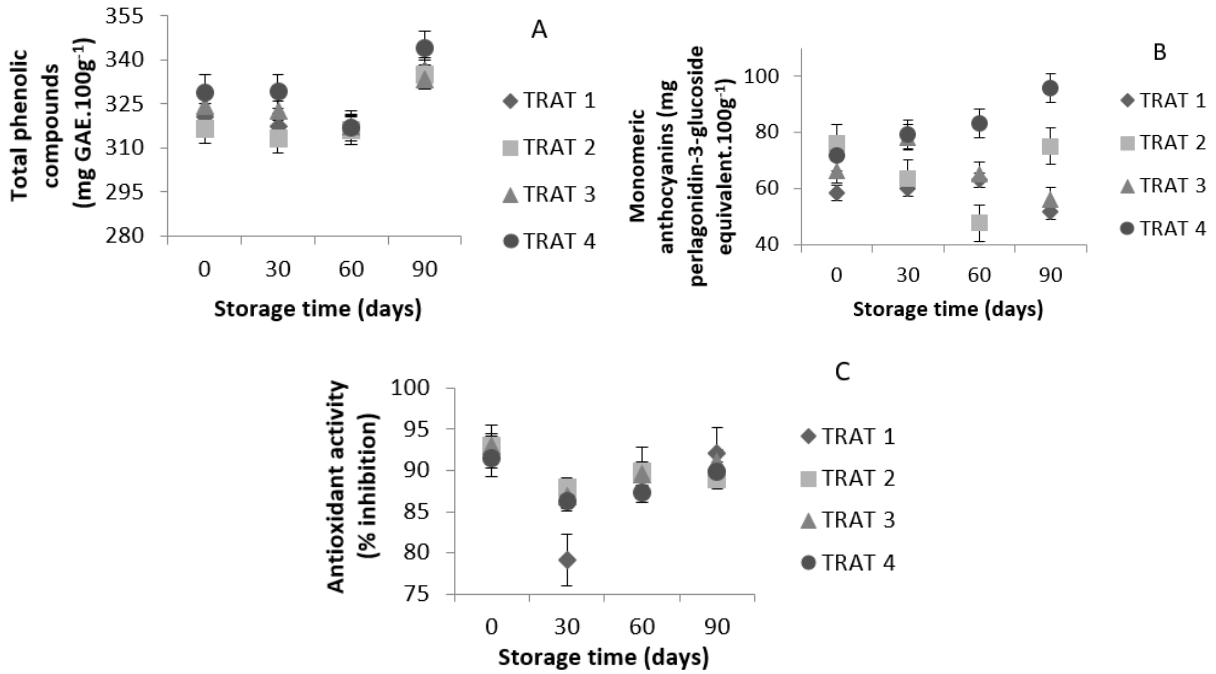

Figure 2. Total phenolic compounds (mg GAE 100 g^{-1}) - A, monomeric anthocyanins (mg perlagonidin-3-glucoside equivalent 100 g^{-1}) - B and antioxidant activity (% inhibition) - C of frozen strawberries stored for 90 days. Treatment 1: Control 1 - strawberry stored at -18ºC; Treatment 2: Control 2 - strawberry stored at -80ºC; Treatment 3: strawberries added with citric acid, calcium chloride and *S. boulardii*, stored at -18ºC; Treatment 4: strawberries added with gelatin, citric acid, calcium chloride, glycerol and *S. boulardii*, stored at -18ºC. Vertical bars represent the 95% confidence interval.

Different behaviors were observed in the content of anthocyanins in strawberries throughout the freezing period (Figure 2B). In treatment 1, values were maintained for up to 60 days ($p > 0.05$), with a subsequent significant reduction ($p \leq 0.05$). In treatment 2, a reduction ($p \leq 0.05$) was observed in 60 days, after which there was a significant increase in values. In treatment 3, an increase can be seen in 30 days, but with reduction in 60 days ($p \leq 0.05$). In treatment 4, there was a significant increase in values ($p \leq 0.05$) in 90 days of storage.

