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Novel strategies for fortifying vegetable matrices with iron and *Lactobacillus casei* simultaneously

C. Genevois [a, b], M. de Escalada Pla [a, b, *], S. Flores [a, b]

[a] Departamento de Industrias de la Facultad de Ciencias Exactas y Naturales de la Universidad de Buenos Aires (UBA), Intendente Güiraldes 2160 (C1428EGA), Ciudad Universitaria, Ciudad Autónoma de Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

The aim of the work was to study the effects of simultaneous fortification, iron and *Lactobacillus casei* (*L. casei*) on a vegetable matrix. Pumpkin tissue was used as vegetable matrix for iron fortification in a dry infusion process and an edible coating containing *L. casei* was applied. Three systems were assayed: A) pumpkin fortified with iron and probiotic, B) pumpkin fortified with iron, and C) pumpkin fortified with probiotic. The chroma and b* changes during storage followed a first-order kinetic model. The combined presence of iron and probiotic delayed the textural change. The final product presented 0.35 mg of iron/g, where 50–58% was bioaccessible after the *"in vitro"* digestion. The probiotic concentration remained $>10^7$ CFU g^{-1} for 14 days and the viability was affected by the mineral incorporation. The presence of *L. casei* tended to improve the iron bioaccessibility by reducing insoluble iron content in simulated lumen conditions. The untrained sensory panel did not perceive differences due to iron presence, and both formulations received punctuations above 5- using a 7-point hedonic scale for overall acceptability being rated as *"like slightly"*.

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1. Introduction

Anaemia due to iron deficiency is a global public health problem affecting both developing and developed countries, being more prevalent in pregnant women and young children (Organization World Health & Food and Agricultural Organization of United Nations, 2006).

The food fortification refers to a process whereby one or more essential micronutrient are added improving their nutritional quality to prevent or correct a micronutrient deficiency (Ottaway, 2008).

In a previous work, *Cucurbita moschata* Duchesne ex Poiret tissue had been successfully fortified with iron and ascorbic acid applying a dry infusion process (Genevois, Flores, & de Escalada Pla, 2014; Genevois, de Escalada Pla, & Flores, 2015). In addition, it was concluded that when an edible coating (EC) technology was performed, the colour and ascorbic acid retention were improved and the iron accessibility at pH of lumen intestinal tended to be higher.

An extensive literature supports the actions of probiotics in maintaining the balance of intestinal flora (Oelschlaeger, 2010) and it was suggested that a healthy gut could perform a better mineral absorption (Scheers, Rossander-Hulthen, Torsdottir, & Sandberg, 2015). In this sense, dairy products with probiotics have the most developed market at the moment.

According to our knowledge, scarce information about the iron fortification of vegetable matrices as well as the effect of iron presence on probiotic survival has been published so far. In this context, the research of iron fortification combined with the probiotic vehiculization in minimally processed fruits or vegetables is a matter of pending investigation.

The objective of this work was to develop and characterize a pumpkin based food fortified with iron and simultaneously covered with a HPMC coating containing *L casei*. The effects of mineral fortification on sensory characteristics and on probiotic survival as well as, the influence of the probiotic presence on the iron bioaccesibility after *in vitro* simulated gastrointestinal digestion were evaluated.

* Corresponding author. Departamento de Industrias de la Facultad de Ciencias Exactas y Naturales de la Universidad de Buenos Aires (UBA), Intendente Güiraldes 2160 (C1428EGA), Ciudad Universitaria, Ciudad Autónoma de Buenos Aires, Argentina.
E-mail address: marina@di.fcen.uba.ar (M. de Escalada Pla).

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2. Material and methods

2.1. Chemicals

Food grade sucrose, glucose (Anedra, Argentina) and HPMC (Methocel® E4M Dow Chemical, USA) containing 28–30% of methoxyl groups were employed. The additives $FeSO_4 \cdot 7H_2O$ (Merck, USA), potassium sorbate (Sigma, USA), citric acid (Sintorgan, Argentina), glycerol (Sintorgan, Argentina) and other used chemicals were of analytical grade.

