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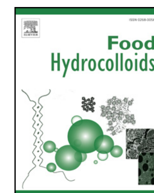

Microencapsulation of *Bifidobacterium animalis* subsp. *lactis* INL1 using whey proteins and dextrans conjugates as wall materials

P.A. Loyeau[a,*], M.J. Spotti[a], N.L. Vanden Braber[b], Y.E. Rossi[b], M.A. Montenegro[b], G. Vinderola[c], C.R. Carrara[a]

[a] *Área de Estudios Fisicoquímicos de Alimentos, Instituto de Tecnología de Alimentos (ITA), Facultad de Ingeniería Química, Universidad Nacional Del Litoral, Santa Fe, Argentina*

[b] *Centro de Investigaciones y Transferencia de Villa María (CIT), Universidad Nacional de Villa María - CONICET, Córdoba, Argentina*

[c] *Instituto de Lactología Industrial (INLAIN), Universidad Nacional Del Litoral - CONICET, Santa Fe, Argentina*

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ABSTRACT

The incorporation of probiotic bacteria to food products is restricted by their instability, so microencapsulation could provide them better protection during storage and gastrointestinal digestion. In this study *Bifidobacterium animalis* subsp. *lactis* INL1 was microencapsulated by spray drying using whey proteins isolate (WPI) and dextrans (DX of 6, 70 and 450 kDa) conjugates obtained by Maillard reaction as wall materials. The stability during storage time and temperature, the viability after simulated gastrointestinal digestion and the antioxidant capacity of the microcapsules were assayed. The cell viability was negatively affected by the gastrointestinal digestion and also over the storage time (12 months). Conjugate with DX 6 kDa was the most stable system at 25 °C and showed improved antioxidant capacity whit $ABTS^{\cdot +}$ technique; meanwhile with $^{\cdot}OH$ technique no differences were found among the samples. Free cells also showed antioxidant activity in all their fractions analyzed using the same techniques.

1. Introduction

In the last years, worldwide food trends aimed at the consumption of food that in addition provides benefits beyond nutrition. In this sense new foods called "functional foods" have emerged, which enhance health conditions, wellness or reduce the risk of certain diseases (Kaur & Pal Singh, 2017). Functional foods not only can incorporate micronutrients, vitamins, essential oils and antioxidants but also, live benefic bacteria. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002).

The incorporation of probiotic bacteria to food products is conditioned by bacterial instability and possible unwanted impacts in organoleptic properties of foods. Consequently, the preservation of viable cell and their incorporation in foods products in the required amounts (10^6–10^8 CFU/g of product) is challenging (Champagne, Paul Ross, Saarela, Flemming Hansen, & Charalampopoulos, 2011). The protection of food-sensitive strains of probiotic bacteria may be achieved by microencapsulation, employing spray drying and encapsulating wall materials, such as: maltodextrin (Slavutsky, Chávez, Favaro-Trindade, & Bertuzzi, 2016), milk (Páez et al., 2013) or whey proteins (Doherty et al., 2011).

Whey proteins are widely used in food industry for different purposes (Beaulieu, Savoie, Paquin, & Subirade, 2002; Wang, Xiong, Rentfrow, & Newman, 2013). These proteins have demonstrated to have good functional properties. These properties can be improved by different approaches, being Maillard reaction one of them. Maillard reaction, also known as non-enzymatic browning reaction or glicosilation reaction consist on the reaction between not protonated amino group of a protein and a carbonil group of a reducing sugar. Maillard reaction products (MRP) may have better functional properties than the starting compounds, for example, better emulsifying properties (Zhu, Damodaran, & Lucey, 2011), antioxidant capacities (Chawla, Chander, & Sharma, 2009) as well as improved nutritional quality and solubility (Wang & Ismail, 2012). In this way, MPR are considered as wall materials with potential to encapsulate not only probiotic bacteria, but also other compounds of interest in food (Choi, Ryu, Kwak, & Ko, 2010; Qu & Zhong, 2017).

The aim of this work was to microencapsulate the breast-milk derived probiotic strain *Bifidobacterium animalis* subsp. *lactis* INL1 using whey protein and dextrans conjugates, obtained by Maillard reaction, through spray drying and to evaluate the stability and antioxidant

* Corresponding author.
E-mail address: loyeau.p@gmail.com (P.A. Loyeau).

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activity of the microcapsules.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) (BIPRO™) was kindly provided by Davisco Foods International Inc. (Minnesota, USA). The composition was 0.4% (w/w) fat, 2.0% (w/w) ashes, 4.8% (w/w) moisture and less than 0.5% (w/w) lactose. According to Kjeldhal method, the protein content was 97.9% (w/w) (dry basis). Dextrans (DX) of 6, 70 and 450 kDa molecular weight were obtained from Sigma-Aldrich (Germany).

The strain used in this work was *Bifidobacterium animalis* subsp. *lactis* INL1, which was isolated from human breast milk (Zacarías, Binetti, Laco, Reinheimer, & Vinderola, 2011) and showed probiotic potential in animal studies (Burns et al., 2017; Zacarías, Reinheimer, Forzani, Grangrette, & Vinderola, 2014).