In general, the gelatin-coated strawberries (treatment 4) had the highest anthocyanin levels in relation to the other treatments (data not shown), possibly due to the gelatin coating that minimizes the presence of oxygen and decreases the polyphenoloxidase activity. It is noteworthy that in treatment 3 (with the use of citric acid and calcium chloride, but without the presence of gelatin), the same effect was not observed. The

strawberries submitted to treatment 1 had the lowest anthocyanin levels possibly due to the higher activity of the polyphenoloxidase enzyme.

The reduction in the acidity of strawberries in treatments 1, 2 and 3 may have contributed to the reduction in the content of anthocyanins. However, the biosynthesis of anthocyanins follows a complex enzymatic pathway (Tanaka, Sasaki, & Ohmiya, 2008), whose activity is not fully inhibited under freezing temperature (Alcarde et al., 2006).

It is suggested that the higher concentration of anthocyanins observed in treatment 4 is due to the presence of the coating, which acts as a gas barrier, thus modifying the internal atmosphere in fruits (higher levels of CO_2 and low levels of O_2). This can delay biochemical reactions, leading to anthocyanin synthesis (Tzoumaki, Biliaderis, & Vasilakakis, 2009).

The stability of anthocyanins is highly variable, depending on the strawberry cultivar, the structure and concentration of anthocyanins, pH, storage temperature, among others (Abd-Elhady, 2014; Oliveira et al., 2015).

According to Figure 2C, it is observed that in treatments 1, 3 and 4, there was a significant reduction in the antioxidant activity of strawberries in 30 days of storage, with subsequent increase up to 90 days ($p \leqslant 0.05$). In treatment 2, there was a reduction in values in 30 days ($p \leqslant 0.05$), followed by maintenance at the other evaluated times ($p > 0.05$).

Regardless of treatment, high values of antioxidant activity were observed. At the end of storage, the highest values were obtained in strawberries submitted to treatments 1 and 3, followed by treatment 4 and 2 (data not shown).

Strawberries are rich in bioactive substances such as phenolic compounds and vitamin C, which are related to the antioxidant activity of the fruit (Turmanidze et al., 2017). There was no clear relationship between the content of vitamin C and phenolic compounds in the antioxidant activity values, however, according to the literature, other factors can influence these values, such as cell destruction due to ice crystals, which would result in the thawing that extraction of compounds (Bulut et al., 2018), presence of antioxidants and consequently the activity of the enzyme polyphenoloxidase (Holzwarth, Wittig, Carle, & Kammerer, 2013). According to the results, possibly in treatment 1 and 3 the highest values obtained are due to greater extraction of compounds due to the damage to cells by ice crystals, it is noteworthy that in treatment 3 there is the presence of citric acid, which may also have caused some effect. In treatment 2, the lowest values of antioxidant activity were obtained, possibly by rapid freezing to preserve cell integrity. In treatment 4, the values observed may have been a function of lesser cell damage, due to the presence of gelatin, or even greater activity of the polyphenoloxidase enzyme due to the composition of gelatin (results shown in the discussion of the activity of the polyphenol oxidase enzyme)

Bulut et al. (2018) observed changes in the antioxidant activity of strawberries stored at -27ºC for 90 days. According to the authors, the increase in antioxidant activity is due to cell disruption caused by thawing the fruit before analysis, which improves the extraction of phenolic compounds. In the study carried out by Oliveira et al. (2015), the antioxidant activity of strawberries subjected to freezing at -20ºC for 24 hours was not changed, however, freezing for 360 days reduced the antioxidant activity by 20%.

According to Figure 3A, it can be seen that the values of the L* coordinate of strawberries submitted to treatments 1 and 3 increased significantly in 30 days of storage, with subsequent reduction ($p \leqslant 0.05$). Values were maintained ($p > 0.05$) in strawberries subjected to treatment 2. In treatment 4, there was a reduction in values in 60 days, with a subsequent significant increase ($p \leqslant 0.05$).

However, despite the different behaviors in relation to time, there was no significant influence of treatments on the values obtained ($p > 0.05$) (data not shown). Thus, there was not evident influence of the addition of citric acid and gelatin in the lightness values of strawberries submitted to treatments 3 and 4.