2.2. Preparation of snack based on pumpkin

2.2.1. Dry infusion process of pumpkin cylinders

The dry infusion process was carried out as previously reported (Genevois et al., 2014). Briefly, the cylinders were blanched with water vapour for 7 min, rapidly cooled for 1 min by immersion in water at 0 °C and then submitted to a dry infusion. Blanched pumpkin cylinders were placed in a plastic bowl and glucose (33 g/100 g of raw pumpkin); sucrose (23 g/100 g of raw pumpkin); citric acid (0.18 g/100 g of raw pumpkin); potassium sorbate (0.13 g/100 g of raw pumpkin) and $FeSO_4 7H_2O$ (0.6 g/100 of raw pumpkin, equivalent to 119 mg of iron) were added. At the same time, another batch was performed as a control system (C), under the same conditions but without the $FeSO_4 7H_2O$ addition. The dry infusion was carried out at 20 °C up to equilibrium on an orbital shaker (Vicking S.A., Argentina) at 0.045× g. After 48 h, the dry infusion was concluded and the cylinders were drained through a stainless steel strainer.

Afterwards, the pumpkins cylinders fortified with iron were divided in two batches, one of them was coated by dipping with an edible coating based on HPMC containing *L. casei* (system A) as described in section 2.2.4, and the other one was coated with HPMC based EC without probiotic (system B) as described in section 2.2.2. Meanwhile, the pumpkins cylinders without iron fortification were coated with HPMC based EC containing *L. casei* (system C).

The pumpkin pieces were immersed for 1 min into the corresponding HPMC based EC solution; thereafter, they were removed from the solution and the residual fluid was allowed to drop off for 1 min. The systems were dried in a convection chamber (FAC, Argentina) at 35 °C for 4 h. Finally, the dried pumpkin cylinders were packed in plastic bags of low density polyethylene with an easy-to-close Ziploc® closing and stored for 21 days at 8 °C.

2.2.2. Edible coating preparation

HPMC (1.5%, w/v) was dissolved in distilled water under constant stirring for 20 min. Afterwards; glycerol (0.5%, w/v) and potassium sorbate (0.025%, w/v) were added to the biopolymer aqueous solution. The solution was stirred at room temperature till obtaining a proper homogenization.

Lastly, the coating solution was sterilized for 15 min at 121 °C and reserved at room temperature under magnetic stirring, until use.

2.2.3. Incorporation of Lactobacillus casei into the edible coating based on HPMC

The *L. casei* inoculums were prepared by transferring 1 mL of the probiotic stock culture (Genevois, Flores, & de Escalada Pla, 2016) into conic tubes (Eppendorf, Hamburg, HH, Germany) containing 30 mL of sterile MRS broth and the cell growth was carried out at 37 °C for 18–20 h with orbital shaking. Then, *L. casei* cells were harvested by centrifugation (Eppendorf 5804R, Germany) for 10 min at 12.000× g at 6 °C, followed of two washes with sterile physiologic solution. Finally, the probiotic pellet obtained was resuspended in sterile distilled water and mixed with sterile HPMC solution (see section 2.2.2). The solution was stirred until obtaining a proper homogenization. The viable *L. casei* count was 10.1 ± 0.1 log CFU per mL of HPMC based EC.

2.3. Physicochemical characterization of pumpkin snack after drying

The superficial pH was determined with a surface combined glass electrode Ag°/AgCl connected to a pH meter (Cole-Parmer, USA).

The water loss of pumpkin cylinders during the drying process was determined as the difference between initial and final mass.

The water activity (a_w) was measured with a hygrometer (Aqualab, USA) at 20 °C, and the moisture content was determined with a moisture analyzer (Ohaus MB-45, USA).

All measurements were performed at least in duplicate and the mean values ± standard deviations (SD) were reported.

2.4. Colour evaluation and texture analysis

Colour as well as texture analysis were performed according to Genevois et al., 2015. After the drying process and during the storage, colour was measured with a colorimeter (Konica Minolta, Japan) in the CIE L* a* b* colour space, using D65 illuminant and 2° observer angle.

Texture was evaluated trough a compression test using an Instron Universal testing machine (Model 3345, USA) provided with a 500 N-load cell and an upper steel plate of 30-mm diameter. The compression rate used was 5 mm/min. Force (N) vs displacement (mm) plots were recorded up to 80% of the sample deformation. The strain ($\varepsilon\% = D/H \times 100$, being D the displacement and H the initial height of the pumpkin cylinder; %) and the stress ($\sigma = F/A$, being the F the force and A the cross section of the pumpkin cylinder; MPa) were evaluated from the force vs displacement profile. The elastic modulus (E_m; MPa) was the slope at the beginning of the plot, and the firmness (MPa) was calculated as the ratio between σ (MPa) and ε (%). At least, four replicates were assayed for each system and the average values ± SD were reported.

