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Incorporation of *Lactobacillus delbrueckii* subsp *lactis* (CIDCA 133) in cold-set gels made from high pressure-treated soybean proteins

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ABSTRACT

The ability of freeze-dried high pressure-treated soybean proteins to form cold-set gels and their aptitude to act as a protective matrix to *Lactobacillus delbrueckii* subsp *lactis* strain CIDCA 133 were evaluated. Gels were obtained by dispersing denatured soybean protein isolates (SPI) in water or in a lactobacilli suspensions, and further incorporation of calcium. When SPI were dispersed in water, ordered, adhesive, and with high water holding capacity gels were obtained at a calcium concentration of 0.015 mol L^{-1}. Increase in calcium concentration to 0.020 mol L^{-1} increased elastic modulus (small deformation rheology) and maximum force (texture profile) and made gels become opaque. When protein concentration was 90 g L^{-1}, and denaturation of SPI was carried out at 600 MPa gels with higher values of elastic modulus were obtained as compared with those from gels denatured at 400 MPa. After dispersing denatured SPI in lactobacillus suspensions the gels were turbid and with a pink-like color. Elastic modulus and tan δ of gels prepared in lactobacilli suspensions were higher than those dispersed in water, revealing that matrix was modified by lactobacilli presence. The lactobacilli included in these cold-set gels survived better than in coagulated milk to a modeled gastric challenge. Moreover, bacteria remained viable without damaging the matrix during a 28-days storage at 4 °C. The high soybean protein concentration would be responsible for lactobacilli protection. Our results suggest that SPI cold-set gels may be used as carriers for lactobacilli protecting them from the effect of simulated gastric juice and enabling their incorporation in functional foods.

1. Introduction

Soybean storage proteins are widely used in food industry because of their nutritional value, functional properties, low cost and health-improving effects of themselves and/or associated compounds. They are mainly represented by β-conglycinin, a trimeric globulin made up of the combination of the α, α′ and β polypeptides, and glycinin, an hexameric globulin made up of AB subunits (Badley et al., 1975; Thanh & Shibasaki, 1977). These proteins are prepared as soybean protein isolate (SPI) with approximately equal quantities of β-conglycinin and glycinin. Protein denaturation, that is often a prerequisite to improve functionality, may be achieved by different treatments. High pressure (HP) processing is an emerging technology in food industry. This treatment denaturates soybean proteins leading to degrees of denaturation that depend on pressure level (100% for glycinin and near 70% for β-conglycinin after a treatment at 600 MPa) (Speroni, Añón, & de Lamballerie, 2010). Dehydrated SPI is an interesting product because it may be cheaply conserved and transported, and may be re-dispersed in different media, conferring functional, nutritional and health-promoting properties to foods. Wang et al. (2008) studied some functional properties of freeze-dried SPI, that were HP-treated before freeze-drying. These authors found that aggregation phenomena were dependent on protein concentration during HP treatment, and that freeze-drying from different protein concentration affected protein solubility.

Gelation is one of the most outstanding properties of soybean proteins. Heat-induced gelation was studied (Hermansson, 1986; Puppo & Añón, 1998). Cold-set gelation of soybean proteins was achieved from heat-denatured (Maltais, Remondetto, Gonzalez, & Subirade, 2005) and from high pressure-denatured (Speroni & Añón, 2013). The strategy in cold-set gelation consisted in incorporate $CaCl_2$ at low temperature to a fluid dispersion of denatured proteins. $CaCl_2$ shields electrostatic charge of polypeptides and form bridges (crosslinking) enhancing protein association and gel

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formation (Marangoni, Barbut, McGauley, Marcone, & Narine, 2000). Cold-set gelation provides the possibility of incorporate thermally unstable compounds or microorganisms to the matrix during the gelation step.