Distinct behavior in relation to the presence of citric acid and calcium was obtained by Abd-Elhady (2014), who observed higher luminosity values for control strawberries frozen at -40ºC and kept at -18ºC for 12 months, compared to treated strawberries with citric acid and/or calcium lactate. According to the author, the lighter color can be attributed to the occurrence of bleaching.

On the other hand, lower values of the L* coordinate of strawberries subjected to cryomechanical freezing (liquid nitrogen and forced air -25ºC) and air blast (forced air -25ºC) were observed by Yu and Liao (2016), in relation to the samples pre-treated with lemon juice, alginate and calcium chloride and frozen under the same conditions as the control samples. According to the authors, the citric acid present in lemon juice acts as a copigment, minimizing the instability of anthocyanins.

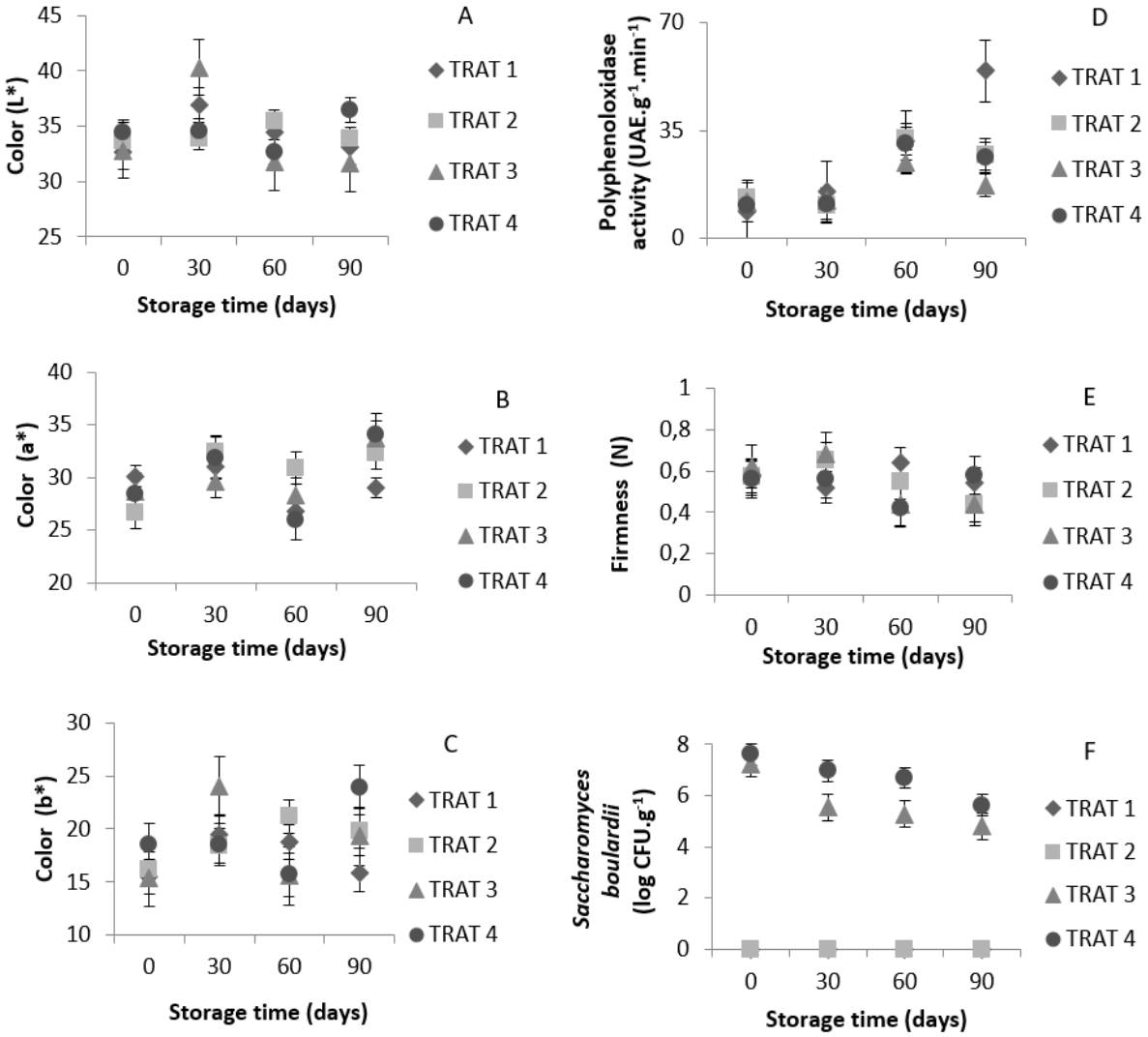

Figure 3. Color (L*) - A, (a*) - B, (b*) - C, polyphenoloxidase activity (UAE g^{-1} $min.^{-1}$) - D, firmness (N) - E and viability of *Saccharomyces boulardii*, expressed by counting fungi (log CFU g^{-1}) - F of frozen strawberries stored for 90 days. Treatment 1: Control 1 - strawberry stored at -18ºC; Treatment 2: Control 2 - strawberry stored at -80ºC; Treatment 3: strawberries added with citric acid, calcium chloride and *S. boulardii*, stored at -18ºC; Treatment 4: strawberries added with gelatin, citric acid, calcium chloride, glycerol and *S. boulardii*, stored at -18ºC. Vertical bars represent the 95% confidence interval.

Regarding the a* coordinate (Figure 3B), values were maintained for up to 30 days of storage, with a subsequent reduction ($p \leqslant 0.05$) in treatment 1. There was a significant increase ($p \leqslant 0.05$) of values in strawberries submitted to treatment 2 during the 90 days of storage. In treatment 3, the a* coordinate values remained constant up to 60 days, with a subsequent increase ($p \leqslant 0.05$). There was an oscillation of the values in strawberries submitted to treatment 4, although with a significant increase ($p \leqslant 0.05$) when comparing the first and last days of storage.

