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Bioconversion of pumpkin by-products in novel supplements supporting *Lactobacillus casei*

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ABSTRACT

The aim of the work was to bio-convert pumpkin by-products in novel supplements supporting probiotics. The physicochemical and functional characterizations as well as the shelf life of the new product were analysed. Lower alcohol insoluble residue (AIR), 47 g/100 g, and higher protein, 12.2 g/100 g, were observed in the pumpkin powder supporting *L. casei* as the result of the probiotic growth. Water and oil holding capacities (23 g/g and 4.55 g/g, respectively) were not modified substantially due to probiotic presence. High non cellulosic carbohydrates content, ≈60 g/100 g AIR, as well as porous microstructure, helps to explain the remarkable hydration properties. The shelf life was determined by a *L. casei* count > 10^6 CFU g^{-1} after the *in vitro* gastrointestinal digestion resulting 28 days at 22 °C. The supplement was tested in a commercial hot drink showing an increase in the probiotic *survival* of almost 4 log cycles respect to the free cells after 90 min at 63 °C. It could represent a relevant advantage for probiotic supplementation in hot preparations. Therefore the process shows potentiality for adding value to pumpkin by-products. In addition, it was successfully scaled up in one order of magnitude.

1. Introduction

About 1.3 million tons of food are lost or wasted each year around the world, representing near 30% of the global food supply (FAO, 2016). Estimations reported by FAO (2016) showed that 45% of vegetables and fruits produced globally are lost or wasted; being considered this group with the greatest impact on food losses compared to other commodity food groups. *Cucurbita moschata* or butternut pumpkin production is widely dispersed in the worldwide, being one of the vegetables most consumed (USDA, 2017). If food processing only used the fleshy part of the pumpkin, which is the case with most pumpkin processing, then the waste produced would be between 18% and 21% (Norfezah, 2013).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). For instance, it was reported that *L. casei* ATCC®393™, reduces cholesterol absorption in the intestinal lumen, modulates the intestinal microbiota and adheres to the colon epithelium within levels compatible with the physiological effect (Lye, Rusul, & Liong, 2010; Saxami et al., 2012). The probiotic strains should survive to technological processes and to the passage through the gastrointestinal tract to act as probiotic at intestinal level (Reale et al., 2015). In general, dairy products were selected as food vehicles for probiotic delivery because the characteristic buffer against the high acidic environment in the gastroic tract (Champagne, Gomez Da Cruz, & Daga, 2018). For instance, *L casei* has been used with success in ripened cheese *in vivo* studies (Sperry et al., 2018). Nevertheless, fewer studies have been reported according to non-dairy matrices (de Souza Leone et al., 2017; Vijaya Kumar, Vijayendra, & Reddy, 2015). To date, scarce studies have been published regarding the use of the vegetable by-products, in particular pumpkin, as substrate for the production of *L. casei* biomass. In this context, the authors Genevois, Flores, and de Escalada Pla (2016) have demonstrated that *L. casei* (ATCC® 393™) could use the by-products from pumpkin *Cucurbita moschata* Duchesne ex Poiret as substrate to increase the cellular biomass and the remnant vegetal matrix served as support of the probiotic. The authors concluded that the proposed process was a promising way to supply probiotic through new food supplements and, in addition, the vegetable matrix conferred to probiotic resistance to low pH environmental. Nevertheless, one of the main challenges not addressed, were to determine if major changes would be produced on the vegetal matrix and on its physicochemical and functional characteristics, due to probiotic presence. Other

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important properties as well as the shelf life were pending of study.

Therefore, the objectives of the present work were to carry out a complete characterization of a novel food supplement based on pumpkin (*Cucurbita moschata*) by-products containing *Lactobacillus casei* ATCC® 393™, and to study the effect of the probiotic presence on the physicochemical and functional properties of the dried vegetable matrix. Different storage conditions were assayed. The *L. casei* resistance to thermal stress was also studied. Complementary data about the process scale up was provided.