Probiotics are defined as food products containing viable microorganisms that, when administrated in adequate amounts, confers a beneficial effect on the health of the host (FDA, 1995). Traditionally, probiotics have been included in the formulation of dairy products such as fermented milk or yoghurt. However, there is an increasing interest in the development of non-diary probiotics products destined to individuals suffering from milk proteins allergy or lactose intolerance (Khalf, Dabour, Kheadr, & Fliss, 2009; Yoon, Woodams, & Hang, 2005, 2006). Viability of microorganisms in probiotic products is highly relevant for the health promoting effects (Succi et al., 2005), thus protection of the microorganisms through encapsulation or nano-encapsulation was investigated (Gbassi & Vandamme, 2012).

Lactobacillus delbrueckii subsp *lactis* (CIDCA 133) is a potentially probiotic strain that have shown interesting properties such as the ability to antagonize growth of food spoilage and pathogenic microorganisms as well as the inhibition of harmful enzymatic activities (Hugo, De Antoni, & Pérez, 2006; Hugo, Kakisu, De Antoni, & Pérez, 2008; Kociubinski, Pérez, Añón, & De Antoni, 1996; Kociubinski, Pérez, & De Antoni, 1999). However, this strain has a low resistance to simulated gastric conditions (Hugo, 2007) that could drastically reduce its viability after oral administration. Among the principal criteria to select a probiotic strain is its ability to survive in the gastrointestinal tract and the capacity to remain viable during storage (Tamime, Saarela, Korslund, Mistry, & Shah, 2005). The inclusion of these lactobacilli in a protective matrix could allow them to reach the intestinal tract in high concentrations to exert their beneficial effects.

Taking into account these findings, the aims of the present work were to study the cold-set gel-forming ability of HP-denatured and freeze-dried SPI, and to use these gels as a protective matrix for *L. delbrueckii* subsp *lactis* strain CIDCA 133.

2. Materials and methods

2.1. Preparation of soybean protein isolate

Soybean protein isolate (SPI) was prepared by alkaline extraction, followed by isoelectric precipitation from defatted soybean flour (ADM, Decatur, USA) according to Speroni, Jung, & de Lamballerie, 2010. The freeze-drying system consisted in a Heto FD 4 apparatus (Waltham, Massachusetts, USA), connected to a Vacuubrand RZ5 vacuum pump (Wertheim, Germany). As previously reported (Speroni, Jung, et al., 2010), SPI mostly contained β-conglycinin and glycinin in similar quantities and small amounts of lectin and Kunitz trypsin inhibitor. Protein content was 86.6% (N-factor 5.8); values for moisture and ash were 5.8 and 4.7%, respectively, whereas that for lipids and carbohydrates, mainly dietary fiber (by difference) was 3%.

2.2. High pressure treatment

Aqueous dispersions of SPI at 90.0 g L^{-1} and pH 8.0, underwent high pressure (HP) treatment at 400, or 600 ± 5 MPa for 10 min in a 2.0 L reactor unit model FPG 9400:922 (Stansted Fluid Power Ltd, United Kingdom). Prior to pressure processing, protein dispersions were vacuum conditioned in polyethylene bags. A mixture of propylene glycol and water (30:70) was used as pressure-transmitting medium. The target pressure was reached at 6.5 MPa s^{-1} and released at 20 MPa s^{-1}. The adiabatic heating was manifested as an increase in temperature that reached a maximum at 600 MPa. In that case, a transient increase of temperature of reactor was verified, reaching up to 33.5 °C. After HP treatment, samples were frozen at −80 °C and freeze-dried in the previously mentioned freeze-dryer system.

2.3. Bacterial strains and growth conditions

L. delbrueckii subsp *lactis* strain CIDCA 133 belongs to the culture collection of the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina). The strain was conserved frozen at −80 °C in de Man, Rogosa, Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) with 10% (v/v) glycerol and was reactivated twice in liquid medium before the experiments. Lactobacilli were grown in anaerobic conditions in MRS broth. For gel or coagulated milk preparation, lactobacilli were harvested by centrifugation at 4000 × *g* for 10 min at 10 °C and dispersed in phosphate buffered saline (PBS), composed by (g L^{-1}) 0.144 KH_2PO_4; 9.00 NaCl; 0.795 Na_2HPO_4 at pH 7.0 and at a concentration of 2.5 × 10^8 CFU mL^{-1}.

