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Edible Biofilms Formulated with Whey Protein Isolate and *L. casei* Probiotic Culture: Characterization and Application in Tomatoes and Grapes

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This study aimed to produce and characterize edible biofilms based on whey protein isolate (WPI). Two formulations were prepared: (1) without and (2) with probiotic *Lactobacillus casei*. Subsequently, the effect of the biofilms as a fruit cover on the characteristics of *Cherry* tomatoes and *Thompson* grapes was evaluated. The films (with or without probiotic culture) were transparent, malleable, homogeneous, continuous and without cracking. In the infrared spectroscopy, characteristic bands of the N-H bonds (protein structure), the amide bonds (1640 to 1560 cm^{-1}) and the C = O and C-N bands were observed. The addition of the probiotic culture had no effect on the density (1.272-1.303 g/cm^3) and water vapor permeation (0.28-0.35 $g.mm/m^2.day.kPa$) of the films. However, the film containing probiotic culture was yellowish, thicker (16.18 *vs* 13.15 µm), more soluble (42.8 *vs* 34.8%), had higher resistance (higher tensile strength, 23.3 *vs* 12.6 N) and was less flexible (lower elongation at break, 5.27 *vs* 45.4%). Scanning Electron Microscopy images evidenced that the probiotic biofilm presented agglomerates in all superficial extension and a higher number of orifices. The *L. casei* remained viable (5.70 to 7.77 log cfu/g) throughout the storage period of the films (25 °C/28 days), however, recommended counts (> 6 log cfu/g) were observed up to the 14th day. The application of the films did not result in positive impact on the shelf life of the tomatoes but reduced the mass loss and TSS of grapes. The application of the film with probiotic culture resulted in lower TSS values in tomatoes and grapes and higher mass loss in grapes. It can be concluded that the use of WPI originated films with suitable characteristics and could increase the shelf life of grapes. The addition of *L. casei* resulted in alterations on the mechanical properties of the films and suitable probiotic counts for 14 days, with positive effect on the ripening process of the tomatoes and grapes (lower TSS values).

1. Introduction

Whey protein isolate (WPI) is a valuable by-product of the cheese industry and it presents high protein content (> 90%) (Sukyai et al., 2018). Whey proteins are characterized as proteins of high biological value because of the presence of essential amino acids. They also have bioactive peptides as exorphins, immunopeptides and phosphopeptides, besides important functional properties, such as, the high gelation capacity. The utilization of whey proteins in the development of edible films is one of the alternatives for the use of whey (Gallus and Kadzinska, 2016). Edible films can be defined as a thin film produced from polysaccharides, proteins, lipids or derivatives. These films are intended to be a "green" substitute for petroleum-based films, can be consumed along with the product and are usually applied onto the surface of food products by brushing, spraying or dipping (Soukoulis et al., 2014, Saadan et al., 2017). The films based on whey proteins are characterized by

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transparency, flexibility and absence of odor and flavor, allowing their wide utilization (Ket-on et al., 2016). The application of biofilms in fruits can be effective in the increase of the shelf life period, as it can slow detrimental reactions by raising a physical or thermodynamically barrier (Soukoulis et al., 2014), and reducing the rates of gas and moisture transfer between the environment and the fruit (Espitia et al., 2016). In this way, it can maintain the freshness of the fruits for longer time, the quality characteristics (color, acid, sugar and flavor) and the nutritional components (Sessa et al., 2015, Feng et al., 2018).

The addition of chemical substances, enzymes or probiotics to whey protein films can favor their intra and intermolecular bonds, which can result in the improvement of their physical, functional or texture characteristics (Soukoulis et al., 2014). Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts (Hill et al., 2014). The consumption of *L. casei* probiotic culture has been associated to health effects, such as anticarcinogenic properties (Liu et al., 2011), healthier lipid profile (Sperry et al., 2018), reduction of the incidence of antibiotic-associated diarrhea and *Clostridium difficile* infections (Auclair et al., 2015), among others.

Research on the development of edible films incorporated with probiotic cultures is still emerging, therefore, more studies should be conducted (Espitia et al., 2016). The objective of this study was to produce and characterize edible biofilms based on WPI and added or not with probiotic *L. casei*. Subsequently, the effect of the biofilms on the characteristics of *Cherry* tomatoes and *Thompson* grapes was evaluated.

