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Byproduct from pumpkin (*Cucurbita moschata* Duchesne ex poiret) as a substrate and vegetable matrix to contain *Lactobacillus casei*

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ABSTRACT

Pumpkin waste was used as a substrate and support of *Lactobacillus casei* (ATCC-393). A complete factorial design (2^4) was performed in order to study the effect of water content, whey, inoculum concentration and the incubation period on cell growth and viability. According to the results of the experimental design, *L. casei* could use pumpkin waste as a substrate successfully and the system with 10 mL/g of water and without whey was selected to continue with further assays. The kinetics of the *L. casei* growth were determined. The maximum specific growth rate (μ_{max}) was 0.6 h^{-1}. After washing, centrifugation, and vacuum drying, 87% of viable cells were recovered. *L. casei* adhered to pumpkin powder was tested as a dietary supplement in chocolate milk or in a soy and apple juice based beverage. Approximately 80% of cells resisted gastrointestinal simulated conditions. Both supplemented beverages were well accepted by consumers.

1. Introduction

Vegetable processing often generates a large amount of solid waste that is usually used for animal feed or as organic fertilisers. At the same time, this waste constitutes a rich but yet underutilised source of valuable components which may find an application as ingredients, food additives or as dietary supplements.

In another aspect and towards improving the sustainability of processes, other authors have proposed to reduce agro-industrial by-products (wheat bran fibre, corn flour, lentils, peas and others) using them as a substrate in the production of L (+) lactic acid by solid state fermentation (SSF) (Naveena, Altaf, Bhadrayya, Madhavendra, & Reddy, 2005; Naveena, Altaf, Bhadriah, & Reddy, 2005).

The *Cucurbita moschata* or butternut pumpkin is one of the vegetables most consumed in Argentina, available during the whole year and throughout the country (National Agricultural Technology Institute [INTA], 2013). Pumpkin postharvest handling is important due to losses that may reach 100% of the crop as a result of mechanical damage, and/or dehydra-

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Abbreviations: PW, Pumpkin wastes; SSF, Solid state fermentation; MRS, De Man, Rogosa and Sharpe; PP, Pumpkin powder containing probiotics; SD, Standard deviation; a_w, Water activity; ChM, Chocolate milk; SM, Soy milk and apple juice; ChMc, Control chocolate milk; SMc, Control soy milk and apple juice

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tion (United States Agency for International Development [USAID], 2004). On the other hand, 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).

Previously, fibre fractions could be obtained from pumpkin by-products (de Escalada Pla, Ponce, Stortz, Gerschenson, & Rojas, 2007; de Escalada Pla, Rojas, & Gerschenson, 2013). In that opportunity, the authors concluded that these fractions could be used as food ingredients for nutritional and technological applications, while upgrading pumpkin waste (PW).

Nowadays, there is a general consensus about the importance of intestinal microflora in health and the welfare of humans and animals (Ranadheera, Evans, Adams, & Baines, 2012). Modulating the host microflora is achieved by the intake of probiotics and/or prebiotics. Probiotic microorganisms must have the ability to survive the harsh conditions of the food processing, resist gastric juices and the exposure to bile salts, and must be able to grow and colonise the intestinal tract. Moreover, they must also keep their effectiveness and potency over the lifetime of the product (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013) and be present in the final formulated product in a viable counting of at least of 10^6 CFU per gram to comply with the local legislation (National Administration of Drugs, Food and Medical Technology [ANMAT], 2011); as well as with the Codex Standard for Fermented Milks recommendations (Codex Alimentarious, 2003; Farnworth & Champagne, 2016).

Currently, commercial probiotic bacteria are provided to the food industry mainly as frozen cultures and/or freeze-drying. Spray drying is a low-cost technology that could be used in the production of probiotics. However, this process results in loss of viability because the cells are subjected to high temperatures, shear effects, osmotic pressure and dehydration (Paéz et al., 2012; Peighambardoust, Golshan Tafti, & Hesari, 2011). On the other hand, vacuum drying has been described as a useful, simple and inexpensive method for microorganism preservation (Tymczyszyn et al., 2008).

Some authors have proposed the immobilisation of probiotic strains in fibre from different vegetable sources (oat, banana, apple, passion fruit) in order to improve the stability and viability of the strains during storage (do Espírito Santo et al., 2012; Guergoletto, Magnani, Martin, Andrade, & Garcia, 2010).

The aim of the present work was to evaluate the performance of PW as a substrate and/or a vegetable matrix to contain *Lactobacillus casei*. The final product obtained was tested as a dietary supplement in chocolate milk and in a soy milk and apple juice based beverage. The probiotic cell recovery was assayed in both supplemented commercial beverages and also in the supernatants obtained after a simulated gastric intestinal digestion. In addition, a sensory evaluation of supplemented beverages was also carried out by an untrained panel.

2. Materials and methods

2.1. Materials

2.1.1. Microorganism and culture stock preparation

The commercial strain of *L. casei* (ATCC-393) was purchased in Microbiologics (St. Cloud, MN, USA). The freeze-drying cell culture was activated following the protocol suggested. Briefly, the freeze-drying cells were resuspended in sterile De Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, OI, France) and incubated at 37 °C for 48 hours (h). Subsequently, 100 μL of the grown broth were transferred to tubes containing 10 mL of sterile MRS broth and incubated at 37 °C for 15–18 h. From this culture, stock cultures were prepared by adding 100 μL of this last grown broth into Eppendorf tubes of 1.5 mL containing 1 mL of sterile cryopreservation broth; tryptone (2%, v/v) (Biokar Diagnostics, Beauvais, OI, France), skim milk (5%, v/v) (Mastellone, Buenos Aires, BA, Argentina), glycerol (20%, v/v) (Syntorgan, Buenos Aires, BA, Argentina) in MRS broth. Finally, the *L. casei* stock culture was stored at –20 °C, until use within 18 months.