2.4. Gel formation

Freeze-dried SPIs were dispersed in water or lactobacilli culture. Protein concentration was 85.0 or 90.0 g L^{-1}, dispersions were stirred for 2 h, calcium chloride was incorporated from a stock solution of 1.0 mol L^{-1} of $CaCl_2 \cdot 2H_2O$ (Sigma, St Louis, USA), at a concentration of 0.015 or 0.020 mol L^{-1}. Samples were kept 12 h at 4 °C in acrylic cylinders of 14 mm diameter. The height of gels ranged between 9 and 14 mm. The bottom of the cylinder was removable and gels were pulled out with the aid of a piston before testing.

Samples were identified by the values of protein concentration (g L^{-1}) − HP level (MPa) and − calcium concentration (mol L^{-1}). For example 90−600−0.020 means that the sample contained 90 g L^{-1} protein, was treated at 600 MPa and 0.020 mol L^{-1} $CaCl_2$ was then incorporated.

2.5. Water holding capacity of gels

Portions of gels of about 1 g were centrifuged at 19,000 × *g* for 20 min at 4 °C. Supernatant was separated and the pellet was weighted. Water loss was determined by weighing before and after centrifugation. Water holding capacity (WHC) was expressed as follows:

WHC = Wa/Wo × 100; where Wa is the water weight in the gel after centrifugation and Wo is the water weight in the original gel.

2.6. Color of gels

Color of gels was determined by using a Minolta Chroma meter (CR 300, Minolta Chroma CO., Osaka, Japan). The instrument was standardized using a white calibration plate. A CIE Lab color scale was used, and L^* (lightness), a^* (related with the red (positive values) − green (negative values) opposition) and b^* (related with the yellow (positive values) − blue (negative values) opposition) were determined. Since some gels were transparent, the determinations were carried out on 5 mm thick portions of gels placed on a white surface, so that the effect of transparence could be standardized.

2.7. Small deformation rheology

Dynamic rheological measurements (storage modulus (G') and loss modulus (G'') *vs.* frequency) were carried out at 20 °C using a

serrated plate-and-plate geometry (35 mm diameter, 1 mm gap) using a Controlled Stress Rheometer Haake RS 600 (Thermoelectron, Karlsruhe, Germany). Frequency of oscillations ranged from 0.01 to 10 Hz. The linear viscoelasticity region was determined through stress sweep tests at a fixed frequency (1 Hz), all samples were analyzed at 1 Pa, which was within the range of linear viscoelasticity. Tan δ was calculated as G''/G'. Before starting the corresponding measurement, samples were allowed to rest for 10 min after positioning the sample on the sensor system. Rheological behavior was measured in lactobacilli containing gels every 7 days during 28 days.

2.8. Texture profile analysis

Texture profile analysis was performed on gels using a TA.XT2 Texture analyzer (Stable Micro Systems Ltd., Surrey, United Kingdom) in the compression mode. Compression was exerted by a cylindrical probe with a flat section (75 mm diameter) at a displacement speed of 0.5 mm s^{-1}. The force (N) at 20% compression was measured. Since the gels were relatively soft and differed in strength depending on the formulation and HP level, their shape was modified after being removed from the cylindrical cast. This modification was verified as an increase in the area of the top face of the gel cylinder and a decrease in height, which was greater in the softest gels. This fact was accompanied by the exposure of a higher area to the probe in the cases of the softest gels. In order to normalize this measurement, the force at 20% compression (maximum force) was divided by the area of the gel at the moment of 20% compression. Thus, maximum force/area ratio was used as indicator of gel hardness.

The measurements of gel adhesivity were performed with two compression cycles. Gel adhesivity was calculated as the negative force *vs.* displacement area (N mm) obtained after the first compression cycle, representing the work necessary to pull the compressing plunger away from the sample.

Texture profile was analyzed every 7 days for 28 days in lactobacilli-containing gels that were stored at 4 °C.