2. Material and methods

2.1 Material

L. casei 01 (Christian Hansen®, Valinhos, SP), WPI (Artesana Doces e Chocolates LTDA®, Novo Hamburgo, RS), glycerol (99.5 % purity, Sigma Aldrich®), magnesium nitrate ($(MgNO_3)_2$, Sigma Aldrich ®), ammonium hydroxide (NH_4OH, Sigma Aldrich ®) and *Cherry* tomatoes and *Thompson* grapes (local market) were used in the experiment.

2.2 Processing of the edible films

The films were produced by the casting methodology. Two types of films were prepared: (1) control and (2) with probiotic culture. For that, the WPI (10% w/v) was dispersed in distilled water and stirred until fully hydration. Then, 5% (w/v) glycerol was added, the pH was adjusted to 9 with NH_4OH (50% w/v) and the solution was heated in a water bath at 90°C for 15 min. The solution was cooled down to 30°C and, for the probiotic film, the *L. casei* was added (0.5% w/v). The film-forming solution was dispersed in Petri dishes (90x15 mm) and placed in a controlled environment (30 ºC for 24 h) for drying. To control the film thickness, the volume of the film-forming solution poured onto the dish was the same, 10 mL. Then, the films were conditioned in a hygrostat environment containing saturated solution of $(MgNO_3)_2$ at 25 °C for 48 h. Equilibrium Relative Humidity was approximately 53.2% (Ket-on et al., 2016). The concentration of WPI, glycerol and *L. casei* and the pH and heating temperature were determined in preliminary tests.

2.3 Characterization of the edible films

The films were analyzed for visual and tactile aspects, verifying the presence of non-solubilized particles, bubbles, color alterations, and the presence of ruptures or brittle zones. The thickness of the films was determined using a micrometer (Series 293-805, Digimatic Micrometers, Mitutoyo, USA, exactness of 0.001 mm) at 15 different points of the film (Feng et al., 2018). The density of the films (pieces of 1 cm^2 area) was calculated as the ratio between the weight and volume (Area x thickness) (Aydogdu et al., 2018). The films were weighed in an analytical balance. The results were reported as averages of ten measurements.

The water solubility (WS) of the films, in triplicates, was measured according to the methodology of Galus et al. (2016). Pieces of the films (1cm x 1cm) were dried in an oven at 105 °C for 24 h to obtain the initial film dry weight (Wi, g). The film was placed in an Erlenmeyer with 50 mL distilled water and stirred for 24 h at 25 °C. Then, the undissolved remnants were filtered out, dried at 105°C for 24 h and weighed (final dry weight, Wf, g). The WS (%) of the films was calculated according to Eq. 1.

$$WS = \left(Wi - \frac{Wf}{Wi}\right) x100 \qquad (1)$$

The Water Vapor Permeability (WVP) was determined in triplicates and according to ASTM E96 (ASTM, 1995). Pieces of the films (3 cm diameter) were analyzed at 24 h intervals for 7 days. The WVP was calculated using Eqs. 2 and 3:

$$WVTR = (\frac{G}{t})/A \qquad (2)$$

$$WVP = \frac{WVTR\ x\ thickness}{(\rho A1 - \rho A2)} \quad (3)$$

Where WVTR is the water vapor transmission rate(g/m^2.day), G/t is the ratio of loss of weight per time (g/day), A is the surface area of the sample (m^2), WVP is the water vapor permeability (g.mm/m^2.d.kPa) and ρA1 and ρA2 are the water vapor partial pressure inside and outside the cup, respectively (kPa).

The Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) spectra of the films were recorded using a spectrophotometer FT-IR (IRPrestig-21, Shimadzu) at wavenumbers in the range of 400–4000 cm^{-1} (Sukyai et al., 2018). Three measurements were taken for each film. The structure of the films was measured using Scanning Electron Microscopy (SEM, FEI Quanta 200). For that, the film was sputtered with a thin layer of gold (20 μm, BALTEC SDC 050 Sputter Coater). The cross-sectional micrographs of the films were imaged with a magnification of 12000x. The mechanical properties of the films (Tensile strength, TS and elongation at break, %E) were determined by TexturePro CT V1.4 Build 17 equipment (Brookfield Engineering Labs, Inc) using the standard method (ASTM, 2010). The films were cut into 60 x 20 cm rectangles. The initial distance between the grips was 40 mm. The measurements were taken at room temperature (25 ^{o}C), and ten replicates were carried out per type of film. The viability of the probiotic culture was determined using Man Rogosa and Sharp (MRS) agar and anaerobic incubation at 37 ^{o}C for 24 h. The films were analyzed in triplicates and weekly for 28 days.

2.4 Tomatoes and grapes storage stability

The fruits were visually checked for uniformity of size and absence of defects. After washing, the selected fruits were immersed in a chlorinated solution (0.1 g/L) for 15 min. Three conditions were tested (n=15 for each): (0) without biofilm, (1) with control biofilm, and (2) with probiotic biofilm. The fruits were dipped into the film-forming solution for 10 s and removed. The excess of coating was removed by draining the fruits for 15 min. The fruits covered with the edible films were stored for 21 days at 25 ^{o}C. The TSS content was determined in an ABBE refractometer (Serie 911705, model I-107-B, Quimis, SP) on days 1, 7, 14 and 21 of storage and the results were expressed in ^{o}Brix. To measure the weight loss, the fruits were weighed in a digital semi-analytical balance, and their mass loss was determined by the difference between the initial mass (day 1) and the final mass (day 21).

2.5 Statistical analysis

Data were analyzed by Student's t-test for comparison of means ($p \leq 0.05$) using Statistica 10 software (Statsoft, Inc. 2011).

3. Results and Discussion

3.1 Characteristics of the films

The film of WPI was transparent, malleable, homogeneous, continuous, without cracking and slightly yellowish (Figure 1A). The film added with the probiotic culture had the same characteristics, except for the coloration, as it presented a dark yellow color (Figure 1B). The characteristics of the films are presented in Table 1. The addition of the probiotic culture had no effect on the density (1.27-1.30 g/cm^3) and WVP (0.28-0.35 g.mm/m^2.day.kPa) of the films ($p > 0.05$). However, the film containing probiotic culture was thicker (16.18 *vs* 13.15 μm), more soluble (42.77 *vs* 34.77%), had higher resistance (higher TS, 23.34 *vs* 12.60 N) and was less flexible (lower %E, 5.27 *vs* 45.38%) ($p \leq 0.05$). The alterations can be associated to the presence of the probiotic culture in the films. In fact, SEM images evidenced that the probiotic film presented agglomerates in all superficial extension and a higher number of orifices (Figure 2B). The control film was uniform without the formation of agglomerates, with a surface with small holes, and with regular and homogeneous superficies (Figure 2A).

Figure 1: Visual appearance of the films. (A) Control film, (B) with L. casei

Table 1: Characteristics of the WPI films

Parameter	Control	Probiotic
Density (g/cm^3)	1.27 ± 0.07^a	1.30 ± 0.06^a
Thickness (µm)	13.15 ± 1.63^b	16.18 ± 1.28^a
WVP (g.mm/m^2.d.kPa)	0.28 ± 0.05^b	0.35 ± 0.09^a
WS (%)	34.77 ± 3.82^b	42.77 ± 1.92^a
TS (N)	12.60 ± 2.28^b	23.34 ± 2.99^a
%E	45.38 ± 12.06^a	5.27 ± 2.34^b

WVP (water vapor permeation, n=3), WS (water solubility, n=3), TS (tensile strength, n=10), %E (elongation at break, n=10), Thickness (n=15), Density (n=10). Mean ± standard deviation with different letters in the same row indicates significant differences ($p \leq 0.05$) among control and probiotic films.