2.1.2. Inoculum preparation

Inoculums were prepared by transferring 1 mL from the *L. casei* stock culture into a 250 mL Erlenmeyer flask containing 30 mL of sterile MRS broth. Cell growth was performed at 37 °C for 18–20 h in an incubation chamber (Ingelab I-290, Buenos Aires, BA, Argentina). Inoculums were considered adequate for the assays when presented at least ~9 log (CFU mL^{-1}) of viable *L. casei* by plate counting.

2.1.3. Sample preparation

PW containing peel and pulp (70:30) was 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. Pumpkin powder was weighed (≈10,000 g) and introduced into a 50 mL conical tube (Eppendorf, Hamburg, HH, Germany) and mixed (MS1 minishaker IKA, Staufen, BW, Germany) with different volumes of water (10–20 mL) and cheese whey (0–0.4 g) according to the experimental design (Table 1). Immediately, all systems were sterilised at 121 °C for 15 min. The systems were cooled and inoculated with 100 μL of MRS containing a suspension of *L. casei* ATCC-393 (10^3–10^9 CFU mL^{-1}), according to the proposed design. Subsequently, the systems were incubated (24–72 h) at 37 °C with orbital shaking (Vicking S.A., Buenos Aires, BA, Argentina) at low rate (0.045× *g*), in order to assure a homogeneous distribution of nutrients. At the end of incubation period, the viable *L. casei* was determined by plate counting. Subsequently, the samples were submitted to centrifugation (Eppendorf, Hamburg, HH, Germany) at 8000× *g* for 10 min. Titratable acidity and pH were determined on the supernatants. On the other hand, pellets were submitted to washing twice with a sterile physiological solution (0.9% NaCl, 5% dextrose, Anedra, Buenos Aires, Argentina) and vacuum drying at 25 °C and 4.5 Pa for 24 h.

One system was selected from the experimental design and two other batches were performed, in order to determine the growth curve of the *L. casei*.

Finally, the effect of the stabilisation process (washing, centrifugation and vacuum drying) on the probiotic cell survival was determined. A third batch was performed and the viable *L. casei* counting was analysed in each step of the process and on the final dried pumpkin powder (PP). The percentage of recovered probiotic cells after drying was calculated according to Eq. (1). Cell counting was referred to dry PP in all cases.

Table 1 – Complete factorial design (2^4) with four factors, two levels per each factor and central points.

Systems	Whey (g)		Incubation period (h)		Initial inoculum (Log (CFU g^{-1} PW))		Distilled water (mL)	
	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded
0	0	0.2	0	48	0	4.68	0	15
0	0	0.2	0	48	0	4.68	0	15
0	0	0.2	0	48	0	4.68	0	15
0	0	0.2	0	48	0	4.68	0	15
1	1	0.4	1	72	1	7.68	1	20
2	1	0.4	1	72	–1	1.68	–1	10
3	1	0.4	–1	24	1	7.68	–1	10
4	1	0.4	–1	24	–1	1.68	1	20
5	–1	0	1	72	1	7.68	–1	10
6	–1	0	1	72	–1	1.68	1	20
7	–1	0	–1	24	1	7.68	1	20
8	–1	0	–1	24	–1	1.68	–1	10
9	1	0.4	1	72	1	7.68	–1	10
10	1	0.4	1	72	–1	1.68	1	20
11	1	0.4	–1	24	1	7.68	1	20
12	1	0.4	–1	24	–1	1.68	–1	10
13	–1	0	1	72	1	7.68	1	20
14	–1	0	1	72	–1	1.68	–1	10
15	–1	0	–1	24	1	7.68	–1	10
16	–1	0	–1	24	–1	1.68	1	20

$$\%\ \text{Recovered cells} = \frac{\text{Final log(CFU g}^{-1}\text{)}}{\text{Initial log(CFU g}^{-1}\text{)}} \times 100 \quad (1)$$

2.2. Methods

2.2.1. L. casei *bacteria counting*

The number of viable *L. casei* cell was determined by plate counting of the samples by decimal serial dilutions with 0.1% (w/v) peptone water (Biokar Diagnostics, Beauvais, OI, France). Aliquots of 0.1 mL were plated in depth using MRS agar (Biokar Diagnostics, Beauvais, OI, France) followed by incubation at 37 °C for 72 h under aerobic conditions. The results were expressed as log (CFU g^{-1} PP) (dry weight). The increase of *L. casei* during the incubation period (ΔCFU) was expressed as the difference between the final and initial counting cell, and was calculated with Eq. (2). Each determination was performed at least in duplicate. Mean values and standard deviation (SD) were reported.

$$\Delta\text{CFU} = [\log(\text{CFU g}^{-1})]_{\text{Final}} - [\log(\text{CFU g}^{-1})]_{\text{Initial}} \quad (2)$$

2.2.2. *Determination of acidity in the supernatant*

Titratable acidity was determined with 0.01 M NaOH and phenolphthalein as an indicator, and the results were expressed as the milliequivalent of lactic acid g^{-1} PW. The pH was determined with a combined glass electrode Ag°/ AgCl connected to a pH meter (Cole-Parmer, Vernon Hills, IL, USA).

Both determinations were performed at least in duplicate for each system. The mean value and SD were reported.

2.2.3. *Determination of growth kinetics of* L. casei *in the medium proposed*

Based on the results of the complete factorial design, one system was selected and a second batch was prepared and analysed. The growth was checked determining the number of viable *L. casei* cell every 3 h for 72 h by plate counting in MRS agar. Determinations were performed at least in duplicate.