2. Material and methods

2.1. Sample preparation

The pumpkin wastes (PW) containing peel and pulp (70:30 w/w) were shredded (D-56 Moulinex, Buenos Aires, BA, Argentina) for 2 min; vacuum dried (Christ 1–4 LD, Osterode am Harz, NI, Germany) for 24 h and stored at −20 °C until use. Approximately 10.0 g of dried (PW) were mixed with 100 mL of distilled water and sterilized at 121 °C for 15 min. Then, the system was cooled and inoculated with 1 mL of de Man, Rogosa and Sharpe (MRS) broth containing a suspension of *L. casei* (ATCC® 393™) (Microbiologics®, St. Cloude, MN, USA) ($\approx 10^3$ CFU mL^{-1}) followed by 24 h of incubation at 37 °C with orbital shaking (Vicking S.A., BA, Argentina) at 60 rpm. Subsequently, the system was centrifuged, washed with a sterile physiological solution and vacuum dried at 25 °C and 4.5 Pa for 24 h. Finally, the dried pumpkin power supporting the *L. casei* cells (PP supporting *L. casei*) was milled and sieved with a mesh with a pore size of 840 μm and packaged into low density polyethylene bags of 40 μm thickness, provided with an easy-to-close Ziploc® closing type. Additionally, a control system was carried out under the same conditions but without the addition of *L. casei* suspension, in order to analyse the effect of probiotic on functional properties of the vegetable matrix. The samples were stored protected from the light under controlled temperature of 8 or 22 °C for 35 days.

In order to stablish if data observed at laboratory scale could be reproduce in major scale, one order scale up step was assayed as detailed in Appendix A.

2.2. Methods

2.2.1. Physical and functional characterization

The water activity (a_w) was measured on the PP using a hygrometer (Aqualab, Pullman, WA, USA) at 20 °C. The functional characterization were determined as detailed in previous works de Escalada Pla et al. (2012). Briefly, the apparent density and specific volume were determined by measuring the volume of a weighed sample in a graduated and calibrated cylinder. For oil-holding capacity (OHC), the sample was mixed with sunflower oil and after 18 h at 25 °C, it was centrifuged at 3600 rpm 20 min (Eppendorf, HH, Germany) and the supernatant was discarded and the weight of the sample was recorded to determine the oil retained. The hydration properties were determined keeping the sample in contact with distilled water for 18 h at 25 °C. Swelling capacity (SC) was determined measuring the final volume attained by the hydrated sample. Water holding capacity (WHC) and water retention capacity (WRC) were determined recording the weight of water retained by the sample after free or accelerate decantation (30 min at 4800 rpm, Eppendorf, HH, Germany), respectively. The water soluble fraction (WSF) was measured by freeze drying (Christ 1–4 LD, Osterode am Harz, NI, Germany) the supernatant from WRC determination.

2.2.2. Chemical analysis

The cell wall compounds were determined according to Latorre, de Escalada Pla, Rojas, & Gerschenson (2013). Briefly, alcohol insoluble residue (AIR) was obtained by treating the sample of PP with boiling ethanol (USP grade) for separation of its AIR. Subsequently, the AIR was frozen and freeze-dried (Christ 1–4 LD, Osterode am Harz, NI, Germany). The AIR fractions were submitted to three different types of hydrolysis with sulfuric acid. On the hydrolysis residue, the content of lignin and cellulose was determined gravimetrically. Subsequently, on the neutralized supernatants, the protein, the non-cellulosic carbohydrates (NCC) and the uronic acids (UA) contents were quantified by spectrophotometry.

All measurements were performed at least in duplicate from independent samples and the mean values ± SD are reported.

2.2.3. L. casei bacteria counting

The number of viable *L. casei* cell was determined by plate counting in depth using MRS agar (Biokar Diagnostics, Beauvais, OI, France), followed by incubation at 37 °C for 72 h under aerobic conditions.

2.2.4. Simulated gastrointestinal digestion conditions

The simulated gastrointestinal digestion (SGID) procedure was conducted according to Genevois et al. (2016). Briefly, ~5 mL of sample were mixed with 5 mL of artificial saliva solution [NaCl (6.2 g L^{-1}), KCl (2.2 g L^{-1}), $CaCl_2$ (0.22 g L^{-1}), $NaHCO_3$ (1.2 g L^{-1})] for 2 min in a vortex (MSI minishaker IKA®, Brazil). Then, 30 mL of the gastric solution [0.3%, w/v pepsin (Merck, 0.7 FIP-U/mg) in 0.01 M HCl] was added followed by incubation at 37 °C for 2 h. Subsequently, the pH was adjusted at 7.5–8.0 with sterile 2 M NaOH, and finally, 30 mL of the intestinal solution [0.6%, w/v bile salts (Saporitti, Buenos Aires, BA, Argentina) in 0.05 M KH_2PO_4] were added, followed by incubation at 37 °C for another 2 h.

The percentage of recovered probiotic cells after gastric or intestinal digestion was calculated as the ratio between final and initial plate counting in MRS agar.