2.9. Survival of lactobacilli during cold storage

Survival of the lactobacilli included in SPI gels, coagulated milk, or phosphate buffered saline (PBS), during cold storage was analyzed. Coagulated milk was obtained as a result of bacterial growth and metabolic activity by inoculating lactobacilli at 10% (v/v) in sterilized milk and incubating at 37 °C for 16 h. Coagulated milk was used as a typical protective matrix of lactic acid bacteria. Samples were stored at 4 °C for 42 days. Viable bacteria were determined by plate counts on MRS agar after different times of storage.

2.10. Susceptibility of lactobacilli to simulated gastric conditions

The tolerance of the lactobacilli included in SPI gels, coagulated milk or PBS to acid and pepsin was evaluated. Bacterial suspensions in PBS or coagulated milk obtained after 18 h of lactobacilli growth in sterilized milk were used as controls. Artificial gastric juice was adapted from Mozzi, Gerbino, Font de Valdez, and Torino (2009). It was composed of (g L^{-1}) 1.012 KCl; 2.00 NaCl; 0.11 $CaCl_2$; 0.40 KH_2PO_4, adjusted at pH 2.0 with HCl and sterilized at 121 °C for 15 min. Pepsin (Sigma, St Louis, USA) was used at 0.1 g L^{-1} final concentration and was added prior to the assay. Samples were added at a ratio of 1:2 to the artificial gastric solution and were incubated at 37 °C for 2 h with constant shaking. The pH of the mixtures was controlled every 30 min and HCl was added when necessary to keep a pH of 2.0. Viability of bacteria at different time points was assessed by plate counts.

The microbial survival was analyzed using **equation 1:** $2.303 \ln N_0/N = k\ t$ and **equation 2:** $D = 2.303/k$ where N_0: CFU mL^{-1} of samples at the beginning of the assay; N: CFU mL^{-1} after the incubation with the gastric solution; k: rate constant of microbial inactivation; t: time of incubation; D: Decimal reduction time (when $N = 0.1\ N_0$).

2.11. Bacterial plate counts

Gels containing bacteria or bacterial suspensions were diluted 1/10 in PBS. Suspensions were gently vortexed to allow for matrix disintegration. Then bacterial suspensions were serially diluted in 8.5 g L^{-1} NaCl aqueous solution. Bacterial counts were determined after 48 h of incubation in MRS agar at 37 °C.

2.12. Statistical analysis

Gel formation and characterization of gel and survival and susceptibility of lactobacilli were performed at least in triplicate. The statistical analysis was completed using the Origin software (OriginLab Corporation, Northampton, MA, USA). Analyses of variance were conducted. Differences between the sample means were analyzed by Tukey's test ($P < 0.05$).

3. Results and discussion

3.1. Cold-set gelation of HP-treated and freeze-dried SPI dispersed in water

The ability of HP-treated and freeze-dried SPI to gellify at low temperature was evaluated by dispersion of SPI in water and further calcium addition. Cold-set gels were obtained in all the conditions assayed. Transparent gels, with an amber-like color were obtained when calcium was 0.015 mol L^{-1} ($L^* = 62.11 \pm 1.26$, $a^* = 3.39 \pm 0.18$, $b^* = 25.92 \pm 1.16$); while opaque gels, with a whitish aspect were obtained when calcium was 0.020 mol L^{-1} ($L^* = 53.70 \pm 0.78$, $a^* = 2.96 \pm 0.24$, $b^* = 13.68 \pm 1.23$). These results indicate that calcium concentration affected the values of L^* and b^*. Opacity was due to the presence of light-scattering insoluble aggregates in the gel matrix, which formation is favored by the low protein/calcium ratio. The visual aspect of gels is related to the formation of ordered (transparent) and aggregated (opaque) gels (Hermansson, 1986). The results from small deformation rheology showed that G' at 1 Hz increased when calcium concentration increased from 0.015 to 0.020 mol L^{-1} for every protein concentration and HP level assayed (Table 1). The increase in pressure level increased G' when protein concentration was 90.0 g L^{-1} (Table 1). The values of tan δ ranged from 0.20 to 0.32, although G' was higher than G'', the values of tan δ indicate a considerable viscous character. The data from texture profile analysis showed that the maximum force/area ratio was dependent on HP level and sample composition, the hardest gel was obtained at 90–600–0.020, the differences between the values of hardness under other conditions were not of great magnitude (Table 1). Adhesivity was different from zero when calcium concentration was 0.015 mol L^{-1}. The values of adhesivity were inversely related with the values of G', being the most adhesive gels those formed at 85–400–0.015 (Table 1). This fact agrees with the observations of Dobraszczyk (1997) who stated that the bulk rheological properties of a material govern its adhesivity. In addition, these results agree with those reported by Speroni and Añón (2013) who obtained gels from HP-treated SPI and calcium, but without freeze-drying SPI after HP treatment. Tang, Chen, and Foegeding (2011) studied the influence