2.2. Production and characterization of WPI/DX conjugated systems

The conjugates were synthesized by mixing WPI and DX of different molecular weight (6, 70 and 450 kDa), in a WPI:DX weight ratio of 1:0.6. All the solutions were dehydrated in a laboratory-scale spray dryer ADL311S (Yamato, Japan) at a constant inlet air temperature (T_{in}) of 130 °C and outlet air temperature (T_{out}) of 71 ± 1 °C. The obtained powders were incubated for 5 days at 60 °C, and 65% relative humidity to develop the Maillard reaction (Spotti et al., 2013). Four systems were obtained: WPI control (WPI native incubated without DX) called WPIc, and WPI/DX conjugates of DX 6, 70 and 450 kDa called: C-WPI/DX 6, C-WPI/DX 70 and C-WPI/DX 450, respectively.

2.2.1. UV–visible absorption spectroscopy

Early and intermediate compounds of Maillard reaction (called Schiff bases and Amadori products) can be determined at 284 nm (Chawla et al., 2009) and at 304 nm (Amadori products) (Wang & Ismail, 2012).

Late Maillard reaction products (called melanoidins, which produce browning effect) (Chawla et al., 2009; Lertittikul, Benjakul, & Tanaka, 2007) were determined at 420 nm (Jimenez-Castaño, Villamiel, & Lopez-Fandiño, 2007; Miralles, Martinez-Rodriguez, Santiago, van de Lagemaat, & Heras, 2007).

All the samples were dissolved at protein concentration of 0.5% (w/w) and the absorbance measurements were carried out in a Lambda-20 spectrophotometer (Perkin-Elmer, USA). For this analysis a WPI native (WPIn) was also used as a reference.

2.2.2. Free amino groups detection using O-phthaldialdehyde (OPA) technique

To detect free amino groups of WPI (not conjugate to polysaccharides) the *O*-phthaldialdehyde test (OPA, Sigma-Aldrich, Germany) was used according to Sun et al. (2011) with some modifications. Samples were prepared at protein concentration of 4% (w/w). To 200 μl of each sample, 100 μl β-mercaptoethanol (Sigma-Aldrich, Germany), 100 μl of 20% (w/v) SDS and 800 μl of 0.1 M sodium tetraborate buffer solution were added. Samples were heated in a water bath for 5 min at 90 °C. OPA reagent was prepared as follow: 200 mg SDS and 7.62 g disodium tetraborate decahydrated were dissolved in 150 mL of demineralized water. Then, 160 mg of OPA were dissolved in 4 mL of ethanol and this solution was mixed to 400 μl of β-mercaptoethanol. Finally, the solution prepared before was taken to 200 mL with demineralized water. The reaction between 2 mL of OPA and 50 μl of sample solutions was carried out for 7 min. Absorbance at 340 nm was measured in a spectrophotometer 7305 (Jenway, U.K.). To calculate the decrease in free amino groups (FAG) of conjugate samples, WPI native (WPIn) was used as a reference (100%) using equation (1):

$$FAG\,(\%) = \left(\frac{A_S}{A_{WPIn}}\right) x\ 100 \tag{1}$$

Where A_S is the total absorbance of the sample and A_{WPIn} the absorbance of WPI native.

2.3. Production of B. animalis subsp. lactis INL1 microcapsules

B. animalis subsp. *lactis* INL1 was encapsulated using WPIc or the WPI/DX conjugates as wall material. Fresh cultures of the probiotic bacteria were harvested by centrifugation (5836 × g, 10 min, 8 °C) at the end of the exponential growth phase in MRS-C broth (MRS broth with cysteine; Biokar Diagnostic, France). The pellet was washed twice with phosphate-buffered saline (PBS) solution pH 7.4 and resuspended in different solutions: 10% (w/v) of WPI/DX conjugates and WPIc. Cell suspensions were dehydrated in a laboratory scale spray dryer ADL311S (Yamato, Japan). Drying conditions were: feeding rate: 270 mL/h, T_{in} 170 °C, T_{out} 81 ± 1 °C and air flux: 0.1 MPa. The microcapsules (WPIc or WPI/DX conjugates with probiotics) were called: M-WPIc, M-WPI/DX 6, M-WPI/DX 70 and M-WPI/DX 450.

2.3.1. Characterization of B. animalis subsp. lactis INL1 microcapsules

Moisture content of the microcapsules was determined by gravimetry at 105 °C until constant weight. Water activity (a_w) was measured at 25 °C using $a_w$meter Aqualab Systems (USA). These determinations were carried out in triplicates.

The morphology of encapsulated cells was analyzed by scanning electron microscopy (SEM) with Phenom World ProX microscope.

2.3.2. Survival to spray drying

Cell viability was determined before and after spray drying. Microcapsules obtained were reconstituted at 10% (w/v) in 0.1% (w/v) peptone water. Serial dilutions were plated on MRS-C agar and plates were incubated for 72 h at 37 °C in anaerobiosis (AnaeroPack-Anaero, Japan). Counts were expressed in Log CFU/mL units.