2.2.5. Survival of L. casei supported in the PP to heat stress conditions

PP was reconstituted (0.8%, w/v) in a chocolate milk followed by homogenisation (IKA® Ultra-Turrax®, Werke Inc., Germany). In parallel, a control system was assayed adding the free cells of *L. casei* re-suspended in physiological solution after being grown in MRS broth. Based on the survival curves (log reduction vs. time), the Weibull distribution equation (Eq. (1)) was used to model the probiotic survival during the heat stress (50, 63 and 75 °C).

$$\mathrm{Log}\,\frac{N_t}{N_0} = -b\,t^n \tag{1}$$

Where N_0 is the probiotic count at initial time; Nt represents the dependent variable, the probiotic count at a determined time, t, which is the time treatment in min and represents the independent variable. Parameters of the fitting were: b, the scale factor, which represents the probiotic inactivation kinetics, and the parameter n which characterizes the shape factor of the curves (dimensionless) ($n > 1$ and $n < 1$ describes convex or concave curves, respectively) (Van Boekel, 2002).

2.2.6. Environmental scanning electron microscope (ESEM)

Dried samples of pumpkin tissue, *L. casei* grown in MRS broth and PP were observed by ESEM (XL-30 ESEM, Philips, Amsterdam, NH, Netherlands) according to Dianawati, Mishra, and Shah (2012). The observations were performed at least in five optical fields and in two different opportunities, and the most representative micrographs are shown. The samples were coated with gold and examined under vacuum with an acceleration voltage of 20 kV and magnifications up to 6500X. ImageJ (1.51j8, National Institute of Health, USA) program was used for image processing.

2.2.7. Statistical analysis of data

The linear and non-linear regression models were corroborated with the statistical parameter of coefficient of determination (R^2) greater than 70% and the Durbin Watson parameter (DW) higher than 0.7 were also considered in the latter case. The statistical analysis of results was performed by the analysis of variance (ANOVA) for a level of

Table 1
Physical and functional properties of pumpkin powder supporting *L. casei* and control.

Systems	Physical Properties		Functional Properties			
	ρ (g/cm^3)	υ (cm^3/g)	OHC (g/g)	SC (mL/g)	WHC (g/g)	WRC (g/g)
PP L. casei	0.18 ± 0.01 [a]	5.50 ± 0.30 [b]	4.55 ± 0.06 [c]	22.00 ± 1.00 [e]	23.00 ± 1.00[f]	17.10 ± 0.70 [g]
PP control	0.19 ± 0.01 [a]	5.30 ± 0.20 [b]	4.90 ± 0.20 [d]	21.90 ± 0.80 [e]	23.00 ± 1.00 [f]	17.80 ± 0.50 [g]
Pumpkin[a]	0.91 ± 0.02	1.10 ± 0.02	NR	25.00 ± 0.20	24.00 ± 6.00	23.00 ± 6.00
Quince[b]	0.56 ± 0.01	1.79 ± 0.02	1.26 ± 0.02	6.40 ± 0.10	13.00 ± 1.00	5.60 ± 0.30
Peach[c]	0.49 ± 0.01	2.06 ± 0.04	1.81 ± 0.02	29.00 ± 2.00	24.00 ± 2.00	14.30 ± 0.40

PP *L. casei*: pumpkin powder supporting *L. casei*. PP control: pumpkin powder without probiotic addition. ρ: apparent density. υ: specific volume. OHC: oil holding capacity. SC: swelling capacity. WHC: water holding capacity. WRC: water retention capacity.
Mean values ± standard deviation (SD) are reported (n = 2).
Different letters denote significant ($p < 0.05$) differences.
NR: not reported.
[a] Fiber fraction (FF) from pulp pumpkin dried in a convection oven at 50 °C for 4 h (de Escalada Pla et al., 2007).
[b] FF from quince by-products dried in a convection oven at 80 °C for 4 h (de Escalada Pla, Uribe, Fissore, Gerschenson, & Rojas, 2010).
[c] FF from pulp peach treated with ethanol and dried in a convection oven at 30 °C for 7 h (De Escalada Pla et al., 2012).

significance (α) of 0.05, followed by a Least Significant Differences (LSD) test. Statgraphics Centurion XV software (V 2.15.06, 2007, Statpoint Technologies, Inc., Warrenton, VA, USA) was used.