Table 1
Characterization of cold-set gels obtained from calcium-added, high pressure-treated, freeze-dried soybean protein isolates, dispersed in water (W) or in lactobacilli culture (LB). Elastic modulus (G'), maximum force/area ratio, adhesivity, and water holding capacity (WHC).

Gel formulation and high pressure level	G' Pa	Max force/area ratio N mm^{-2}	Adhesivity N mm	WHC %
85–400–0.015–W	23.5 ± 3.4^{a}	0.00027 ± 0.00001^{a}	0.08090 ± 0.01104^{e}	99.7 ± 0.2^{a}
85–400–0.020–W	52.4 ± 3.6^{bc}	0.00032 ± 0.00001^{bc}	0.00157 ± 0.00151^{ab}	61.7 ± 2.8^{c}
85–600–0.015–W	38.8 ± 3.3^{ab}	0.00031 ± 0.00001^{ab}	0.01515 ± 0.00061^{d}	99.8 ± 0.1^{a}
85–600–0.020–W	63.1 ± 4.6^{c}	0.00036 ± 0.00002^{c}	0.00002 ± 0.00002^{ab}	92.6 ± 0.9^{b}
90–400–0.015–W	43.5 ± 4.4^{b}	0.00031 ± 0.00001^{ab}	0.01952 ± 0.00480^{d}	99.7 ± 0.1^{a}
90–400–0.020–W	72.9 ± 2.7^{c}	0.00034 ± 0.00001^{bc}	0.00342 ± 0.00266^{ab}	93.4 ± 0.5^{b}
90–600–0.015–W	59.2 ± 1.3^{c}	0.00032 ± 0.00000^{abc}	0.00771 ± 0.00123^{bc}	99.8 ± 0.1^{a}
90–600–0.020–W	96.7 ± 3.2^{d}	0.00045 ± 0.00001^{d}	0.00000 ± 0.00000^{a}	92.7 ± 1.2^{b}
90–600–0.020–LB	116.8 ± 5.3^{e}	0.00047 ± 0.00005^{d}	0.00000 ± 0.00000^{a}	91.0 ± 1.3^{b}

Samples were identified by the values of protein concentration (g L^{-1}) – HP level (MPa) and – calcium concentration (mol L^{-1}). Values are expressed as averages ± standard error. Different superscripts indicate that means are significantly different ($p < 0.05$).

of thermal treatment in soybean protein emulsion gels induced by $CaCl_2$ and observed that without thermal treatment the gels were soft. Our cold-set gels were also soft, reflecting the lack of certain interactions induced by thermal treatment. The data from water holding capacity reflected a difference with those of gels formed without freeze-drying of SPI after HP treatment (where only a loss of water of 5% had been found for 85–400–0.020 and 85–600–0.020, but not for the other conditions) (Speroni & Añón, 2013). When freeze-drying was carried out after HP treatment, the gels that contained 0.020 mol L^{-1} calcium significantly lost water, the amount of released water was dependent on HP level when protein concentration was 85.0 g L^{-1} (Table 1). The unfolding achieved at 600 MPa was possibly accompanied by a higher number of binding sites for water than those achieved at 400 MPa. Proteins may exhibit a partial decrease in the ability to interact with water as a consequence of freeze-drying (Wang et al., 2008). In our work, the cold-set gels that lost water were opaque, whereas the cold-set gels with WHC higher than 99% were transparent. This fact is in accordance with Hu et al. (2013) who stated that uniformity, presence of fine strands, and increase in protein solubility could result in the improvement of WHC. Taking into account our findings, we conclude that freeze-dried HP-treated SPI may be used to formulate cold-set gels when dispersed in water, and that calcium concentration is an important factor in the properties of these gels.