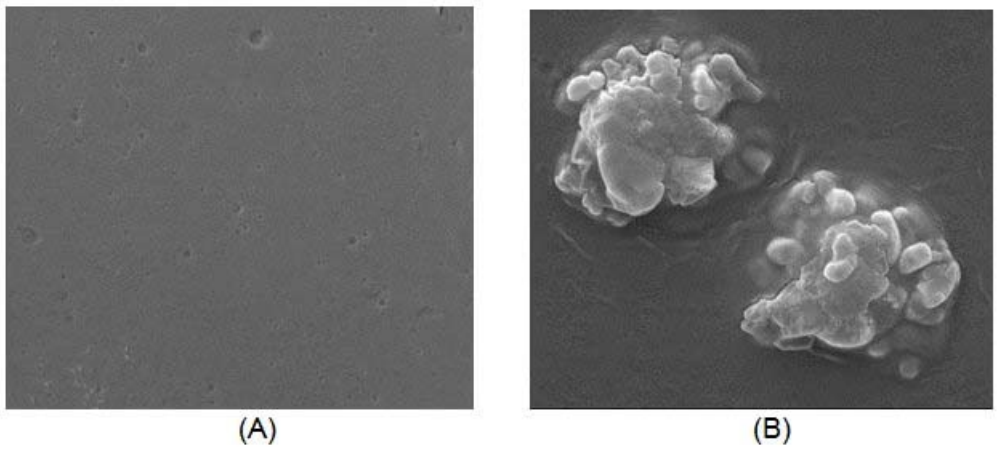

Figure 2: SEM of the films. (A) Control film, (B) with L. casei (12000 x of magnification)

The spectrum of the films is shown in Figure 3. Characteristic bands of the N-H bonds related to protein structures, the amide bonds (1640 to 1560 cm $^{-1}$) and the C = O and C-N bands were observed in both films.

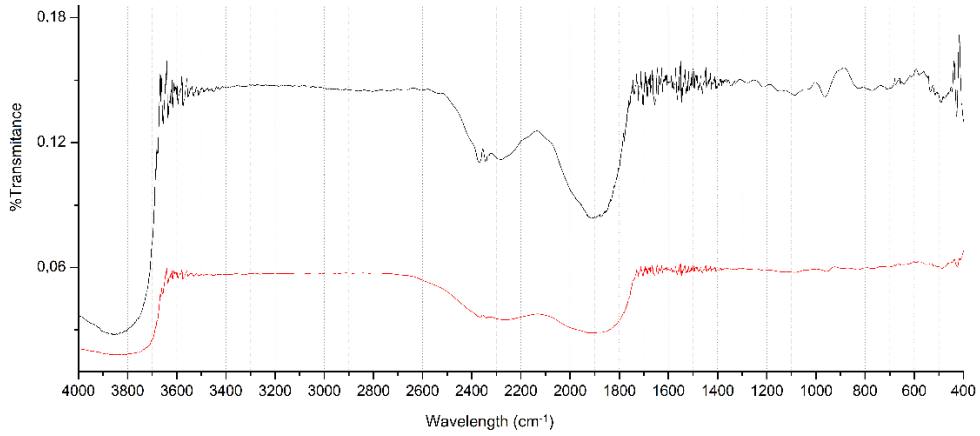

Figure 3: Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) spectra of the films (400–4000 cm^{-1}). Black: control film. Red: probiotic film.

The probiotic viability in the films is presented in Figure 4. The *L. casei* remained viable (5.70 to 7.77 log cfu/g) throughout the storage period of the films (25 ^{o}C/28 days), however, recommended counts (> 6 log cfu/g) were observed up to the 14th day. Loss of viability can be attributed to the stress environment caused by the storage at room temperature and the drying process of the films.

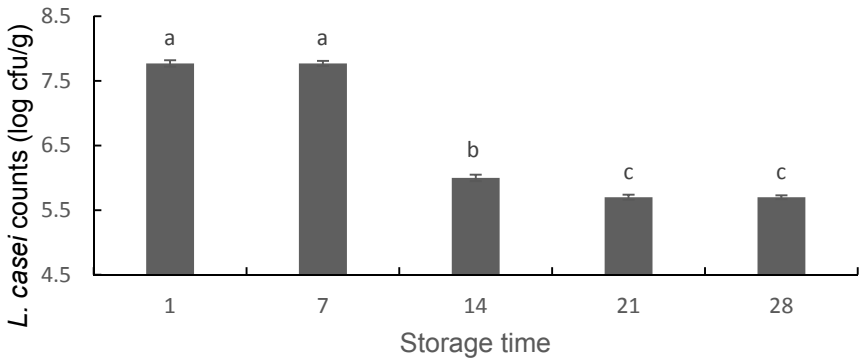

Figure 4: L. casei viability during storage of the films (25 °C) for 28 days.