3. Results and discussion

With the proposed process, a dried powder with a_w around 0.12 ± 0.05 was obtained. At this a_w value, the product storage results stable from microbiological point of view (Adams & Moss, 2000). The results corresponding to the physical and functional characteristics of the systems assayed are detailed in Table 1. For comparative purposes, values obtained in fractions rich in dietary fibre reported in previous works are also included. The presence of *L. casei* did not affect ($p > 0.05$) the specific volume (υ), while it reduced slight but significantly ($p < 0.05$) the OHC compared to the control system (Table 1). The OHC depends on the surface properties and the hydrophobic nature of the components of the plant matrix and it is also associated with the adsorption and retention of fats in the gastrointestinal tract (Mora et al., 2013). The values obtained for the OHC were in the same order than those reported, 1.07 g oil/g dry sample, in dried pumpkin powders through hot-air by Roongruangsri and Bronlund (2016), while other fibre-rich fractions, such as quince and peach, showed lower OHC values (Table 1). As regards the hydration properties, SC indicates how much water is weakly retained in the capillary structures of the fibre through surface tension forces and bonds with the molecular components through hydrogen bonding or dipole-dipole forces. The SC, WHC and WRC were not affected significantly ($p > 0.05$) by the presence of *L. casei* in the PP and the values obtained herein are in the order of that reported for dietary fibre concentrates of good functionality (Table 1). From a food industry point of view, these properties are relevant because they describe the ability of vegetables matrix to retain water in their structure. The authors had concluded previously that dietary fibre from pumpkin presented remarkable hydration properties (de Escalada Pla, Ponce, Stortz, Gerschenson, & Rojas, 2007) and it is important to highlight that these properties were maintained despite the process here in proposed.

In general, chemical composition was not significantly ($p < 0.05$) changed due to the probiotic presence (Table 2). Nevertheless, PP supporting *L. casei* presented a significantly ($p < 0.05$) lower AIR content, while proteins in the AIR, was significantly ($p < 0.05$) higher respect to the control system.

The growth of the probiotic cellular mass in the vegetal matrix during the incubation period could explain the lower trend in WSF, lower AIR and higher protein content in PP supporting *L. casei* (Table 2). It has been reported 13.4–24.0% of non-cellulosic glucose in AIR from pumpkin tissue (de Escalada Pla et al., 2005), it could be used, in part, as substrate by *L. casei* cells (Genevois et al., 2016). The high content in NCC reveals the hydrophilic chemical nature of the vegetable matrix rich in fibre, which in turn, is consequent with the results obtained for the hydration properties for the PP (Table 1). In addition, pumpkin tissue presented a porous microstructure (Fig. 1a) that enhances oil as well as water absorption. The presence of *L. casei* adhered to vegetal matrix, can be better observed in Fig. 1b. In order to corroborate the presence of *L. casei*, the diameter was measured (0.73 ± 0.04 μm) and compared with free cells of probiotic strain growth in MRS broth (0.64 ± 0.07 μm) (Fig. 1c).

Table 2
Chemical properties of the pumpkin powder and of its alcohol insoluble residue.

	PP *L. casei*	PP control
WSF[a]	22.00 ± 4.00[a]	24.80 ± 0.80[a]
AIR[b]	47.00 ± 3.00[b]	52.00 ± 1.00[c]
Protein[c]	12.20 ± 0.10[d]	10.80 ± 0.40[e]
Lignin[c]	5.50 ± 0.50[f]	5.50 ± 0.70[f]
Cellulose[c]	5.30 ± 0.50[g]	5.14 ± 0.08[g]
NCC[c]	61.00 ± 5.00[h]	58.00 ± 3.00[h]
UA[c]	15.80 ± 0.70[i]	17.00 ± 1.00[i]

NCC: total non-cellulosic carbohydrates.
UA: uronic acids.
Mean values ± standard deviation are reported (n = 2).
Different letters denote significant ($p \leq 0.05$) differences.
[a] WSF: water soluble fraction (g WSF/100 g PP db).
[b] AIR: alcohol insoluble residue. PP *L. casei*: pumpkin powder supporting *L. casei*. PP control: pumpkin powder without probiotic addition. (g AIR/100 g PP db).
[c] g/100 g AIR.

3.1. Shelf life characterization: study of the probiotic stability

The viability of *L. casei* supported in the pumpkin power (PP) was assessed at room (22 °C) and refrigerated (8 °C) temperatures for 35 days as Fig. 2 shows. At the beginning of storage, the probiotic count in the PP showed an average of 7.80 ± 0.20 (log CFU g^{-1} PP). During the storage, the viability of *L. casei* did not present significant ($p > 0.05$) changes up to 28 days, but it showed a significant ($p < 0.05$) reduction at 35 days. Meanwhile, the system stored at 8 °C presented a significant ($p < 0.05$) reduction in the probiotic viable count after 21 days. Despite of it, after 35 days of storage, the systems kept 6.61 ± 0.04 and 7.30 ± 0.10 (log CFU g^{-1} PP) for 8 and 22 °C storage respectively, which fit to the minimum concentration (10^6 CFU g^{-1}) established by the international organizations for a product to be considered as probiotic at the moment of consumption (Sperry et al., 2018). The viability of *L. casei* in the PP was affected by the time and temperature of storage, being the PP stored at room temperature the system with higher