3.2. Cold-set gelation of HP-treated and freeze-dried SPI dispersed in lactobacilli suspensions

The ability of HP-treated and freeze-dried SPI to gellify at low temperature was evaluated by dispersing SPI in a suspension of lactobacilli and adding calcium. The 90–600–0.020 gels were selected because in these conditions strongest gels were obtained when dispersing in water. The lactobacilli-containing gels were turbid and their color was pink-like (L^* = 55.11 ± 0.68, a^* = 3.05 ± 0.12, b^* = 4.38 ± 0.41). Turbidity was probably due to the contribution of insoluble protein, aggregated gel structure and high bacteria concentration. It is possible that interaction between components led to lower values of b^*. The presence of lactobacilli had a great impact in visual aspect. Indeed, lactobacilli-containing gels showed increased values of G' (Table 1), and a shift in the range of tan δ (the values ranged from 0.23 to 0.45). These data suggest that bacteria were retained in the matrix exerting a load effect (increase in G') but perturbed the structure increasing the viscous character. The texture profile and WHC were similar to those of gel with same composition and HP level without bacteria (Table 1). Yang, Liu, and Tang (2013) analyzed the effects of the relative amount of dispersed phase (oil) in transglutaminase-set soy protein-stabilized emulsion gels. They found that "active fillers" alter the gels because of interactions with protein matrix, leading to a more particulate structure with increased G' values.

Our results show that self-standing gels can be obtained by calcium addition to HP-treated and freeze-dried SPI dispersed in water or in lactobacilli suspensions.

3.3. Storage of gels

The rheological behavior and texture profile of gels were analyzed at different time points. G' at 1 Hz and maximum force/area ratio were constant during the 28 days of assay (Fig. 1). These findings are important in order to design a bacteria-containing food, because microorganisms could interact with the matrix, e.g. hydrolyzing the polypeptides thus weakening the network.

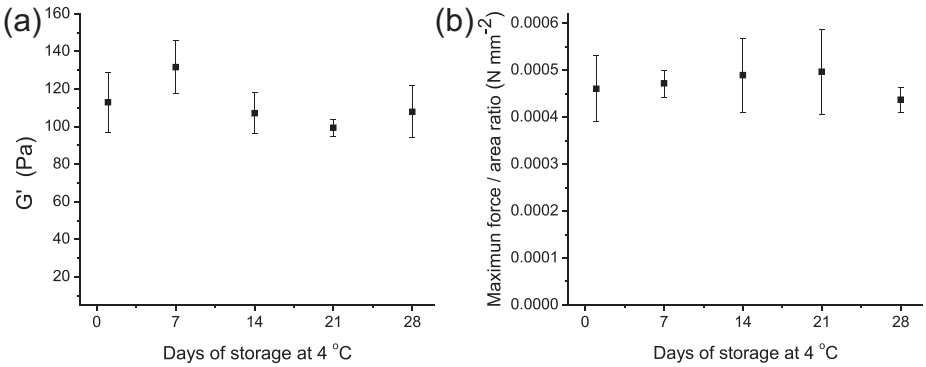

Fig. 1. Stability of gel matrix during cold storage. G' at 1 Hz from small deformation rheology (a) and maximum force/area ratio from texture profile analysis (b). Values are expressed as means ± standard deviation (a).

However, our results suggest that strain CIDCA 133 can be loaded in SPI cold-set gels without damaging the matrix.