2.3.3. Survival to simulated gastrointestinal digestion

This assay was carried out according to Saito et al. (2014). 1 g of encapsulated was mixed with 20 mL of a simulated saliva-gastric solution (NaCl (16.2 g/L), $CaCl_2$ (0.22 g/L), KCl (2.2 g/L), $NaHCO_3$ (1.2 g/L)). Bovine pepsin (Merck, Darmstadt, Germany) was added to a 0.3% (w/v) final concentration. A cell count was performed (t_0) and pH was quickly lowered down to 2.5, with 0.5 N HCl. Samples were brought to 37 °C in a water bath for 90 min. Cell counts were performed as described before every 30 min (Steps named GD t_{30}; GD t_{60} and GD t_{90}). After 90 min of simulated saliva-gastric digestion, the samples were centrifuged (6200 × g, 5 min, 8 °C) (Thermo Scientific, Sorvall Legend Micro 21R, Massachusetts, USA) and the pellets were washed twice with PBS (pH 7.4), and it was resuspended to the original volume in 1% (w/v) bovine bile (Sigma-Aldrich, Germany) at pH 8.0. The cell suspensions were incubated in a water bath for 10 min at 37 °C (step called "Duodenal shock of bile", DSB). A sample was collected for cell viability assessment and another sample was centrifuged (6200 × g, 5 min, 8 °C), the pellet was washed twice with PBS (pH 7.4) and resuspended to the original volume in a solution containing 0.3% (w/v) bovine bile (Sigma-Aldrich, Germany) and 0.1% (w/v) pancreatin (Sigma-Aldrich, Germany) at pH 8.0. Cell counts were performed after an incubation of 90 min (Step named: t_{end}). The test was performed in duplicate and cell viability was expressed in Log CFU/g.

2.3.4. Survival of B. animalis subsp. lactis INL1 during storage

Microcapsules were fractionated, vacuum-sealed, and stored protected from the light at 4 °C and 25 °C. Cell counts were performed every 2 months for one year. Cell counts were performed on MRS-C agar plate (72 h, 37 °C, anaerobic incubation) and expressed as Log CFU/g units.

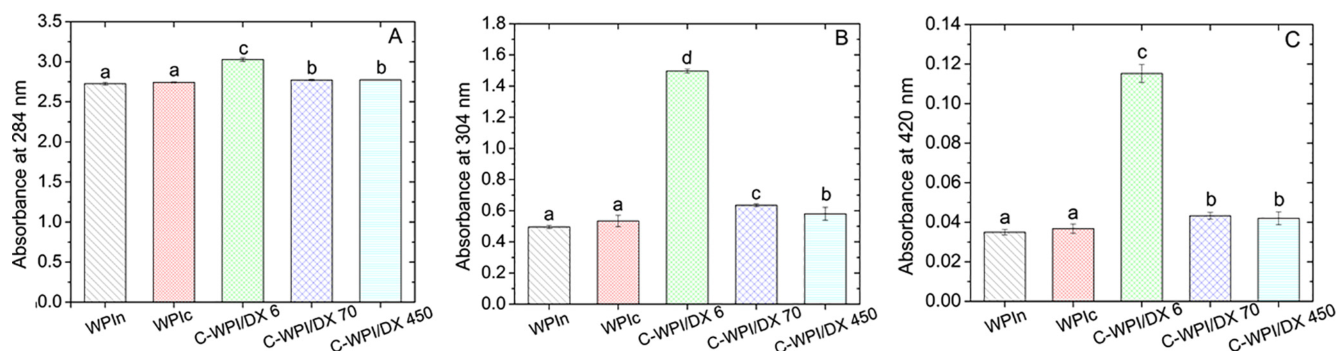

Fig. 1. UV–visible absorbance at 284 (A), 304 (B) and 420 nm (C) of WPIn (WPI native,), WPIc (WPI Control,) and conjugated systems: C-WPI/DX 6 (), C-WPI/DX 70 () and C-WPI/DX 450 kDa (). The values plotted correspond of three independent experiments with its standard deviation. The different small letters indicate significant differences according to the least significant differences test (LSD) ($p < 0.05$).

2.3.5. Antioxidant capacity

The antioxidant activity was evaluated through the study of free radicals scavenging activity of free cells, conjugates (wall materials) and microcapsules. Scavenging activity was evaluated against 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\cdot +}$) and hydroxyl radical ($^{\cdot +}OH$) techniques.

The radical scavenging percentage RS (%) for $ABTS^{\cdot +}$ and $^{\cdot +}OH$ was calculated with equation (2):

$$RS\,(\%) = (A_0 - A_x)\,x\,100 \quad (2)$$

Where A_0 is the absorbance of the control and A_X is the absorbance in presence of conjugates, microcapsules and free cells samples.

The EC_{50} value, defined as the concentration of the sample (mg prot./mL) leading to 50% reduction of free initial radical concentration was obtained from the linear regression of plots (slope) of RS (%) vs. protein concentration.