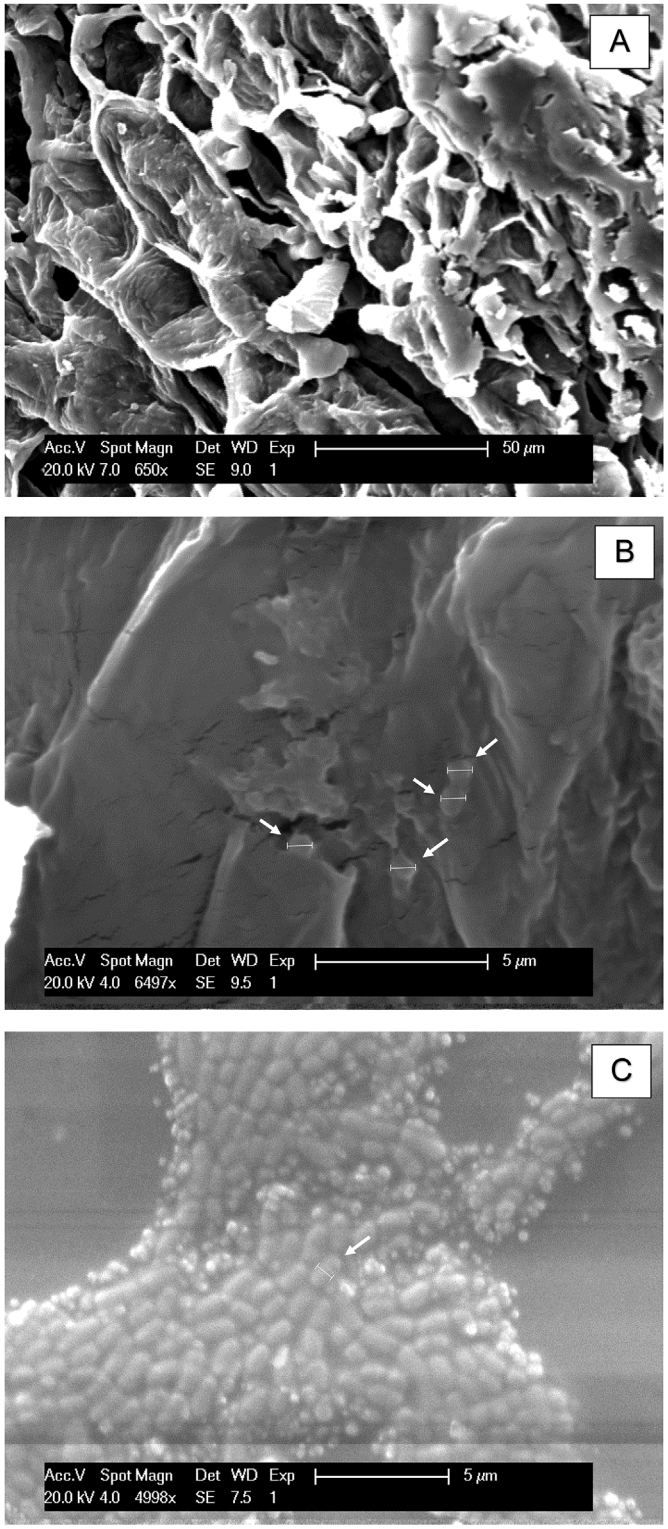


Fig. 1. Microstructure of dried samples of: A. Pumpkin tissue. B. The supplement (PP supporting *L. casei*). C. Free cells of *L. casei* growth in De Man, Rogosa, and Sharpe broth. Bars and raws indicate the adhered probiotic cells.

probiotic count. It is important to highlight that transport and storage costs of probiotics powders are notably reduced when these products do not require storage at refrigeration temperature; thus, improving the accessibility to the population in general, especially in developing countries (Coghetto, Hickman Flores, Brusch Brinques, & Záchia Ayub, 2016).