3.2 Tomatoes and grapes storage stability

The characteristics of the fruits are presented in Table 2.

Table 2: Characteristics of the fruits

Fruit	Parameter	Storage time (Days)	Without film	Control film	Probiotic film
Tomato	TSS (°Brix)	1	3.87 ± 1.24^{Ba}	4.12 ± 0.53^{Ba}	4.50 ± 0.10^{Aa}
		7	4.63 ± 0.53^{Aa}	5.37 ± 0.53^{Aa}	3.88 ± 0.18^{Ab}
		14	4.12 ± 0.18^{ABb}	5.38 ± 0.18^{Aa}	4.13 ± 0.53^{Ab}
		21	4.25 ± 0.71^{Ab}	5.39 ± 0.18^{Aa}	2.63 ± 0.53^{Bc}
	Mass loss (%)	21	25.92 ± 5.46^{a}	26.01 ± 6.88^{a}	20.58 ± 5.92^{a}
Grapes	TSS (°Brix)	1	21.25 ± 2.47^{Ca}	19.38 ± 0.53^{Ca}	18.62 ± 0.53^{Ca}
		7	23.00 ± 2.47^{Ca}	23.00 ± 1.06^{Ba}	23.63 ± 3.30^{Ba}
		14	28.63 ± 3.36^{Ba}	23.63 ± 1.94^{Ba}	26.25 ± 2.48^{ABa}
		21	32.38 ± 2.30^{Aa}	27.75 ± 1.06^{Ab}	27.25 ± 0.71^{Ab}
	Mass loss (%)	21	34.91 ± 0.25^{b}	32.80 ± 0.98^{c}	43.63 ± 0.66^{a}

TSS (Total soluble solids). Mean ± standard deviation with different lowercase letters in the same row indicates significant differences ($p \leq 0.05$) among films. Mean ± standard deviation with different capital letters in the same column indicates significant differences in TSS ($p \leq 0.05$) during storage time. n=15.

The application of the WPI films did not result in positive impact on the shelf life of the tomatoes. It is possible to observe the maintenance of mass loss similar to the products without film ($p > 0.05$) and increased TSS from 14th day of storage ($p \leq 0.05$) but reduced the mass loss of the grapes and the TSS values (day 21) ($p \leq 0.05$). The mass loss during storage is a factor associated with quality loss, and the main objective of the application of edible films is its control. The protection exerted by the film can be related to the filmogenic properties of the WPI, acting as a barrier to water vapor and gas exchange, reducing water loss through transpiration, the respiratory rate and metabolic activity, the use of metabolites and the hydrolysis of carbohydrates to sugars (Hajji et al., 2018). Therefore, the application of the WPI edible film contributed to the maintenance of the characteristics of the grapes, which shows a behavior different from the biofilms, depending on the material covered.

The application of the film with probiotic culture resulted in lower TSS values in tomatoes and grapes (day 21) but higher mass loss in grapes ($p \leq 0.05$) than the product without film. Therefore, the addition of the probiotic films did not contribute to decrease the mass loss of the fruits but retarded the increase in the TSS values.

4. Conclusion

It can be concluded that the use of WPI originated films with suitable characteristics and could increase the shelf life of grapes but not tomatoes. Therefore, the applicability of the film is dependent on the fruit used. The addition of *L. casei* resulted in alterations on the mechanical properties of the films as higher strength and

lower elasticity besides the suitable probiotic counts for 14 days, with a positive effect on the fruit ripening process evidenced by the lower SST. This study proved that the *L. casei* probiotic culture was able to survive in the WPI films for 14 days at 25 ^{o}C and could be incorporated in WPI films. The incorporation of a probiotic edible film in fruits could be an alternative to prolong the ripening process and provide health benefits to the consumer.

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