Experimental data were fitted to the modified Gompertz model (Eq. 3) (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990) expressing in the equation the microorganism population change [*ln* (N/N_0)] in function of time (*t*).

$$ln\left(\frac{N}{N_0}\right) = C \cdot exp^{\left\{-exp\left[\left(\frac{\mu max}{C}\right)(lag-t)+1\right]\right\}} \quad (3)$$

Where *N* is defined as the viable *L. casei* bacteria at a given time (hours) during the incubation period (CFU g^{-1} PW); N_0 is the viable *L. casei* bacteria at the initial incubation period (CFU g^{-1} PW); *C* is maximum logarithmic sustained *L. casei* growth, μ_{max} is the maximum specific growth rate (h^{-1}); *lag* is the duration of the adaptation phase (expressed in h); *t* is the time of the incubation period (expressed in hours). The curve was performed with two different initial inoculum concentrations: 1.8 ± 0.2 and 3.4 ± 0.1 log (CFU g^{-1} PW). All measurements were performed at least in duplicate and the average value ± SD was reported for each inoculum concentration.

2.2.4. *Physiochemical characterisation of PP*

The pH was determined at the final period of fermentation with a pH meter. The water activity (a_w) and moisture content were performed on the dried pellet of PP; a_w was measured with a hygrometer (Aqualab, Pullmann, WA, USA) at 20 °C, and the moisture content was determined with a moisture analyser (Ohaus MB-45, Parsippany, NJ, USA) using a drying time profile of 5 min at 130 °C followed by a drying step at 105 °C until reaching a moisture loss less than 1 mg in 30 seconds. The results were expressed in the percentage of moisture on a dried basis of PP. All measurements were performed at least in duplicate and the average value ± SD was reported.

2.2.5. *Environmental scanning electron microscope (ESEM)*

The presence of *L. casei* was observed by ESEM (XL-30 ESEM, Philips, Amsterdam, NH, The Netherlands). The dried PP samples were coated with gold and examined under vacuum with an acceleration voltage of 20 kV and magnifications up to 6500×.

2.2.6. *Simulated gastrointestinal conditions*

The resistance of *L. casei* to simulated gastrointestinal conditions was assessed in the PP reconstituted in chocolate milk (ChM) or in a soy milk and apple juice based beverage (SM). In parallel, control systems, ChMc and SMc respectively, were assayed adding the free cells of *L. casei* resuspended in physiological solution after being grown in MRS broth. The assay was conducted according to Guergoletto et al. (2010), with some modifications.

The simulated gastrointestinal procedure included three steps: mouth, gastric and intestinal digestion. Briefly, 0.12 g of PP containing 9.0 ± 0.1 log (CFU g^{-1}) of *L. casei* was reconstituted with 15 mL of ChM or with 25 mL of SM. Subsequently, the supplemented beverages 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. The gastric digestion was verified by the addition of 30 mL of pepsin-HCl solution [0.3%, v/v pepsin (Merck, 0.7 FIP-U/mg) in 0.01 M HCl] followed by incubation at 37 °C with orbital shaking at 0.32× *g* for 2 h. Subsequently, the pH of the systems was adjusted at 7.5–8.0 with sterile 2 M NaOH, and finally, 30 mL of the intestinal solution (0.05 M KH_2PO_4) containing 0.6% of bile salts were added, followed by incubation at 37 °C with orbital shaking at 0.32× *g* for another 2 h.

The simulated gastrointestinal process was performed at least in duplicate and the *L. casei* viability was determined by plate counting in MRS agar at the end of gastric and intestinal digestion.

2.2.7. *Sensorial evaluation*

The sensory attributes of the commercial beverages supplemented with the PP containing *L. casei* (see section 2.2.6) were evaluated by an untrained sensory panel.

The beverages were purchased in a local supermarket and their commercial brands will be kept undisclosed for confidentiality reasons.

One hundred and twenty volunteers between the ages of 19 and 55, frequent or not frequent beverage consumers participated in the test. They were selected at random in the Industry Department of the University of Buenos Aires. The beverages were stored at room temperature before the supplementation in order to obtain homogeneous conditions during the sensory analysis sessions. The panellists were offered 10 mL of the sample in plastic cups coded with a three-digit random number in individually partitioned booths. The panel was instructed to evaluate the supplemented beverages judging the degree of liking or disliking to the odour, texture in the mouth, and overall acceptability using an unstructured 9-point hedonic scale. Consumers were instructed to rinse their mouths with water and eat a cracker between samples to avoid carryover effects (Lawless & Heymann, 2013). The score on acceptance was calculated for each attribute and the averages ± SD were reported.

2.2.8. *Experimental design and statistical analysis*

The present work was performed according to a complete factorial design (2^4) that included the effect of four factors: partially demineralised cheese whey (Mageral, Buenos Aires, BA, Argentina), the incubation period, the initial inoculum concentration and the distilled water volume with two levels for each factor and central points. The levels of the factors were selected according previous assays and are detailed in Table 1. The experimental design was conducted in one block and central points were performed in quadruplicate. All determinations were performed at least in duplicate for each system. The experimental design was carried out twice. The same trend for the effects was observed in both cases. The results of the last experimental design were reported.

The statistical parameter R-square (R^2_{adj}) greater than 70% and the lack of fit ($p > 0.05$) were used to report the experimental design factors. In order to validate the non-linear regression model, (R^2_{adj}) greater than 90 and Durbin Watson (DW) higher than 1 were considered.

The findings are informed on the basis of their average and SD. The statistical analysis of results was performed by use of the analysis of variance (ANOVA) at the 95% confidence level. Additionally, the Pearson product–moment correlation between the variables was also evaluated.

The experimental design, regression curves, correlations and statistical analysis of the results were carried out using the Statgraphics Centurion XV (V 2.15.06, 2007, Statpoint Technologies, Inc., Warrenton, VA, USA).

3. Results and discussion

3.1. *Effect of four factors on* L. casei *growth*

The results corresponding to the viable *L. casei* counting, ΔCFU, pH and titratable acidity are shown in Table 2 while in Table 3 the regression coefficients of the four factors (cheese whey, incubation period, initial inoculum and water volume) are summarised. In general, the signs of the coefficients agree with those observed for the respective standardised effects.