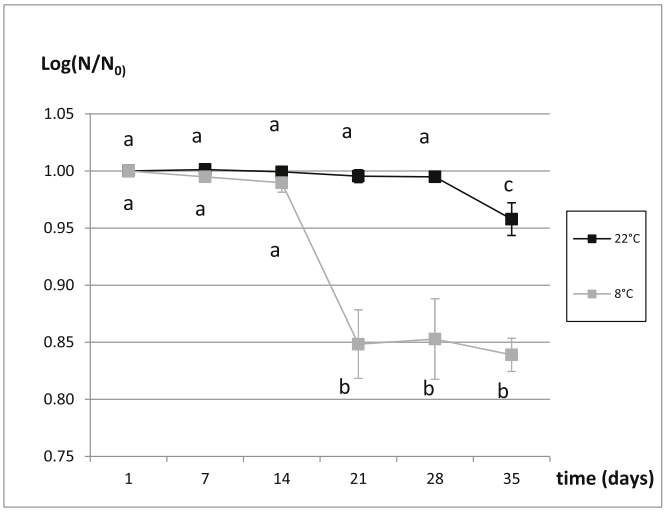


Fig. 2. Viability of *L. casei* supported in the pumpkin powder stored at 8 °C and 22 °C for 35 days of storage. PP supporting *L. casei* stored at 8 °C (grey squares) and 22 °C (black squares). *L. casei* viability expressed by the ratio between the final and initial logarithmic cell count (N/N_0). Values are the means ± standard deviation (n = 2) of duplicate analysis. Different letters denote significant ($p < 0.05$) differences.

The health benefits are only obtained when a probiotic strain reaches the target site in a metabolically active state and in a sufficient number (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013).

The survival to the SGID of *L. casei* supported in PP and stored at 8 °C is shown in Fig. 3a. After the gastric digestion, the *L. casei* survival remained without significant ($p > 0.05$) changes until 28 days of storage in the PP stored at 8 °C. Meanwhile, the probiotic resistance to bile salts was stable until 14 days of storage.

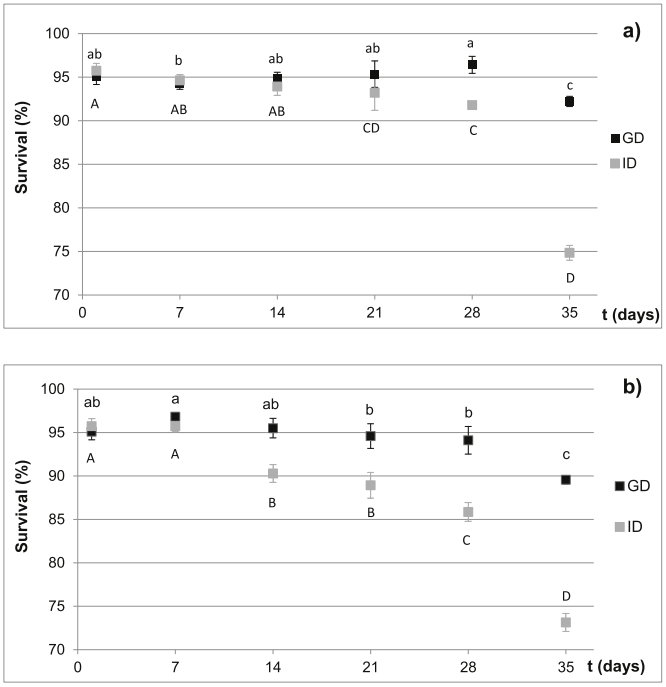


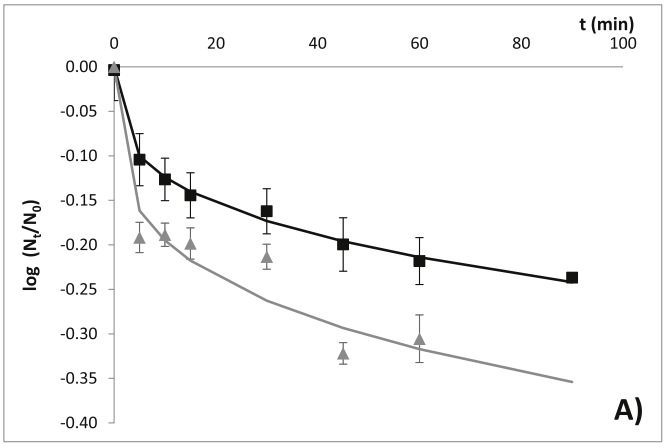
Fig. 3. Survival to *in vitro* simulated gastric and intestinal digestion of *L. casei* supported in the pumpkin powder stored at 8 °C (a) and 22 °C (b) for 35 days of storage. Black and grey squares represents the *L. casei* survival after the *in vitro* simulated gastric and intestinal digestion, respectively. Survival expressed as percentage (%). GD: gastric digestion; ID: intestinal digestion. Values are the means ± standard deviation (n = 2) of duplicate analysis.