2.5. L. casei bacteria counting

Viable cell count was determined by preparing decimal serial dilutions from pumpkin samples with 0.1% (w/v) peptone water (Biokar Diagnostics, France). Aliquots of 0.1 mL were plated in depth using MRS agar (Biokar Diagnostics, France) followed by incubation at 37 °C for 72 h under aerobic conditions. Each determination was performed at least in duplicate, and the mean values ± SD were reported.

2.6. "In vitro" assays

The simulated gastrointestinal digestion (mouth, stomach and small intestine) was conducted according to Genevois et al., 2015. Briefly, ~5 g of pumpkin were mixed with 5 mL of artificial saliva solution. The gastric phase was started adding 30 mL of gastric solution, followed of 30 mL of intestinal solution.

The survival of *L. casei* to the simulated gastrointestinal process was performed in systems A and C at least in duplicate, by plate counting in MRS agar.

The iron content was measured with a spectrophotometer (Shimadzu Model UV-1800, Japan) using the method recommended by the AOAC (AOAC official method 937.03, 1990; Nayak & Nair, 2003). The measurements were performed on the supernatant and on the residue after the *in vitro* simulated gastrointestinal digestion, at least in duplicate. The fraction of the bioaccessible iron

was calculated using Eq. (1):

$$\text{Bioaccessibility\%} = \frac{\text{Fe}_{\text{intestinal}}}{\text{Fe}_{\text{intestinal}} + \text{Fe}_{\text{residue}}} \times 100 \quad (1)$$

Where $Fe_{intestinal}$ is the iron fraction (mg of iron g^{-1} of pumpkin; db.) in the gastrointestinal supernatant and $Fe_{residue}$ is the iron fraction (mg of iron g^{-1} of pumpkin; db.) in the gastrointestinal residue.

2.7. HPMC based edible films characterization

In order to analyse the effect of *L. casei* presence in the biopolymeric matrix properties, two edible films were prepared: a) HPMC based edible films containing viable *L. casei* (HPMC *L. casei*) and, b) HPMC based edible films (control). The films were obtained by spreading ~20 g of HPMC solution onto polyethylene Petri dishes of 9 cm diameter and drying in a convection chamber at 45 °C for 20 h. Afterwards they were removed from Petri dishes and equilibrated during 5 days at 75% relative humidity and 25 °C before testing. A tensile test on films trips (6 × 60 mm) was performed with an universal testing machine (Instron, Model 3345, US) according to Espinel, Flores, and Gerschenson (2014). At least, ten specimens were assayed for each system and the mean values ± SD were reported.

2.8. Scanning electron microscopy (SEM)

The presence of *L. casei* was confirmed by SEM (Supra™40, Carl-Zeiss, Germany) under vacuum with an acceleration voltage of 3.00 kV and magnifications up to 10.000×. The samples were maintained in a desiccator with $CaCl_2 \cdot 2H_2O$ during 3 days, and then they were mounted on a bronze stub and sputter-coated (Sputter 108, Cressington Scientific Instruments, UK) with a gold layer prior to imaging.

2.9. Sensory evaluation

The sensory evaluation was assayed in system A and C. Ninety-six frequent or infrequent pumpkin consumers, participated voluntarily as untrained panellists. The samples were offered at room temperature in order to obtain homogeneous conditions during the three sensory analysis sessions. The panellists received 5 g of the sample in a plastic tray coded with a three-digit random number. The test was performed in individual booths. Consumers were instructed to rinse their mouths with water and to eat a cracker between samples in order to avoid carryover effects (Lawless & Heymann, 2010).

Firstly, a discriminative test, triangular test session, was performed to confirm if the samples would be perceived as different as a consequence of iron presence (ISO 4120:2004). Subsequently, a consumer acceptance test was carried out to determine the overall acceptability. The panellists were asked to judge the samples with respect to their degree of liking or disliking of the sample using a structured 7-point hedonic scale (ISO 4121:2003).

2.10. Statistical analysis

Statistical analysis of the results was performed through analysis of variance (ANOVA) for a level of significance (α) of 0.05 followed by a LSD Fisher post-test to identify significant differences between samples. The *t* student post-test was used in the consumer acceptance examination.

Statistical analysis of the results and linear regression were performed using the Statgraphics Centurion XV software (V 2.15.06, 2007, Statpoint Technologies, Inc., USA).