In the present work, the fermentation was carried out at 37 °C since probiotic strains are typically originated from the human gastrointestinal tract. Costa, Fonteles, De Jesus, and Rodrigues (2013) as well as Pereira, Maciel, and Rodrigues (2011) reported a fermentation temperature of 30–31 °C as the optimum operating conditions for the highest *L. casei* viability in a pineapple and cashew apple beverage.

After the fermentation process corresponding to 24, 48 or 72 h, an increase in viability was observed in all systems with respect to the initial inoculum. To test the statistical significance of each effect, its mean square is compared with an estimate of the experimental error. The effects are significantly different from zero with a confidence level of 95.0% when the "p" value results less than 0.05. The incubation period ($p < 0.0150$) and the initial inoculum ($p < 0.02$) had a significant positive effect on the *L. casei* viability. However, the interaction factor had a significant negative effect ($p < 0.0150$), which could be explained due to a lack of nutritive resources.

Therefore, the *L. casei* increase (ΔCFU) was positively affected by the incubation period ($p < 0.015$) and was negatively

Table 2 – Reponses variables from complete factorial design (2^4): *L. casei* viability, pH, titratable acidity and ΔCFU.

Systems	*Lactobacillus casei* viability log (CFU g^{-1} PW)	pH	mEquivalent lactic acid g^{-1} PW	ΔCFU
0	$9.5 \pm 0.2^{a,c,d,e}$	3.83 ± 0.05^{a}	$1.3 \pm 0.1^{a,g}$	$3.6 \pm 0.2^{a,g}$
0	$9.5 \pm 0.2^{a,c,d,e}$	3.85 ± 0.05^{a}	1.36 ± 0.01^{a}	$3.6 \pm 0.2^{a,g}$
0	$9.5 \pm 0.1^{a,c,d,e}$	3.83 ± 0.05^{a}	$1,35 \pm 0.01^{a}$	$3.6 \pm 0.1^{a,g}$
0	$9.6 \pm 0.3^{a,c,d,e}$	3.82 ± 0.05^{a}	$1,35 \pm 0.01^{a}$	$3.7 \pm 0.3^{a,g}$
1	$9.2 \pm 0.4^{a,d,e}$	3.6 ± 0.05^{a}	2.35 ± 0.04^{b}	$1.5 \pm 0.4^{b,e}$
2	$8.9 \pm 0.4^{a,d}$	3.93 ± 0.05^{a}	$1,32 \pm 0.03^{a,g}$	7.2 ± 0.4^{c}
3	$9.2 \pm 0.2^{a,d,e}$	4.02 ± 0.05^{a}	$1.15 \pm 0.07^{a,g,h}$	$1.6 \pm 0.2^{b,e}$
4	5.57 ± 0.05^{b}	6.07 ± 0.08^{b}	0.25 ± 0.07^{c}	$3.89 \pm 0.05^{a,g}$
5	$9.02 \pm 0.13^{a,d}$	4.09 ± 0.05^{a}	0.85 ± 0.07^{d}	1.3 ± 0.1^{b}
6	$9.1 \pm 0.1^{a,d}$	4.09 ± 0.05^{a}	0.80 ± 0.01^{d}	7.4 ± 0.1^{c}
7	8.6 ± 0.2^{a}	4.17 ± 0.05^{a}	0.80 ± 0.01^{d}	1.01 ± 0.05^{b}
8	5.51 ± 0.01^{b}	6.18 ± 0.08^{b}	0.20 ± 0.01^{c}	$3.84 \pm 0.01^{a,g}$
9	$9.5 \pm 0.1^{a,c,d,e}$	3.71 ± 0.05^{a}	1.86 ± 0.07^{e}	$1.8 \pm 0.1^{b,e}$
10	$10.4 \pm 0.1^{c,e}$	3.78 ± 0.05^{a}	1.60 ± 0.01^{f}	$8.7 \pm 0.1^{d,f}$
11	$9.8 \pm 0.5^{d,e}$	3.93 ± 0.05^{a}	1.1 ± 0.1^{g}	$2.1 \pm 0.5^{b,e}$
12	5.74 ± 0.02^{b}	5.82 ± 0.07^{b}	0.25 ± 0.07^{c}	4.07 ± 0.03^{a}
13	$10.31 \pm 0.09^{c,e}$	4.04 ± 0.05^{a}	0.80 ± 0.01^{d}	2.6 ± 0.1^{e}
14	$9.9 \pm 0.6^{c,d,e}$	4.02 ± 0.05^{a}	$1.01 \pm 0.01^{d,h}$	$8.2 \pm 0.6^{c,f}$
15	$9.1 \pm 0.3^{a,d}$	4.15 ± 0.05^{a}	0.75 ± 0.07^{d}	1.4 ± 0.3^{b}
16	4.7 ± 0.5^{b}	6.23 ± 0.08^{b}	0.3 ± 0.1^{c}	$2.9 \pm 0.5^{e,g}$

ΔCFU: increase of *L. casei* during the incubation period.
Mean values and standard deviation were reported: for *L. casei* viability, n = 2, for pH, n = 3, for titratable acidity, n = 2 and for ΔCFU, n = 2.
Different letters in the column indicate significant differences ($p < 0.05$).

affected by the initial inoculum ($p < 0.010$). Nonetheless, the interaction factor had a significant negative effect ($p < 0.02$).

As regards the water and cheese whey content, these factors did not show a significant effect on probiotic viability nor did they on ΔCFU. As a consequence, independently of the presence or absence of cheese whey and the water content, it was observed that systems with a lower initial inoculum increased 3-4-log cycles with reference to the initial counting of cell probiotic for the systems with 24 h of fermentation, while the increase was of 7-8-log cycles for the systems with 72 h of fermentation (Table 2).

Table 3 – Regression coefficients of the effects studied on *L. casei* viability, pH, titrable acidity and ΔCFU in pumpkin waste (PW).