When evaluating the influence of treatment (data not shown), it is observed that there was no distinction between the samples up to 60 days; however, at the end of storage, strawberries coated with citric acid and calcium chloride (treatments 3 and 4) presented higher values ($p \leqslant 0.05$) in coordinate a* (more reddish color), in relation to treatment 1. The values observed in coordinate a* of strawberries submitted to treatment 2 did not differ significantly from those originating from treatments 1, 3 and 4.

Increased a* coordinate values of strawberries stored for 3 months at -27ºC were attributed by Bulut et al. (2018), to the diffusion of pigment from the center of the fruit to the outermost layers of the cells, due to the rupture of cell walls.

Abd-Elhady (2014) also observed higher values (31%) in the a* coordinate of frozen strawberries treated with citric acid (0.4%) and calcium lactate (0.5-1.5%), compared to strawberries control as citric acid inhibits browning reactions and maintains anthocyanin content during freezing.

Different behaviors in relation to time were observed in the values of the b* coordinate of frozen strawberries (Figure 3C). In treatment 1, there was an increase in values in 30 days, with a subsequent reduction in 60 days of storage ($p \leqslant 0.05$). There was a significant increase in the b* coordinate values in the strawberries from treatment 2. In treatment 3, there was an increase in the values in 30 days of storage, with a subsequent reduction in 60 days ($p \leqslant 0.05$). In treatment 4, the values remained constant up to 60 days, with a subsequent increase in values ($p \leqslant 0.05$).

The strawberries submitted to treatment 4 showed higher values ($p \leqslant 0.05$) in the coordinate b* (more yellowish color) when compared to treatment 1, in 90 days of storage (data not shown). There was no significant difference in the b* values of strawberries submitted to treatments 1, 2 and 3.

Higher values in the b* coordinate of frozen strawberries were reported by Abd-Elhady (2014), in the treatments added with citric acid and calcium lactate, compared to the control.

When analyzing the activity data of the polyphenoloxidase enzyme of frozen strawberries submitted to the different treatments, it can be observed that up to 30 days of storage, values were maintained ($p > 0.05$), with a subsequent significant increase ($p \leqslant 0.05$) in relation to the day of processing (point 0), as shown in Figure 3D.