2.3.5.1. Antioxidant capacity of free cells. The antioxidant capacity of the strain was assayed on different fractions: culture supernatant, intact cell and intracellular cell-free extracts. The preparation process was conducted according to the method of Shen, Shang, and Li (2011). In the preparation process, total cell numbers were adjusted to about 1×10^9 CFU/mL. Through centrifugation at 1084 × g for 10 min, culture supernatant was separated from intact cells. After being washed three times, intact cells were obtained and resuspended in deionized and sterile water. For the preparation of intracellular cell-free extracts, 500 μl of buffer SET (25 mM EDTA; 20 mM TRIS; 75 mM NaCl; pH 7.0) were added. Then, three cycles of: heating (boiling bath, 5min) – freezing (−4 °C, 5 min) were applied. After that, 5 μl of lysozyme solution (10 mg/mL) were added and the cell suspension was incubated for 1 h at 37 °C. The sample was brought to its initial volume with sterile distilled water, and after homogenizing, it was centrifuged for 10 min at 3145 × g to obtain two intracellular cell-free extracts: lysate supernatant and lysate pellet. Each fraction (culture supernatant, intact cell, lysate supernatant and lysate pellet) was evaluated at 1×10^9 CFU (1 mL) to determine the $ABTS^{\cdot +}$ and $^{\cdot +}OH$ radicals scavenging activity, according to the methodology described in the next sections. Results were express as Radical scavenging percentage (RS %).

2.3.5.2. Assay of ABTS cation radical scavenging activity. Scavenging capacity against $ABTS^{c+}$ radical was determined by decolorization test as described by Sarkis, Michel, Tessaro, and Ferreira Marczak (2014) with some modifications. $ABTS^{\cdot +}$ radical was produced by mixing an ABTS solution (7 mM) with potassium persulfate (2.45 mM), and keeping the mixture in the dark at room temperature for 12 h. Afterwards, the $ABTS^{\cdot +}$ solution was diluted in water until absorbance at 734 nm reached 0.70 ± 0.02 units. Aliquots from 100 μl to 600 μL of conjugates or microcapsules, and the volume of fresh culture equivalent to 1×10^9 CFU (1 mL) were mixed with 1 mL of the $ABTS^{\cdot +}$ solution, and absorbance was determined after 6 min. Results were express as EC_{50} value.

2.3.5.3. Assay of hydroxyl radical scavenging activity. Hydroxyl radical ($^{\cdot}OH$) was generated via Fenton reaction at pH 7.4. In presence of 2-Deoxy-D-ribose, the $^{\cdot}OH$ reacts producing malonaldehyde (MDA) among other products, which forms a pinkish adduct in presence of thiobarbituric acid (TBA) allowing its quantification by UV–Vis spectroscopy. The $^{\cdot}OH$ scavenging effect of encapsulates and free cells, were determined as previously described by Boiero et al. (2014). The reaction was performed in 50 mM phosphate buffer (pH 7.4) containing 10 mM 2-deoxy-D-ribose, 100 mMH_2O_2, 1 mM $FeCl_3$, 5 mM EDTA, in presence and absence of the samples. The reaction started with the addition of ascorbic acid in a final concentration of 5 mM. The reaction mixture was incubated for 1 h at 37 °C in a water bath; then, 1% (w/v) TBA and 5.6% (w/v) cold trichloroacetic acid were added and heated up to boiling temperature (95–100 °C) for 20 min to cause the colored adduct formation, which was measured at 532 nm. Results were express as EC_{50} value.

2.4. Statistical analysis

Experiments were replicated at least twice in independent assays. Data was analyzed using one-way ANOVA ($p^{<}0.05$) with StatGraphics Centurion XV software (StatGraphics.net, Madrid, Spain). Least Significant Differences Test (LSD) ($\alpha = 0.05$) was carried out.

3. Results and discussion

3.1. Production and characterization of WPI/DX conjugated systems

3.1.1. UV–visible absorption spectroscopy

Early and intermediate products of Maillard reaction absorb at 284 and 304 nm, and later products at 420 nm (Chawla et al., 2009; Jimenez-Castaño et al., 2007; Miralles et al., 2007; Wang & Ismail, 2012).

In Fig. 1 A the absorbance at 284 nm, which corresponds to Schiff's reagent formation and in Fig. 1 B the absorbance at 304 nm, which corresponds to Amadori products, can be observed. In both graphs, the absorbance of the C-WPI/DX 6 system was higher than for the others systems, indicating that DX of 6 kDa is much more reactive than higher molecular weight DX.