Constant	Regression coeficients			
	CFU[a]	pH	mEquivalent lactic acid[b]	ΔCFU[c]
	2.53	7.96	0.153	2.95
A: Whey	2.31	–0.119	–2.31*	0.523
B: Incubation period	0.115*	–0.057*	0.011*	0.096*
C: Initial inoculum	0.852*	–0.547*	0.091*	–0.110*
D: Water	–0.107	0.024	–0.018	–0.113
AB	–0.015	0.020	0.039*	–0.035
AC	–0.237	–0.225	0.224	–0.077
AD	0.046	–0.0034	0.052	0.144
BC	–0.014*	0.0083*	–0.0014	–0.0135*
BD	0.0006	–0.0003	0.0002	0.0014
CD	0.012	–0.002	0.0010	0.0058
R^2	93.7	93.8	93.6	96.1
R^2_{adj}	83.2	83.6	84.6	90.4
Lack of fit	0.09	0.17	0.16	0.08

* Significant ($p < 0.05$) effect with a confidence level of 95%.
[a] *L. casei* viability expressed as log (UFC g^{-1} PW).
[b] Titrable acidity expressed as mEq. of lactic acid g^{-1} PW.
[c] *L. casei* growth during period incubation.

The synthesis of amino acids, purines, pyrimidines, some carbohydrates and lipids, enzyme cofactors, and other substances is associated with the use of nitrogen by the microorganism. In this context, the whey supplementation could serve as a source of carbon as well as nitrogen. By way of illustration, Aguirre-Ezkauriatza, Aguilar-Yáñez, Ramírez-Medrano, and Alvarez (2010) as well as Bernárdez, Amado, Castro, and Guerra (2008) reported that whey can be used to produce a high *L. casei* biomass with the application of fermentation technology. More recently, Horackova, Sedlackova, Sluková, and Plocková (2014) demonstrated that the addition of whey to skim milk did not improve the final counting of *L. casei* after 24 h of cultivation at 37 °C. In the present work, a non-significant effect on *L. casei* viability and on ΔCFU was observed when whey was added to the pumpkin waste, suggesting that a by-product from pumpkin could serve by itself as a substrate to enhance the biomass yield under the conditions herein assayed.

On the other hand, it is highlighted that the effect of water content on the cell counting and ΔCFU was negative and non-significant, showing the same sign in the regression coefficient (Table 3). The fact that a minimum quantity of water could be used without affecting the *L. casei* growth represents an additional advantage from the process sustainability point of view.

Despite the low water content, a variable volume of supernatant was collected after being centrifuged in all the systems

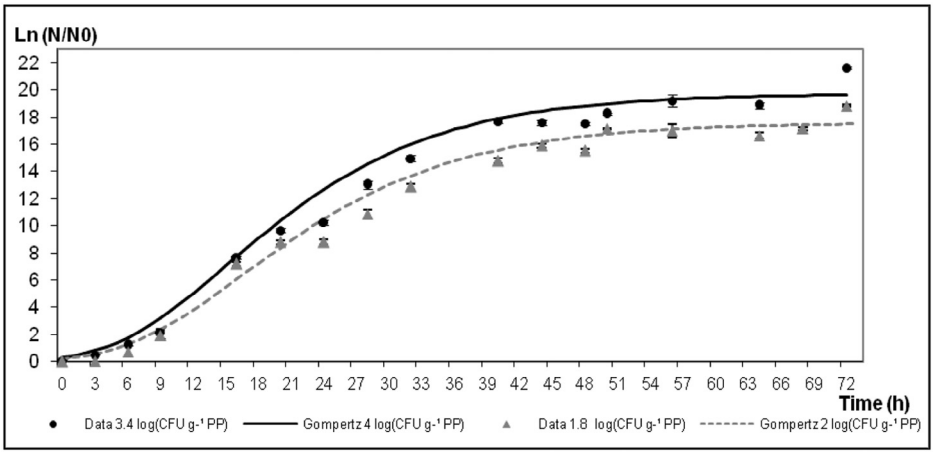

Fig. 1 – Growth curve of *Lactobacillus casei* on pumpkin waste (PW) medium at 37 °C. Black and grey lines represent the fit of the modified Gompertz model to the experimental data. Black lines and dots correspond to initial inoculums of 3.4 log (CFU g^{-1}PW); grey lines and triangles correspond to initial inoculums of 1.8 log (CFU g^{-1} PW). Bars represent standard deviation of n = 2 in each point.

tested. These supernatants may be constituted mainly by products from the fermentable metabolism that are responsible for the decrease in the pH (Pereira et al., 2011; Rivera-Espinoza & Gallardo-Navarro, 2010).

The lactic acid bacteria (LAB) are important because of their ability to transform fermentable sugars into lactic acid. Even though the aim of this work was not to produce lactic acid, the occurrence of this acid is partially coupled with biomass production (Aguirre-Ezkauriatza et al., 2010). Then pH and titratable acidity expressed in milliequivalents (meq) of lactic acid/g of PW was determined and shown (Table 2) and analysed (Table 3). According to our results, the pH of the systems tested was negatively affected by the initial inoculum ($p < 0.015$) and the incubation period ($p < 0.025$). The interaction term had a significant positive effect ($p < 0.015$); nevertheless, this coefficient was one order of magnitude lower than the linear coefficient of the incubation period (Table 3). Consequently with pH, the titratable acidity was positively affected by incubation period ($p < 0.025$) and the initial inoculum ($p < 0.035$). Furthermore, a positive effect of the whey ($p < 0.035$) could also be observed. The buffering capacity of whey could possibly explain that their effect was only observed on titratable acidity but not on the pH variable (Horackova et al., 2014; Salaün, Mietton, & Gaucheron, 2005). A positive effect was also observed for the interaction term between whey and incubation time ($p < 0.05$), which agreed with the regression coefficient sign (Table 3). In relation to water content, no significant effect was observed on titratable acidity. Therefore, more cells in the initial inoculum along with a longer incubation period and the presence of whey would tend to increase the lactic acid production.