As regards the resistance to SGID of *L. casei* contained in the PP stored at 22 °C, presented almost the same tendency compared to the probiotic supported in the PP stored at 8 °C, showing at 22 °C no significant ($p > 0.05$) decrease till 28 and 7 days when the probiotic was exposure to gastric and intestinal solution, respectively (Fig. 3b). The *L. casei* tolerance to the harsh gastric and bile conditions was significantly ($p < 0.05$) affected by the time of storage, showing at the end of storage a survival percentage of 74.8 ± 0.8 and 73.0 ± 1.0 for the PP stored at 8 and 22 °C, respectively (Fig. 3a and b). Comparing the survival % at 8 and 22 °C through a test *t*; it could be observed that there were no significant ($p > 0.05$) differences, suggesting that temperature of storage ranging 8–22 °C did not affect the probiotic rate survival during storage. Even though the enumeration of probiotic microorganism, in the present work, guaranteed the minimum probiotic concentration established (10^6–10^9 CFU) (Fig. 2), the probiotic SGID resistance was affected by the time but not by the temperature of storage in the conditions here assayed. Complementary data about loss of colour was also provided in Appendix B. Although rate of changes in colour were registered, *L. casei* survival controlled the product shelf life. Hence, the product shelf life in both systems was established up to 28 days of storage with a *L. casei* count > 10^6 CFU g^{-1} PP after the *in vitro* SGID, in the conditions herein assayed.

3.2. Thermal resistance of L. casei supported in the PP and supplemented to a dairy beverage

Thermal stress affects the activity of microorganisms causing membrane damage, denaturation and aggregation of proteins, and destabilization of ribosomes and ribonucleic acids. Although the thermal tolerance is specie and strain dependent, in general, temperatures above 50 °C causes thermal stress in lactic acid bacteria (Capozzi, Arena, Russo, Spano, & Fiocco, 2016). Therefore, the knowledge of the thermal resistance of *L. casei* supported in the PP is useful to allow its proper use as a dietary supplement, for instance in beverages that would be consumed as hot drinks. For this reason, the viability of the *L. casei* supported in the PP and subjected to heat stress was studied in chocolate milk (CM) and compared with control system (CMc), with free probiotic cells, in the same conditions. The temperatures studied were at 50, 63 and 75 °C, however, the results recorded at 75 °C are not shown since no viable *L. casei* cells were registered at the end of the test (90 min).

The Weibull distribution model was used to describe the resistance of *L. casei* to the heat stress. In Table 3, the parameters of the Weibull model are shown. The "b" parameter symbolizes the temperature-dependent scale factor, therefore, it presents the same analogy to the D value that represents the decimal reduction time necessary to reduce 90% of a population of microorganisms at a specific temperature, when n = 1 (Van Boekel, 2002). No significant differences were observed for the parameter b between CM and CMc when beverages were heated up to a temperature of 50 °C; however, a tendency to higher inactivation rate was observed when the dairy beverage was supplemented with the free probiotic cells (CMc). On the other hand, it was observed that the



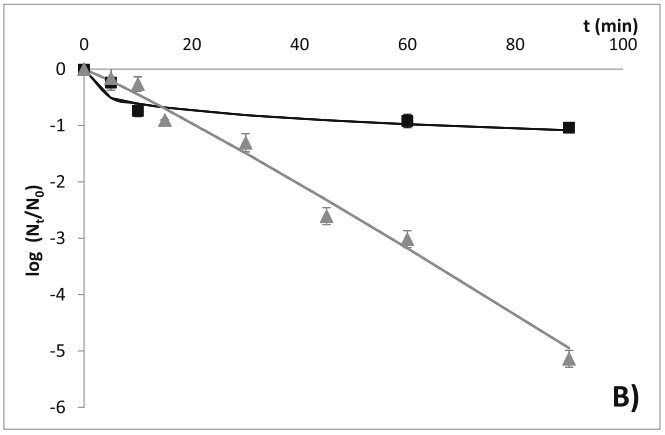


Fig. 4. Survival curves of L. casei supplemented to chocolate milk thermally treated at 50 (A) and 63 °C (B). Black squares: chocolate milk supplemented with the pumpkin powder supporting *L. casei*, and grey triangles: chocolate milk supplemented with free cells of *L. casei*. Black and grey lines show the respective values fitted to Weibull model. Values are the means ± standard deviation (n = 3).

Table 3
Weibull model parameters for L. casei survival supplemented to a dairy beverage thermally treated at different temperatures.