In Fig. 1 C the absorbance at 420 nm of the systems under study is presented. This measurement is indicative of browning in the advanced stages of Maillard reaction (Lertittikul et al., 2007; Sun, Hayakawa, & Izumori, 2004). The C-WPI/DX 6 was the most reactive, meanwhile C-WPI/DX 70 and C-WPI/DX 450 exhibited similar values of absorbance, and WPIn and WPIc were the less reactive systems. Jimenez-Castaño et al. (2007), who studied β-lactoglobulin, α-lactalbumin and bovine

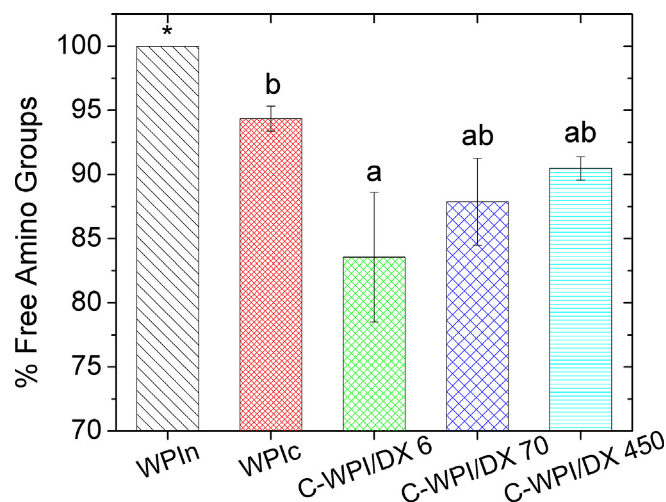

Fig. 2. Quantification of free amino groups respect to WPI native (*consider at 100%; represented by ▧) of WPIc (▩) and conjugated systems: C-WPI/DX 6 (▩), C-WPI/DX 70 (▩) and C-WPI/DX 450 kDa (▤). Results are means of two independent experiments with its standard deviation. The different small letters indicate significant differences according to the least significant differences test (LSD) ($p < 0.05$).

serum albumin (BSA) conjugated to DX of 10 and 20 kDa, found the same tendency, those DX of smaller molecular weight exhibited greater absorbance at 420 nm.

In the three figures WPIc presented slightly higher absorbance that WPIn, probably due to the residual lactose that can react after incubation. However, these systems did not present differences when ANOVA was performed.

3.1.2. Determination of free amino groups

The quantification of free amino groups in conjugate samples (Fig. 2) could provide an estimation of the extent of the Maillard reaction. All WPI/DX conjugates systems presented a decrease in free amino group content with decreasing DX molecular weight, when regarding with WPIn: 90%, 87% and 84% for C-WPI/DX 450, 70 and 6 kDa, respectively. A small decrease in free amino groups was observed for WPIc which could be due to the incubation promotes the chemical reaction between the minimum content of lactose present in the WPI and its proteins during incubation.

As can be observe in Fig. 2, molecular weight of DX significantly affected the percentage of free amino groups, since the smallest DX (6 kDa) is the most reactive one. This result could be explained in terms of the minor steric hindrance and a greater reactivity that smaller molecules could present. Instead, the bulky structures of 70 and 450 kDa DX limit the access at the reactive sites of amino groups. Similarly, Wooster and Augustin (2007) determined the free amino groups of WPI/DX conjugates incubated at 5 days and obtained values of 95, 93 and 87% for conjugates produced with DX of 42.4, 29.4 and 5.9 kDa respectively.

The results of this essay can be correlated with the results obtained in the previous section (3.1.1). C-WPI/DX 6 presented the lowest content in free amino groups and it was the system with the highest absorbances at 284, 304 and 420 nm, indicating that DX 6 kDa was the most reactive DX. Therefore, the lower the molecular weight of DX, the greater extent of Maillard reaction.

3.2. Characterization of B. animalis subsp. lactis INL1 microcapsules

3.2.1. Water activity (a_w), moisture content (%H) and SEM

The water activity (a_w) values of spray-dried microcapsules varied between 0.30 and 0.41, and moisture content (%H) from 7.97 to 10.93%. No tendency in the water activity or moisture content with DX molecular weight was observed in these samples.

Passot, Cenard, Douania, Tréléa, and Fonseca (2012), showed that a_w values close to 0.21 is required to maintain the normal acidification activity of lactic acid bacteria. According to Gardiner et al. (2000), the spray dried powders should have moisture content lower than 4% in order to be stable. Therefore, in line with the bibliography cited, the obtained values of a_w and %H of WPI/DX microcapsules were higher than those recommended.

The SEM microphotographs of microcapsules are shown in Fig. 3. No differences were found in shape or size for the different systems under study. The morphology of the microcapsules was spherical, without cracks or pores, but many of them exhibited typical concavities as a consequence of the spray drying process (Saéns, Tapia, Chávez, & Robert, 2009). It was observed that the size of the capsules was around 10 µm, similar to that reported by Slavutsky et al. (2016), who worked with probiotic encapsulation using low methoxil pectin, maltodextrin and milk power, under comparable operative conditions. Páez et al. (2013), who characterized the functionality of three lactobacilli strains after spray drying in skim milk, also found similar shapes on the encapsulated systems.