It is known that some strains of *L. delbrueckii* subsp *lactis* exhibited proteolytic activity against β-conglycinin and glycinin (Aguirre, Garro, & Savoy de Giori, 2008). However this proteolytic activity was verified after a 6-h incubation at 37 °C at a lower protein concentration than that in our gels (10 g L^{-1}). In the present study the absence of degradation of the matrix could be explained by the low storage temperature of the gels. It is also possible that a small extent of hydrolysis occurred, but without effects on texture profile or rheological behavior because the most of protein was unaffected, due to high protein concentration of gels or low proteolytic activity of the strain CIDCA 133.

3.4. Viability of lactobacillus under simulated gastric conditions

L. delbrueckii subsp *lactis* showed a high susceptibility to simulated gastric conditions in PBS presenting a constant of inactivation (k) of 0.0618 min^{-1} (and a decimal reduction time (D) of 37.3 min) (Table 2). Viability of strain CIDCA 133 in PBS decreased 5 logs after 60 min of incubation and more than 7 logs at the end of the assay (120 min) (Fig. 2). In coagulated milk the survival of the lactobacilli was similar to that in PBS: decimal reduction time in coagulated milk was not different to that in PBS (Table 2). The bacterial viability in milk fell below the detection limit after 120 min of incubation. Interestingly, the inclusion of the lactobacilli in the SPI cold-set gels was the best condition for survival. Viability decreased only 3 logs during the experiment (Fig. 2) and the decimal reduction time was 2 fold higher than the value found for coagulated milk or PBS ($p < 0.05$) (Table 2).

It is important to point out that SPI cold-set gels were more resistant to disintegration than coagulated milk. It was necessary to apply an intense agitation to disperse the gels into the simulated gastric solution. This fact evidenced the presence of a matrix; this matrix might have protected the lactobacilli against acid and pepsin during the assay.

Taking into account that the gastric transit time is approximately 90 min (Berrada, Lemeland, Laroch, Thouvenot, & Piaia, 1991) and that the suggested concentration to provide health benefits for a probiotic formulation is 1×10^6 CFU g^{-1} of product (Succi et al., 2005), we conclude that lactobacilli included in SPI gels (obtained with $2.8 \times 10^7 \pm 1.0 \times 10^7$ CFU mL^{-1}, Fig. 2) could be able to survive the gastric transit thus reach viable the proximal region of small bowel.

3.5. Preservation of lactobacilli during cold storage

Survival of lactobacilli in PBS was constant for 7 days, but after 14 days of storage the bacterial concentration dramatically decreased below 10 CFU mL^{-1} after 21 days (Fig. 3). Viable counts of strain CIDCA 133 in coagulated milk remained constant during 28 days ($p < 0.05$) and then there was a statistically significant decrease of 0.65 and 0.91 log after 35 and 42 days, respectively ($p < 0.05$) (Fig. 3). The capacity to survive of lactobacilli included in SPI cold-set gels were even higher than in coagulated milk. Noteworthy, no changes in bacterial viability were observed after 42 days of storage (Fig. 3).

Several factors have been claimed to affect the viability of probiotic organisms in fermented milk products. During refrigerated storage, postacidification processes take place reducing pH and there is also a gradual increase in the oxygen content and in the redox potential of the products (Dave & Shah, 1997a, 1997b). The pH values at the beginning of storage were 3.4 ± 0.3 and 6.1 ± 0.1 for coagulated milk and SPI cold-set gels, respectively. The higher pH value, buffer capacity due to highly concentrated protein and absence of fermentable sugars in SPI cold-set gels may prevent acidification and/or its effects on lactobacilli. Sulfur-containing aminoacids present in soybean proteins could contribute to maintain low redox potentials and also they can act as oxygen

Table 2
Thermal parameters of the inactivation curves of strain CIDCA 133 in the *in vitro* gastric conditions.