Parameters	50 °C		63 °C	
	CM	CMc	CM	CMc
b	0.062 ± 0.004 [a]	0.100 ± 0.020 [a]	0.340 ± 0.090 [b]	0.030 ± 0.010 [a]
n	0.300 ± 0.020 [c]	0.270 ± 0.060 [c]	0.260 ± 0.080 [c]	1.120 ± 0.070 [d]
R^2	99.3	93.6	87.0	98.9
R^2_{adj}	99.3	92.3	84.9	98.8
DW	2.3	2.3	2.1	3.5

Weibull distribution model: $\text{Log } N_t/N_0 = -b\, t^n$.
b: probiotic rate inactivation expressed as min^{-1}.
n: probiotic survival shape curve factor.
R^2: coefficient of determination (%).
R^2_{adj}: adjusted coefficient of determination (%).
DW: Durbin-Watson statistic.
CM: chocolate milk supplemented with the pumpkin powder with *L. casei*. CMc: chocolate milk supplemented with free cells of *L. casei*.
Mean values and standard deviation (SD) are reported (n = 2).
Different letters denote significant ($p < 0.05$) differences.

value of b increased ($p < 0.05$) with the temperature treatment, demonstrating that the rate of the probiotic inactivation was quintupled at 63 °C in the CM sample (Table 3).

The "n" value describes the type of concavity in the survival curve shape and its deviation from the linearity. The value of n in the beverages heat up to 50 °C was < 1, indicating that the curve of the thermal resistance for the *L. casei* was concave-upwards (tailing) (Fig. 4A). This type of concavity suggests that there is a dependence between the cell inactivation and the time, where the most sensitive cells are rapidly inactivated, followed by a decrease in the rate of inactivation of the cells that are more resistant and present a better adaptation to the thermic stress (Evelyn & Silva, 2015).

The values of n of the heat treated beverages at 63 °C were significantly ($p < 0.05$) different suggesting different susceptibilities of probiotic cells to the heat stress; therefore, the comparison of parameter b between CM and CMc would not be appropriate (Table 3, Fig. 4). The value of $n \approx 1$ in the CMc indicates that the inactivation rate was fitted to the first-order kinetics model, where each cell is equally susceptible to inactivation by heat treatment (Evelyn & Silva, 2015). Whereas, the value of n was < 1 for the CM, presenting a concave-upwards survival curve (Fig. 4B). The higher ($p < 0.05$) resistance of *L. casei* to the heat stress observed in the CM could be explained due to the protective effect of the pumpkin matrix. The thermo-resistance of microorganisms depends on the medium in which they are vehiculized. It is noteworthy, that the viability of free *L. casei* in chocolate milk presented a reduction of ≈5 log cycles after 90 min of heat treatment at 63 °C, and the strain survival was improved in almost 4 log cycles when it was supported in the pumpkin matrix. This result could represent relevant advantages for probiotic supplementation trough hot preparations like soups, purees and hot beverages.

Nowadays, besides maintain probiotic cell viability, there is a tendency to identify the presence of metabolites and/or enzymes (probioactives) which are specifically the result of probiotic activity in the food, and could provide an additional effect on health (Champagne, Gomes da Cruz, & Daga, 2018). It is important to highlight that, this *L. casei* strain supported in PP had also characterized for maintaining a phytase and α-galactosidase activity, which could improve the nutritional characteristics during the manufacture and storage of free-dairy products (Genevois, Castellanos Fuentes, Flores, & de Escalada Pla, 2018). In addition, it is interesting to remark that the overall acceptability of the chocolate milk supplemented with *Lactobacillus casei* supported in PP resulted above 7.0 on a 9-point scale (liked moderately) (Genevois et al., 2016).

4. Conclusions

The present study proposes the development and characterization of new supplements based on pumpkin by-product supporting *Lactobacillus casei*. The functional and physicochemical properties of pumpkin matrix were almost not affected by the probiotic presence and the hydration properties as well as OHC were in the order of that reported for dietary fibre of good performance. High NCC content as well as porous microstructure observed, help to explain the remarkable hydration properties registered. Lower AIR and higher protein content in PP with *L. casei* were observed as the result of the probiotic growth. After 35 days of storage at room temperature (22 °C), 10^6 CFU/g of *L. casei* were counted in the supplement. The probiotic resistance to the SGID was significantly affected by the time but not by the temperature of storage. The supplement was tested in a commercial hot drink showing an increase in the probiotic survival of almost 4 log cycles respect to the free cells after 90 min at 63 °C. It could represent a relevant advantage for probiotic supplementation in hot preparations.

These results confirmed that it is possible to profit pumpkin by-products for developing a supplement enriched in dietary fibre and simultaneously with probiotic through sustainable processes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2019.01.057.

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