3.2.2. Survival of B. animalis subsp. lactis INL1 to spray drying

The cell counts were carried out before and after the spray drying because the heat exposure might affect the probiotic survival. The cell counts of all the microcapsules prior to spray drying were close to 9 ± 0.5 Log CFU/g. The differences between this value and the cell counts after spray drying were: 0.73, 0.50, 0.20 and 0.01 Log orders for M-WPIc, M-WPI/DX 6, M-WPI/DX 70 and M-WPI/DX 450, respectively. M-WPIc, which was the only system that showed a significant difference ($p < 0.05$) between the counts before and after spray drying, was the less protecting system. This results suggests that conjugates maintained the cell viability against of spray drying, probably because they have higher molecular weight. Ramakrishnan, Adzahan, Yusof, and Muhammad (2018) found that higher molecular weight of wall materials resulted in a higher viscosity, which can advance the formation of

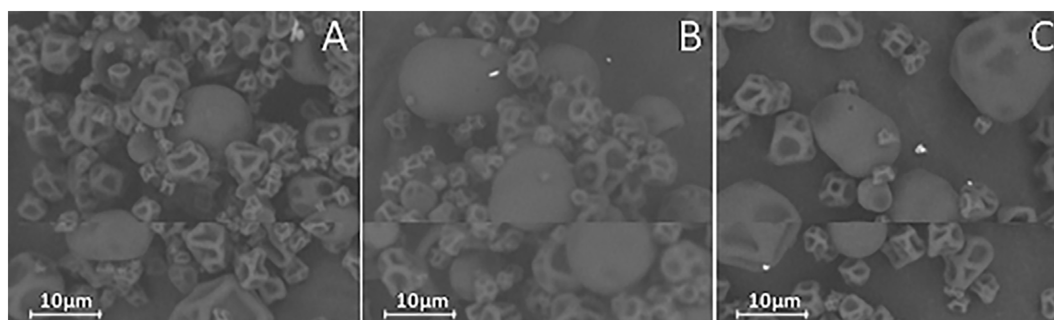

Fig. 3. SEM photomicrographs of spray dried microcapsules (magnification of 5600×), corresponding to the systems: M-WPI/DX 6 (A), M-WPI/DX 70 (B) and M-WPI/DX 450 (C).

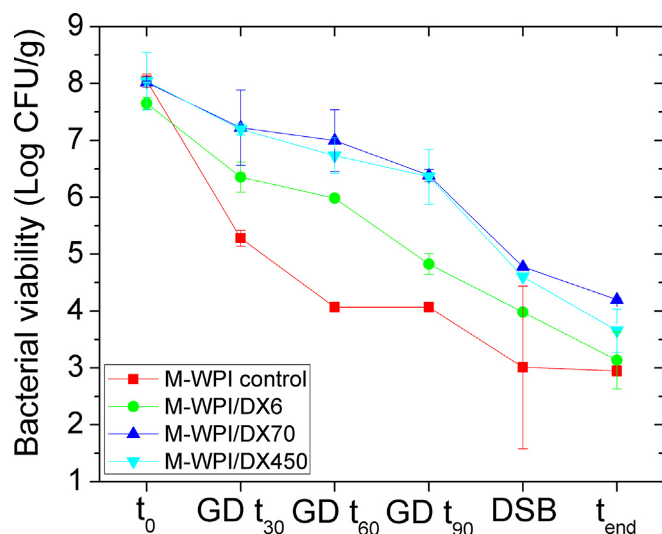

Fig. 4. Cell counts of *B. animalis* subsp. *lactis* INL 1 at the beginning (t_0); after 30, 60 and 90 min of simulated gastric digestion (GD t_{30}, GD t_{60} and GD t_{90}, respectively); then of 10 min of duodenal shock of bile (DSB), and after 90 min of exposure to bile-pancreatin (t_{end}) in M-WPIc (■), E-WPI/DX 6 (●), M-WPI/DX 70 (▲) and M-WPI/DX 450 (▼).

a semipermeable surface during drying and protect the bioactive compounds.

The use of whey proteins or conjugates as encapsulating agents for production of microcapsules can result in different physical properties, depending on the structure and the characteristics of each agent (Fritzen-Freire et al., 2012).

3.2.3. Survival to simulated gastrointestinal digestion

The survival to simulated gastrointestinal digestion of microcapsules is showed in Fig. 4. The microcapsules with WPI/DX conjugates as wall materials were more effective for conferring protection at the strain regarding to WPIc. However, all systems presented a reduction in cell viability at each step assessed. Vinderola et al. (2012), using skim milk as encapsulating system, found similar cell counts (7.66 ± 0.13 Log CFU/ml) at GD $_{t90}$ stage when the same strain was assessed.

Because of the adverse results achieved in the survival to spray drying process and simulated gastrointestinal digestion tests of M-WPIc system, this system was no longer used for the following studies (sections 3.2.4 and 3.2.5).

3.2.4. Survival of encapsulated B. animalis subsp. lactis INL1 along storage

The viability of microcapsules during one year of storage at 4 and 25 °C based on WPI/DX are shown in Fig. 5. Most of the systems showed stable viabilities at 4 °C (Fig. 5 A), being the system M-WPI/DX 450 which displayed a greatest decrease of 1.2 units Log CFU/g at the end of the tested period.

At 25 °C (Fig. 5 B) the reduction in cell counts were pronounced, being M-WPI/DX 6 the most stable system, presenting a decrease of 3.9 units Log CFU/g after one year of storage. The bacterial survival at 25 °C increased whit decreasing DX molecular weight, as can be seeing in Fig. 5 B. The lower probiotic viability at 25 °C could be due to a higher rate in bacteria membrane fatty acids oxidation (Strasser, Neureiter, Geppl, Braun, & Danner, 2009), which damage the probiotic cell.