3. Results and discussion

3.1. Physical-chemical characteristic of the snack pumpkin

After the drying process, the system A, B and C presented a 15 ± 5% of water loss, 4.42 ± 0.05 of surface pH, 20 ± 5% of moisture content and 0.81 ± 0.03 of a_w. No significant differences among the systems were observed (data not shown) regarding the studied parameters. In addition, these parameters remained stable during the storage (data not shown). The *Lactobacillus* spp is characterized by the capacity of producing organic acids from sugar metabolism and, as consequence, the pH of the environment decreases (Willey, Sherwood, & Woolverton, 2011). In the present research, no significant ($p > 0.05$) pH reduction occurred during the 21 days of storage, which could be attributed to the immobilization of *L. casei* in the biopolymer matrix.

3.2. Colour evaluation

The Chr and b* resulted the most sensitive parameters to register the surface colour changes in pumpkin during the storage. In Fig. 1 a and b, the changes in Chr and b* as a function of storage time

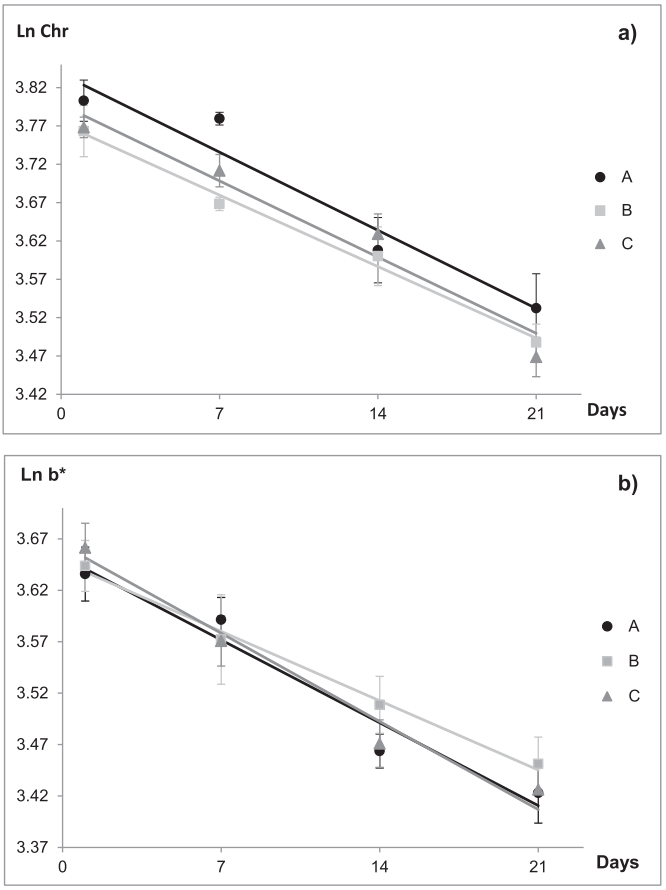

Fig. 1. Chroma **(a)** and b* **(b)** parameters degradation kinetics of pumpkin snacks as function of time. Black, dark grey and grey lines represent the fit of the first-order model to the experimental data. Dots, squares and triangles correspond to the experimental data of the assayed systems.
A) Pumpkin fortified with iron and coated with HPMC based edible coating (EC) containing *L. casei*; B) Pumpkin fortified with iron and coated with HPMC based EC; C) pumpkin without iron fortification and coated with HPMC based EC containing *L. casei*.

are shown. Negative slopes of linear tendency for Chr changes (Fig. 1a), indicate a loss in the colour intensity which are mainly associated to the loss of yellowness (b*) (Fig. 1b). According to Dutta, Dutta, Raychaudhuri, and Chakraborty (2006), there is a direct correlation between visual colour changes and the β-carotene concentration in pumpkin tissue. The corresponding initial colour parameter and the kinetic rate constant are given in Table 1. As could be observed, no significant differences ($p > 0.05$) were detected on the rate of Chr and b loss in the pumpkin during the storage due to the iron presence or to the *L. casei* fortification under the conditions assayed here in. In part, these results are in line with Sánchez-González, Quintero Saavedra, Saavedra, & Chiralt (2013) who reported that no changes in the colour parameters of films based on HPMC occurred as a result of the lactic acid bacteria incorporation. A picture of pumpkin systems tested at the end of storage is shown in Fig. 2.

3.3. Iron content in the pumpkin fortified

The final product showed an average content of 35 ± 8 mg of

Table 1
Initial colour parameters and kinetic constant values for chroma (Chr) and b* obtained from the first order kinetic model.