At the beginning of storage, there was no clear trend on the influence of treatments (data not shown). However, at the end of storage, strawberries submitted to treatment 3 showed the lowest values ($p \leqslant 0.05$) of polyphenoloxidase activity, possibly due to the presence of citric acid and the absence of gelatin, which led to greater contact between the antioxidant and the phenolic compounds. It is suggested that gelatin in treatment 4 acted as a substrate for the enzyme due to the presence of the amino acids tyrosine and phenylalanine (Van Vlierberghe, Graulus, Samall, Van Nieuwenhove, & Dubruel 2014). There was no significant difference in enzyme activity in strawberries submitted to treatments 2 and 4. The highest enzyme activity ($p \leqslant 0.05$) was observed in strawberries submitted to treatment 1.

Thus, there was not evident influence of the addition of citric acid and gelatin in the lightness values of strawberries submitted to treatments 3 and 4.

When the enzyme polyphenoloxidase is in the presence of oxygen, it oxidizes anthocyanins and phenolic compounds, causing the formation of quinones that lead to the darkening of fruits (Abd-Elhady, 2014; Oliveira et al., 2015). Although the results demonstrate the influence of the addition of citric acid and gelatin on the polyphenoloxidase activity, there was no clear relationship with the color results.

Citric acid has been used as an anti-browning agent because it inhibits the activity of polyphenoloxidase (Abd-Elhady, 2014).

According to Sulaiman and Silva (2013), the freezing of strawberries at -18ºC and -70ºC did not affect the enzyme activity in the thawed fruits. This is because although the enzyme activity is slowed by the low temperature, the functionality of the enzyme can be fully recovered when samples are thawed.

According to Figure 3E, although fluctuations in firmness values have been observed during the frozen storage of strawberries, there was no significant distinction between the values in relation to the storage time, as well as depending on the treatment ($p > 0.05$) (data not shown), due to the high standard deviation.

Normally, there is a reduction in the firmness of vegetables subjected to freezing. This is caused by the loss of cell turgidity due to the perforation of the plasma membrane by the ice crystals. In addition, the damage done causes the release of enzymes such as pectinases and hemicellulases, which also contribute to this change during thawing (Jha, Xanthakis, Chevallier, Jury, & Le-Bail, 2019).

Different studies have shown a reduction in the firmness of strawberries subjected to freezing even in the presence of coatings and firmness agents (Han et al., 2004; Yu & Liao, 2016).

It can be seen that there was no growth of *S. boulardii* during the 90 days of storage in strawberries from treatments 1 and 2, since there was no addition of probiotics in these treatments. In treatments 3 and 4 there was a significant reduction in the yeast count ($p \leqslant 0.05$) (Figure 3F). In relation to the treatments, throughout the storage, the yeast counts in strawberries from treatment 4 were higher than those obtained in treatment 3 ($p \leqslant 0.05$) (data not shown).

The minimum amount of viable probiotics that foods must have in order to transmit health benefits varies, but the food industry commonly adopts the recommended level of 6 - 7 log CFU g-1 at the time of consumption (Burgain, Gaiani, Linder, & Scher, 2011). Thus, only strawberries submitted to treatment 4 obtained this value (6.69 log CFU g-1), up to 60 days of storage.

The gelatin coating seems to have provided protection to *S. boulardii* against freezing conditions. This was possibly due to the maintenance of water activity, since in treatment 3 there was a greater drip loss and, therefore, a reduction in water activity and leaching of nutrients.

According to Alcarde et al. (2006), colloidal coatings protect against cold damage, because reduce crystallization and retain the liquid phase inside the cell.

According to Guimarães, Abrunhosa, Pastrana, and Cerqueira (2018), edible films and coatings proved to be a good strategy for carrying live microorganisms, demonstrating an improvement in the viability of the probiotic during storage and processing time of the food product.

Conclusion

The gelatin coating added with citric acid and calcium chloride, in combination with freezing at -18ºC, reduced the drip loss, the metabolic reactions and exerted a protective effect on bioactive compounds, making it possible to use them instead of deep-freezing, mainly for agribusinesses that do not have the equipment. The use of gelatin coating on strawberries minimized the effects of freezing and promoted the protection of *S. boulardii*, obtaining in up to 60 days of storage the minimum viable concentration of the microorganism to exercise its functional property. On the other hand, in the absence of gelatin, *S. boulardii* had a negative influence on the physical-chemical parameters, in addition to showing less viability.

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