3.2.5. Antioxidant capacity of free cells B. animalis subsp. lactis INL1, WPI/DX conjugates and M-WPI/DX

Some authors reported that protection against oxidative stress and the capacity to decrease the risk of accumulation of reactive oxygen species (ROS) are other beneficial effects presented by probiotics (Martarelli et al., 2011). As the viability of the encapsulated strain implies the preservation of its probiotic and antioxidant properties, their antioxidant capacity should be maintained after microencapsulation (Golowczyc, Silva, Teixeira, De Antoni, & Abraham, 2011; Huang et al., 2017). Because of that, the antioxidant effect of strain *Bifidobacterium animalis* subsp. *lactis* INL1 and their microcapsules were evaluated.

In Table 1, deactivation percentages of $ABTS^{\cdot+}$ and $^{\cdot+}OH$ radicals used to evaluate the antioxidant capacity of probiotic strain are presented. As can be observed, all analyzed cell fractions exhibited excellent antioxidant capacity with both radicals. Different techniques presented different tendency since the deactivation mechanism change with the radicals used in each test.

Shen et al. (2011) found that cell supernatants, intact cells and lysate supernatants of another strain of bifidobacteria exhibited radical scavenging activity with the $^{\cdot}OH$ technique. Amaretti et al. (2013) have shown that another strain of *Bifidobacterium animalis* subsp. *lactis* presented antioxidant capacity using diverse techniques such as determination of ascorbic and linolenic acid oxidation, trolox-equivalent antioxidant capacity and intracellular glutathione. Huang and Chang (2012) also found similar results with $ABTS^{\cdot+}$ and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) techniques in *Bifidobacterium adolescentis* strain.

Antioxidant capacity from cell supernatant could be attribute to presence of some molecules, which are produced and liberated to the medium by the probiotic strain. These molecules could be exopolysaccharides, produced by several strains of Bifidobacterium as described by Salazar et al. (2011) and Hidalgo-Cantabrana et al. (2016).

In Table 2, the antioxidant capacities of WPIn, WPI/DX conjugates (C-WPI/DX) and microcapsules (M-WPI/DX) are presented. C-WPI/DX and M-WPI/DX showed better antioxidant capacity than WPIn regarding to $ABTS^{\cdot+}$ technique.

Regarding to the C-WPI/DX systems, the $ABTS^{\cdot+}$ radicals scavenging (reducing power) decreased with increasing in DX molecular weight: C-WPI/DX 6 > C-WPI/DX 70 > C-WPI/DX 450, which was

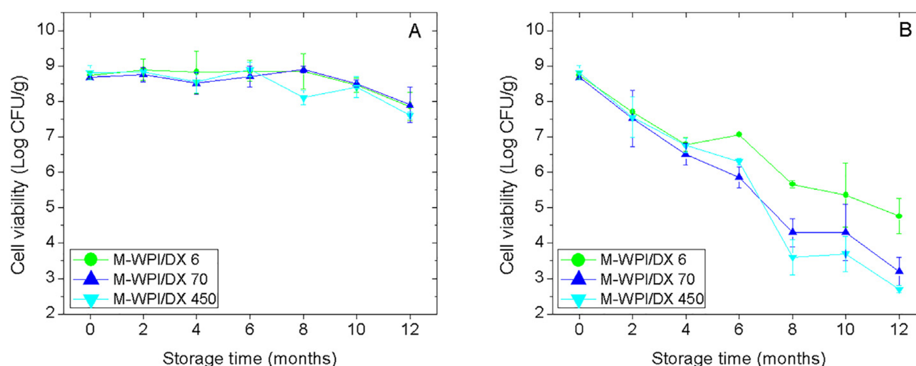

Fig. 5. Probiotic cell viability (Log CFU/g) for encapsulated *B. animalis* subsp. *lactis* INL1 at 4 °C (A) and 25 °C (B).

Table 1
Radical scavenging percentage (RS %) of different radicals evaluated on 1 mL of different fractions obtained from a probiotic cell culture of *Bifidobacterium animalis* subsp. *lactis* INL1.

Samples	RS % $ABTS^{\cdot+}$	RS % $^{\cdot}OH$
Culture supernatant	79,26 ± 20,74	88,42 ± 4,47
Intact cell	12,31 ± 1,43	37,38 ± 6,61
Lysate supernatant	98,90 ± 0,09	65,89 ± 3,30
Lysate pellet	22,32 ± 7,35	57,01 ± 22,47

Table 2
EC_{50} corresponding to $ABTS^{\cdot+}$ and Fenton ($^{\cdot}OH$) techniques, for all systems: WPIn (native), conjugates (C-WPI/DX) and microcapsules (M-WPI/DX).