Systems	Chr				b*			
	C_0	k x 10^2 (day^{-1})	R^2	DW	C_0	k x 10^2 (day^{-1})	R^2	DW
A	45 ± 1 [a]	1.4 ± 0.3 [a]	0.93	3.2	38.6 ± 0.8 [a]	1.1 ± 0.2 [a]	0.96	3.4
B	43.1 ± 0.5 [a]	1.33 ± 0.01 [a]	0.99	3.4	38.4 ± 0.3 [a]	0.96 ± 0.06 [a]	0.99	2.1
C	43 ± 1 [a]	1.4 ± 0.2 [a]	0.96	2.1	39.1 ± 0.7 [a]	1.2 ± 0.1 [a]	0.97	2.1

A) pumpkin snacks fortified with iron and coated with HPMC based edible coating (EC) containing *L. casei*; B) pumpkin snacks fortified with iron and coated with HPMC based EC; C) pumpkin snacks without iron fortification and coated with HPMC based EC containing *L. casei*.
C_0: initial colour parameter and the corresponding standard deviations (SD); k: kinetic rate constant and the R^2: determination coefficients. DW: Durbin-Watson statistic.
Similar letters in the same column denote no significant ($p > 0.05$) differences between the mean values.

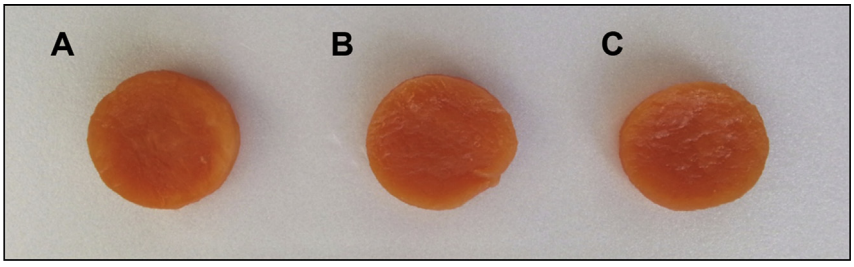

Fig. 2. Picture of the pumpkin snacks at 21 days of storage.
A) Pumpkin fortified with iron and coated with HPMC based edible coating (EC) containing *L. casei*; B) Pumpkin fortified with iron and coated with HPMC based EC; C) pumpkin without iron fortification and coated with HPMC based EC containing *L. casei*.

Table 2
Soluble and insoluble iron content after *in vitro* simulated gastrointestinal digestion of pumpkin snacks and total iron content.

Systems	Soluble intestinal iron content [1]	Insoluble intestinal iron content [2]	Total iron content	Bioaccessibility %
A	0.21±-0.06 [a]	0.14 ± 0.03 [a]	0.35 ± 0.06 [a]	61 ± 6 [a]
B	0.20 ± 0.04 [a]	0.19 ± 0.02 [b]	0.38 ± 0.05 [a]	50 ± 4 [b]
C	0.006 ± 0.005 [b]	0.03 ± 0.01 [c]	0.03 ± 0.01 [b]	16 ± 7 [c]

Iron content in the [1]supernatant and in the [2]residue after the *in vitro* simulated gastrointestinal digestion.
Values represent the averages ± SD of independent samples (n = 4) expressed as mg of iron g^{-1} of pumpkin (dried basis).
A) Pumpkin snacks fortified with iron and coated with HPMC based EC containing *L. casei*; B) pumpkin snacks fortified with iron and coated with HPMC based EC; C) pumpkin snacks without iron fortification and coated with HPMC based EC containing *L. casei*.
Different letters in the same column denotes significant differences ($p < 0.05$) between systems.

Table 3
Texture parameters of pumpkin snacks coated with hidroxypropyl methylcellulose at baseline and final storage time.

Systems	Day 1			Day 21		
	Stress (MPa)	Firmness (MPa)	E_m (MPa)	Stress (MPa)	Firmness (MPa)	E_m (MPa)
A	0,061 ± 0014 [a]	0,12 ± 0,03 [d]	0,14 ± 0,02 [g]	0,08 ± 0,01 [a]	0,17 ± 0,03 [d]	0,18 ± 0,04 [g]
B	0,059 ± 0018 [a]	0,12 ± 0,04 [d]	0,14 ± 0,04 [g]	0,16 ± 0,03 [c]	0,32 ± 0,06 [f]	0,31 ± 0,07 [i]
C	0,016 ± 0003 [b]	0,032 ± 0007 [e]	0,04 ± 0,004 [h]	0,08 ± 0,01 [a]	0,15 ± 0,04 [d]	0,20 ± 0,05 [g]