	Constant of microbial inactivation (k)	Decimal reduction time (D)
	min^{-1}	min
Cold-set gel	0.0249 ± 0.0019[a]	92.5 ± 7.1[b]
Coagulated milk	0.0598 ± 0.0036[b]	38.5 ± 2.3[a]
PBS	0.0618 ± 0.0068[b]	37.3 ± 4.1[a]

Values are expressed as averages ± standard error. Different superscripts indicate that means are significantly different ($p < 0.05$).

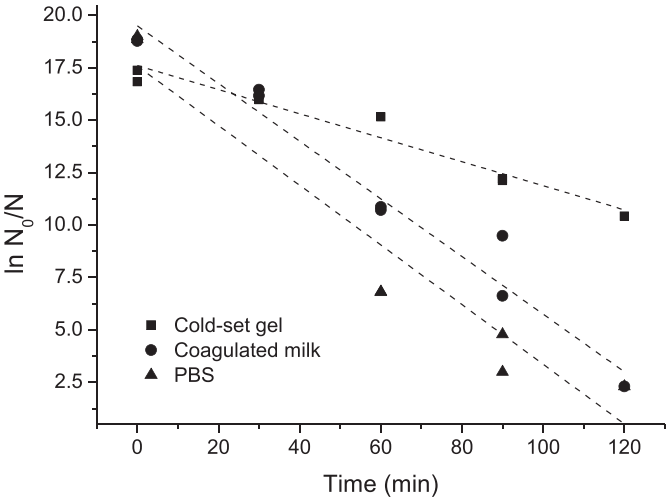

Fig. 2. Death curves of strain CIDCA 133 in modeled gastric digestion. Lactobacilli were contained in PBS (triangles), coagulated milk (circles), or cold-set gels (squares). Figure shows a representative kinetics. Dashed lines represent linear fits.

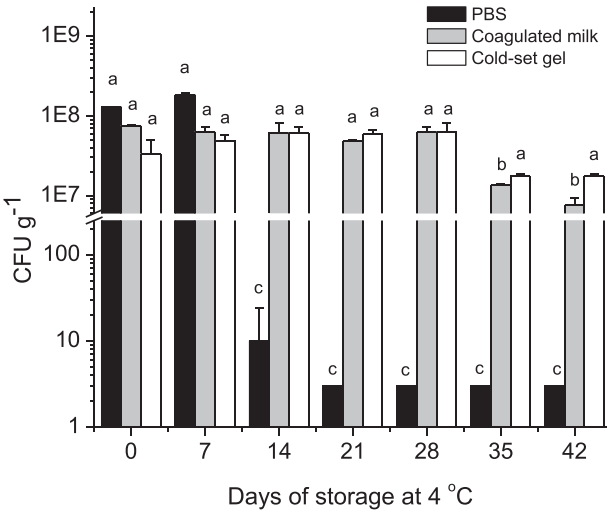

Fig. 3. Survival of strain CIDCA 133 during cold storage. Strain CIDCA 133 was stored in PBS (black bars); coagulated milk (gray bars) or in cold-set gels (white bars). Values are presented as means + standard deviation. Different letters within a storage medium indicate significant difference ($p < 0.05$).

scavengers. Our results indicate that there are factors in milk and in SPI cold-set gels, which are not present in PBS, that improve survival of lactobacilli. In addition, the matrix of SPI cold-set gels (Fig. 1) containing lactobacilli was stable.

4. Conclusions

HP-treated and freeze-dried SPI could be easily transported and stored, representing an interesting functional ingredient. Our results indicate that addition of calcium to HP-treated and freeze-dried SPI dispersed in water or in suspensions of lactobacilli results in the formation of stable gels. These cold-set gels had properties that depended on gel composition and HP level. The lactobacilli included in these cold-set gels remained viable during cold storage and survived better than in coagulated milk to a simulated gastric juice.

Taken together, our results suggest that SPI cold-set gels may be used as carriers of viable lactobacilli in the formulation of non-dairy probiotic products. Our results encourage further research on alternative low-cost technologies, e.g. spray drying, to develop lactobacilli-containing SPI cold-set gels as acceptable food products.

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