There was a negative and significant linear correlation between the pH of the supernatant and the titratable acidity (Pearson product–moment correlation, $r = -0.777$, $p < 0.0015$); and also with the viability of *L. casei* (Pearson product–moment correlation, $r = -0.961$, $p < 0.0005$). This means that the acid lactic production was, in part, associated with the *L. casei* growth when the PW was used as a substrate.

Additionally, the exposure of lactic acid bacteria to acid stress during the fermentation period could result in loss of viability or cell death due to an intracellular accumulation of undissociated lactic acid and structural damages to the DNA and proteins present in the cell membrane (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011; De Angelis & Gobbetti, 2004). The pH at the end of fermentation process in yoghurt and juices has been reported to be the most important factor influencing the growth and viability in probiotics (Champagne et al., 2011). According to our results, a decrease in the final pH values was accompanied by an increase in the probiotic count in all the systems tested. Thus, no negative effect of low pH on the probiotic survival was observed, probably as the result of an intrinsic resistance to the low pH of the *L. casei* strain as well as to the protective microenvironment provided by the pumpkin matrix during the incubation period (Vinderola et al., 2011). On the other hand, Costa et al. (2013), Guergoletto et al. (2010) and Pereira et al. (2011) reported that *L. casei* strains usually added to vegetable matrices exhibit an optimum growth at pH ≥5.5. The pH of the systems 4, 8, 12 and 16 (lowest incubation period and lowest inoculum) were in agreement with these results since they presented a pH value ≥5.5.

Based on the findings obtained from the complete factorial design, the system with a lower cell concentration in the initial inoculum, lower water content and without whey supplementation was selected to continue with further assays (system 8).

When a new medium is proposed, it is useful to provide the characterisation of the microorganism growth in the new conditions. In this sense, the kinetic parameters of *L. casei* growth were determined. The non-linear regression of the data plotted in Fig. 1 describes the *L. casei* growth curve. Additionally to the initial inoculums at the lower level (1.8 ± 0.2), a second growth curve was performed at initial inoculums of 3.4 ± 0.1 log (CFU g^{-1} of PP). As could be observed in Fig. 1, the growth of *L. casei* was slow during the initial hours of fermentation (adaptation period or lag phase), possibly due to the time consumed

Table 4 – Parameters values fitted to modified Gompertz equation, from growth curve of *L. casei* in the medium based on pumpkin waste (PW).

	Initial inoculum	
Parameters	1.8 log (CFU g^{-1} PW)	3.4 log (CFU g^{-1} PW)
C	17.6 ± 0.9[a]	19.7 ± 1.4[a]
μmax	0.6 ± 0.1[b]	0.7 ± 0.2[b]
Lag	5.6 ± 2.8[c]	5.7 ± 4.1[c]
R^2_{adj}	98.5	96.7
DW	1.8	1.9

C is maximum logarithmic sustained *L. casei* growth, *μmax* is the maximum specific growth rate (h^{-1}); Lag is the duration of adaptation phase (expressed in h).
Mean values: ±SD. *C*: dimensionless; *μmax*: (h^{-1}); *Lag*: (h).
Similar letter in the row means no significant difference ($p > 0.05$) between different inoculums for the same parameter.

by the cells to hydrolyse the nutrients or the synthesis of several compounds which are needed to grow (Willey et al. 2011). After approximately 6 h, both systems presented an exponential growing phase until hour 20. From hour 20 to the end of the assay, the exponential growth was partly inhibited probably because of a decrease of essential nutrients resources (stationary phase). No viability loss was observed (decline phase) during the assay (72 h). The viable cell count was 5.61 ± 0.12 and 7.82 ± 0.05 log (CFU g^{-1} of PW) at 24 h of fermentation for the system inoculated with 1.8 ± 0.2 and 3.4 ± 0.1 log (CFU g^{-1} of PW), respectively.

Pereira et al. (2011) reported that the fermentation process of cashew apple juice with *L. casei* presented a slow initial phase followed by a logarithmic phase from 6 h to 14 h and after this period, some viability loss was observed. The logarithmic phase of *L. casei* in pumpkin was between 6 and 24 h; however no viability loss was observed during the 72 h of incubation, possibly as a result of the role of the pumpkin matrix as a substrate and simultaneously as a protection support of *L. casei* cells.

The maximum specific growth rate (μ_{max}) was determined in the new medium proposed. The μ_{max} values duplicated consistently being 0.6 ± 0.1 h^{-1} (R^2_{adj}: 98.5) and 0.7 ± 0.2 h^{-1} (R^2_{adj}: 96.7) for initial inoculums of 1.8 ± 0.2 and 3.4 ± 0.1 log (CFU g^{-1} PW), respectively. The parameters C and lag were also determined (Table 4). These parameters are important when the process needs to be scaled up and for the design of new equipment.

3.2. *Effect of subsequent unit operations on* L. casei *survival*

In order to analyse the effect of subsequent unit operations on *L. casei* survival, a new batch using a higher amount of PW (10 g) was carried out. Thus, 100 mL of water and 2.5 log (CFU g^{-1}) of initial inoculums were added. At the end of fermentation, the product showed a *L. casei* count of 8.8 ± 0.1 log (CFU g^{-1} PW), pH 6.09 ± 0.05 and 0.40 ± 0.01 meq of lactic acid per gram. A higher viable probiotic counting and lactic acid production values were observed when comparing these values with those obtained by prediction with coefficients (Table 3), which could possibly be attributed to the scaling up effect. The experiment with 1 g of pumpkin powder was performed in 50 mL tubes while studies with 10 g of powder were carried out in a 500 mL flask. Although the same shaker was employed in both cases, in the case of the 500 mL flask, the agitation was more effective, as the result of the higher diameter/height ratio (Doran, 2008). Subsequently, the product was washed, centrifuged, vacuum dried, and sieved (ASTM 420 microns) showing a probiotic viability of 7.67 ± 0.06 log (CFU g^{-1} PP). At the end of the vacuum drying process, the percentage of recovered probiotic cells was 87%, representing a reduction of 1.13 log cycles during the process. Guergoletto et al. (2010) reported a recovered *L. casei* of 79% for oat bran, 76% for green banana flour, 64% for apple fibre and 55% for inulin after vacuum drying at 45 °C during 12 or 25 h until a moisture content of 5.6% and a final a_w of 0.35–0.25 were reached. In our study, the final product presented a moisture content of 7.0 ± 0.2% and a_w of 0.20 ± 0.09.