Samples	EC_{50} (mg protein/mL)	
	$ABTS^{\cdot+}$	$^{\cdot}OH$
WPIn	1.17 ± 0.01[F]	1.26 ± 0.01[A]
C-WPI/DX 6	0.89 ± 0.02[aA]	1.53 ± 0.03[aCD]
M-WPI/DX 6	0.72 ± 0.01[bB]	1.30 ± 0.02[bB]
C-WPI/DX 70	1.00 ± 0.03[aC]	1.34 ± 0.16[aBC]
M-WPI/DX 70	1.10 ± 0.02[bE]	1.38 ± 0.03[aBC]
C-WPI/DX 450	1.05 ± 0.02[aD]	1.46 ± 0.25[aBCD]
M-WPI/DX 450	1.04 ± 0.01[aD]	1.65 ± 0.09[aD]

Mean of three independent experiments with its standard deviation (SD), for each sample. [a, b] Indicate significant differences using the least significant differences test (LSD) ($p < 0.05$) between conjugate and microcapsule synthesized with the same DX. [A, B, C, D, E, F] Indicate significant differences using the least significant differences test (LSD) ($p < 0.05$) between all systems analyzed in the same column.

confirmed by statistical analysis (significant differences between conjugates, see capital letters in $ABTS^{\cdot+}$ technique of Table 2). According to Wang, Qian, and Yao (2011), browning compounds formed during the Maillard reaction, which are primarily composed of melanoidins (detected at 420 nm, see section 2.2.1), are the major contributors to the radical scavenging capacity. In the present work, C-WPI/DX 6 showed the highest $ABTS^{\cdot+}$ radical scavenging which also presented the greatest extent of Maillard reaction, as have been seeing by the lowest amount of free amino groups and also the greater amounts of melanoidins (highest absorbance at 420 nm). Liu, Kong, Han, Sun, and Li (2014) found similar results working with WPI/glucose conjugates that showed higher antioxidant capacity than WPI alone, using $ABTS^{\cdot+}$ technique.

With respect to microcapsules, no tendency was found between M-WPI/DX and the DX molecular weight. M-WPI/DX 6 presented the highest $ABTS^{\cdot+}$ radical scavenging of all the systems. Moreover, it was the only one that enhanced the antioxidant capacity regarding to its corresponding conjugate.

In relation to the $^{\cdot}OH$ radical scavenging, it was observed that conjugates and microcapsules showed less deactivation capacity than WPIn (they presented higher values of EC_{50}). Even though these results, EC_{50} values presented the same order of concentration than WPI suggesting that the conjugates can be still considered antioxidants.

The mechanism of deactivation of $^{\cdot}OH$ radical is based on the reaction with H^+ (Dasgupta & Klein, 2014). The reactive groups (H^+ donors) of WPIn and microcapsules are amino and hydroxyl groups, being greater the reactivity of amine groups (Yang, Guo, Miao, Xue, & Qin, 2010). In this sense, the decrease in the $^{\cdot}OH$ radical scavenging activity of conjugates, could be due mainly to the decrease in free amino group's content that these systems underwent during Maillard reaction. As we showed in Fig. 2, C-WPI/DX 6 presented the lowest free amino group content and also the highest EC_{50} value (the lowest radical scavenging activity).

Additionally, due to the high reducing power shown by many MRP (Liu et al., 2014; Wang, Bao, & Chen, 2013) it is possible that the conjugates could interfere in the Fenton reaction, reducing Fe^{3+} to Fe^{2+} and exerting a catalytic effect on the $^{\cdot}OH$ radical generation (Yoshimura, Iijima, Watanabe, & Nakazawa, 1997). This $^{\cdot}OH$ radicals excess would produce a greater amount of malonaldehyde and malonaldehyde - thiobarbituric acid adduct, which are the products that this technique determine. As a consequence, a decrease in the $^{\cdot}OH$ radical scavenging activity of conjugates against WPIn is observed.

4. Conclusions

It was possible to synthesize WPI/DX conjugates using controlled dry-heating. Also, the occurrence of the Maillard reaction was verified in all the systems through determinations of absorbance and by the determination of free amino groups.

Bifidobacterium animalis subsp. *lactis* INL1 was successfully encapsulated using WPI/DX conjugated systems as wall materials.

After the spray drying, all the M-WPI/DX systems showed better cell counts compared to M-WPIc, which was the less protective one. In the simulated gastrointestinal digestion test no differences were found between M-WPI/DX systems, but those presented better protection that M-WPIc.

Respect to stability in storage time and temperature, C-WPI/DX 6 resulted in the best wall material at 25 °C. Meanwhile, at 4 °C all the systems showed the same final cell count.

The antioxidant capacity of different fractions of free cells, conjugates and microcapsules was evaluated by $ABTS^{\cdot+}$ and $^{\cdot}OH$ techniques. All the cells fractions showed radical scavenging activity with both techniques. However, conjugates and microcapsules presented higher antioxidant capacity than WPIn only with $ABTS^{\cdot+}$, but lower with $^{\cdot}OH$ radical. The M-WPI/DX 6 presented the best antioxidant capacity, even better than C-WPI/DX 6 indicating a synergic relation between the strain and the wall material.

Taking into account all these results, C-WPI/DX 6 resulted to be the best system to encapsulate *Bifidobacterium animalis* subsp. *lactis* INL1.

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