During the vacuum drying, the cell damage could be reduced to dehydration damages, leading to a high viability of probiotics in the final product (Foerst & Santivarangkna, 2015). Moreover, the application of the incubation period as a prior step to the drying process could make changes in the technical behaviour of probiotic cells, such as heat resistance, freeze-drying for cell survival and storage stability (Makinen, Berger, Bel-Rhlid, & Ananta, 2012). Bauer, Schneider, Behr, Kulozik, and Foerst (2012) reported higher survival rates for *L. paracasei* ssp. *paracasei* after vacuum drying at a low-temperature when it was previously fermented under controlled pH (6.3) stress. Therefore, the resistance to a vacuum dehydration process along with a higher viability of *L. casei* reported in the present work could be associated with both the use of a pre-incubation step and the protective microenvironment effect of the pumpkin matrix. It is important to remark that these results were obtained in a laboratory scale. Further assays should be conducted in order to scale up the described process.

Fig. 2a and b shows the scanning electronic representative micrograph of free *L. casei* cells and the probiotic cells adhered to the PP after vacuum drying, respectively. These observations confirmed the adherence to the vegetable matrix of probiotic bacteria as well as the absence of morphological changes in cells after the fermentation and the vacuum drying process. Similar results were reported by Kourkoutas, Xolias, Kallis, Bezirtzoglou, and Kanellaki (2005) and Guergoletto et al. (2010).

3.3. *PP as a dietary supplement*

An additional batch with higher final concentration (≈9 log (CFU g^{-1}) was performed in order to test the final product as a dietary supplement. The PP was reconstituted with chocolate milk (ChM) or with a soy milk and apple juice based beverage (SM); followed by homogenisation. In order to achieve a pH < 2.5 during the gastric phase using the same quantity of gastric solution for SM and for ChM, the amount of ChM to be digested had to be reduced due to its high buffer capacity. In Fig. 3, the percentage of recovered *L. casei* cells after the gastrointestinal digestion are shown. In addition, a control assay containing free cells of *L. casei* was performed with both matrices: chocolate milk (ChMc) and with the soy milk beverage (SMc) (Fig. 3). The *L. casei* viability after the homogenisation process was within the range required by the local regulation, showing a

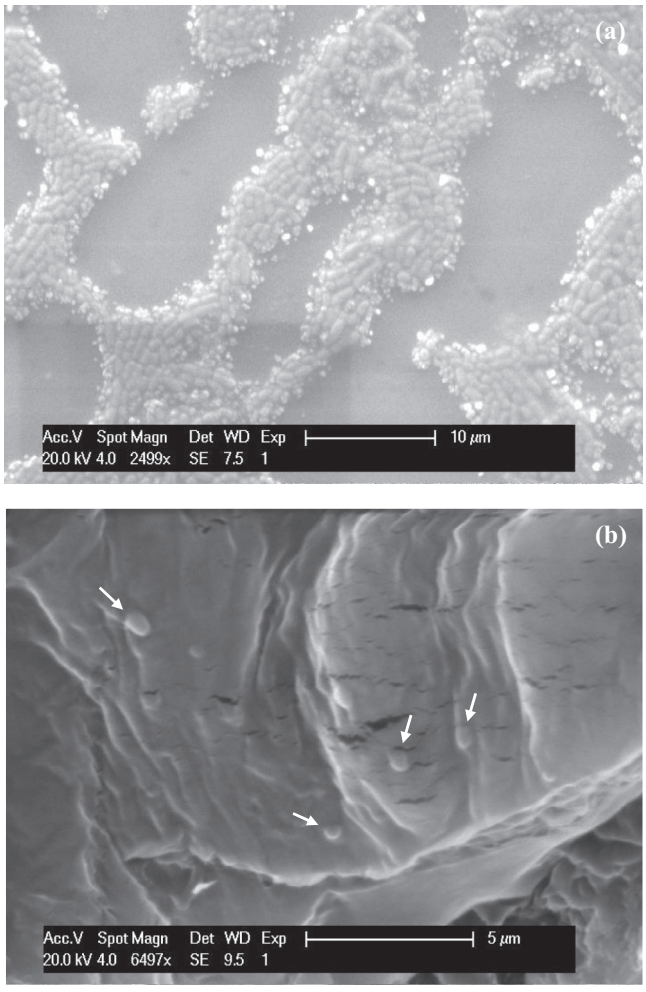

Fig. 2 – Electron micrographs of free *Lactobacillus casei* (a) and of *L. casei* adhered to pumpkin powder containing probiotics (PP) representating a selected system from an experimental design (b). Arrows show *L. casei* cells.

counting of 7.7 ± 0.2, 7.4 ± 0.1, 7.9 ± 0.2 and 7.5 ± 0.2 log (CFU mL^{-1}) for SM, SMc, ChM and ChMc, respectively. After the gastric and intestinal digestion, no differences ($p \geq 0.05$) were found in the percentage of recovered *L. casei* cells from SM or from ChM, where approximately 80% of probiotic cells were viable. These findings agree with Guergoletto et al. (2010), who showed a higher survival rate for the *L. casei* adhered to oat bran without the addition of any protection aid.

In contrast, a significant decrease ($p \leq 0.05$) in probiotic survival was observed when the free cells of *L. casei* were added to SMc and ChMc, where the cell recovery was 44 ± 2% for SMc and 91 ± 1% for ChMc, after gastric digestion, while 34 ± 8% and 68 ± 3% were recovered after the intestinal digestion phase, for SMc or ChMc, respectively. In SMc, a drastic reduction of a 5-log cycle in the *L. casei* viability was observed that could be attributed to the low initial pH (4.1) of the beverage. Opposite results were detected in SM where the *L. casei* viability decreased only 1.3-log cycles; possibly due to the pumpkin microarchitecture, which could contribute to protect probiotics during the harsh gastrointestinal environment (Ranadheera et al., 2012). It is noteworthy that these results are relevant considering the adequacy of the product obtained to be incorporated into dairy or into non-dairy food matrices with low pH.

Table 5 – Sensory acceptance of commercial beverages supplemented with pumpkin power containing *L. casei* (PP).

Attribute	ChM*		SM*	
	Score	Classification	Score	Classification
Odour	7 ± 2^a	Like fairly	7 ± 2^a	Like fairly
Texture	6 ± 2^b	Like slightly	7 ± 3^b	Like fairly
Overall acceptability	7 ± 2^c	Like fairly	7 ± 2^c	Like fairly

Values: mean ± SD; n: 120.
* ChM: chocolate milk; SM: soy milk with apple-juice.
Similar letter in the row means no significant difference ($p > 0.05$) between samples.

The consumer acceptance is considered one of the key issues for the success of functional ingredients in the marketplace (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). Table 5 shows the scores obtained for the sensory attributes of the commercial beverages supplemented with PP containing *L. casei*. Both supplemented beverages, chocolate milk (ChM) and soy milk (SM) were well accepted by consumers. The overall acceptability, in both cases, was above 7.0 on a 9-point scale (liked moderately). Similar results were obtained for the attribute of odour. Although no significant differences were observed between the attributes of the supplemented beverages, the texture score in ChM supplemented tended to be lower than that of SM. The use of a high percentage of PP in chocolate milk might be perceived as unpleasant by the consumers. The amount of PP to be supplemented could be a limiting factor to be taken into account. In this sense, more assays should be performed using the PP as an ingredient in different food formulations and concentrations.

4. Conclusions

The by-products from pumpkin could successfully be used to obtain a functional ingredient with the *L. casei* strain adhered. The process selected involved initial inoculums with the lowest cell density and incubation period, without whey supplementation and with the lowest water addition. These conditions were the most suitable for the *L. casei* growth and also confirm that a by-product from pumpkin could serve by itself as a substrate to enhance the biomass yield. Moreover, by-products generated with this proposed process would be potentially used as a source/substrate for lactic acid production. The *L. casei* adhered in the pumpkin matrix resisted in more than 80% the subsequent operations of water washing, centrifugation and vacuum drying. When PP was tested as a dietary supplement by reconstitution in two commercial beverages, a higher percentage of recovered *L. casei* after simulated gastrointestinal digestion was obtained compared to the addition of free cells. The consumer acceptance test performed on the commercial beverages supplemented with PP received scores above 6.0 on a 9-point scale in odour, texture and overall acceptability.

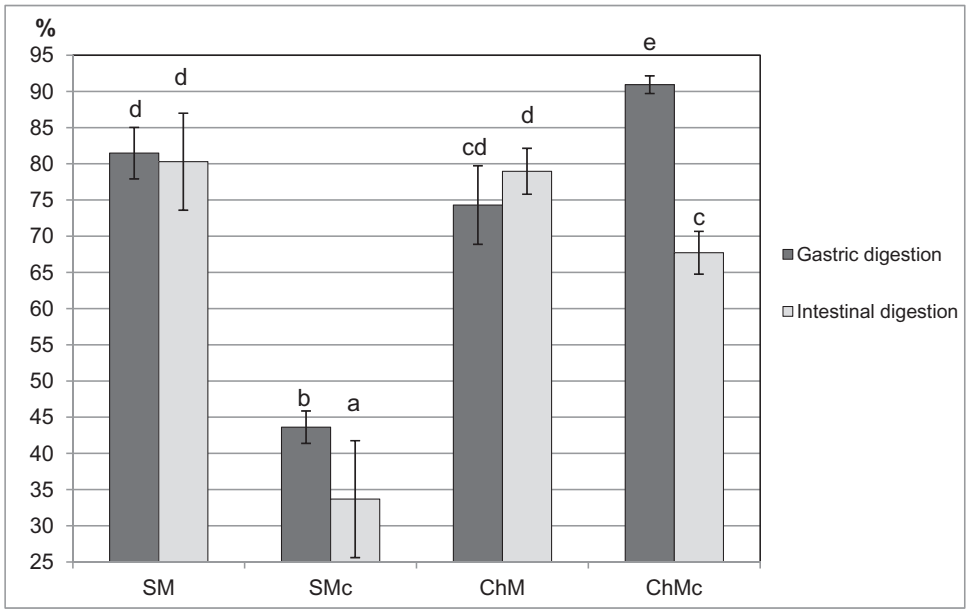

Fig. 3 – Percentage of recovered *Lactobacillus casei* cells after gastric (dark grey bars) and intestinal (grey bars) digestion in soy milk (SM) or in chocolate milk (ChM) supplemented with pumpkin powder containing probiotics (PP). Assays with soy milk (SMc) and chocolate milk (ChMc) with an addition of free cells of *L. casei* were also performed as a control. Means values and standard deviation, n = 2 were reported. Different letters mean significant differences ($p < 0.05$).

Therefore, it can be concluded that by-products from pumpkin are a good source for the preparation of a functional ingredient containing *L. casei*, thus giving added value to this waste. From the results herein obtained, more assays should be carried out in order to complete the characterisation of the ingredient and to look for other applications in food formulations.

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