E_m: elastic modulus.
Values represent the averages ± SD of independent samples (n = 4).
Different letters denote significant ($p < 0.05$) differences between the mean values of the texture parameters at different storage times.
A) Pumpkin snack fortified with iron and coated with HPMC based EC containing *L. casei*; B) Pumpkin snack fortified with iron and coated with HPMC based EC; C) Pumpkin snack without iron fortification and coated with HPMC based EC containing *L. casei*.

iron/100 g of pumpkin (db.). According to the Argentinian Food Code, a fortified food should contain at least 20% of the Recommended Nutrient Intake (RNI) per portion in the final product. Therefore, a size serving of about 25 g of pumpkin fortified with iron (4–5 cylinders) would provide about 48.6% and 30.2% of the RNI established for men (14 mg/day) and women (29 mg/day), respectively (FAO/OMS, 2001, pp. 195–216). On the other hand, by comparing iron content of A and B systems with C it could be observed that process applied here in, allowed to fortify the pumpkin matrix ≈12 times (Table 2). Barrera, Betoret, and Fito (2004) have reported that vacuum impregnation followed by osmotic dehydration increased 5.6 times iron content of apple slices.

The Table 2 also shows the content of soluble and insoluble intestinal iron after the *in vitro* simulated gastrointestinal digestion. The first step towards mineral bioavailability comprises the solubility in the intestinal tract, the bioaccessibility (Cilla et al., 2009). The bioaccessibility has been defined as the fraction of a compound that is released from its food matrix in the gastrointestinal tract and becomes available for intestinal absorption (Alegría, Garcia-Llatas, & Cilla, 2015). As regards to the total and the soluble intestinal content of iron in the pumpkin fortified, no significant differences were observed between systems A and B. However, a slight but significant difference was presented in the iron content in the residue and, consequently, the percentage of mineral bioaccessibility resulted different ($p < 0.05$) (Table 2). System A showed slightly higher ($p < 0.05$) iron bioaccessibility in comparison to the system B. The less insoluble iron content in the system A could be associated to the presence of *L. casei* (Scheers et al., 2015). Scholz-Ahrens et al. (2007) also summarized some evidences regarding probiotic effect on facilitating mineral absorption. They reported that several studies in animals and humans have shown positive effects of calcium metabolism, bone composition and bone architecture, and these effects were not in all cases uniform. One of the mechanisms proposed to explain these effects is the hydrolysing of glycoside bond of the foods in the intestines by *Lactobacillus* and *Bifidobacterium* that leads to a release of mineral and thus increases the bioaccessibility (Parvaneh, Jamaluddin, Karimi, & Erfani, 2014).

3.4. Effect of iron presence on the texture and L. casei surviving

The stress, the firmness and the elastic modulus recorded from forces-deformation curves are presented in Table 3. The texture parameters were determined at 50% of strain since no sharp break point tissue was detected during the measurements, suggesting a high plasticization level of the pumpkin matrix after drying infusion process and coating. At the beginning of storage, no significant differences were noted for stress, firmness and elastic modulus in the pumpkin cylinders fortified with iron coated with HPMC based EC containing *L. casei* (system A) and pumpkin cylinders coated with HPMC based EC (system B). The stress, firmness and elastic modulus were around one order magnitude lower in the pumpkin without iron fortification covered with HPMC containing *L. casei* (system C), which could be attributed to the iron absence in the vegetable tissue.

Very few references about the iron presence on vegetables texture has been reported so far. Mierczynska, Cybulska, Sołowiej, and Zdunek (2015) have recently reported that the iron presence in a food matrix made of modified cell wall polysaccharides from apple pomace, improved the rheological properties and this behaviour could be due to the iron ions may cross-link the pectins in a similar way as calcium, i.e. according to the egg-box model.

After 21 days of storage, the system B and C showed a significant increase in the stress, firmness and elastic modulus respect to the baseline values. Meanwhile, in the system A only a slight and no significant ($p > 0.05$) tendency on these increasing values, was noted.

On the other hand, the probiotic presence in HPMC based EC could introduce discontinuities in the biopolymer network. Thus, a tensile test was carried out to elucidate if probiotic presence in the polymer matrix could affect the film performance. The mechanical properties of the HPMC edible film determined by a tensile plot (data not shown) presented significant higher values for the stress

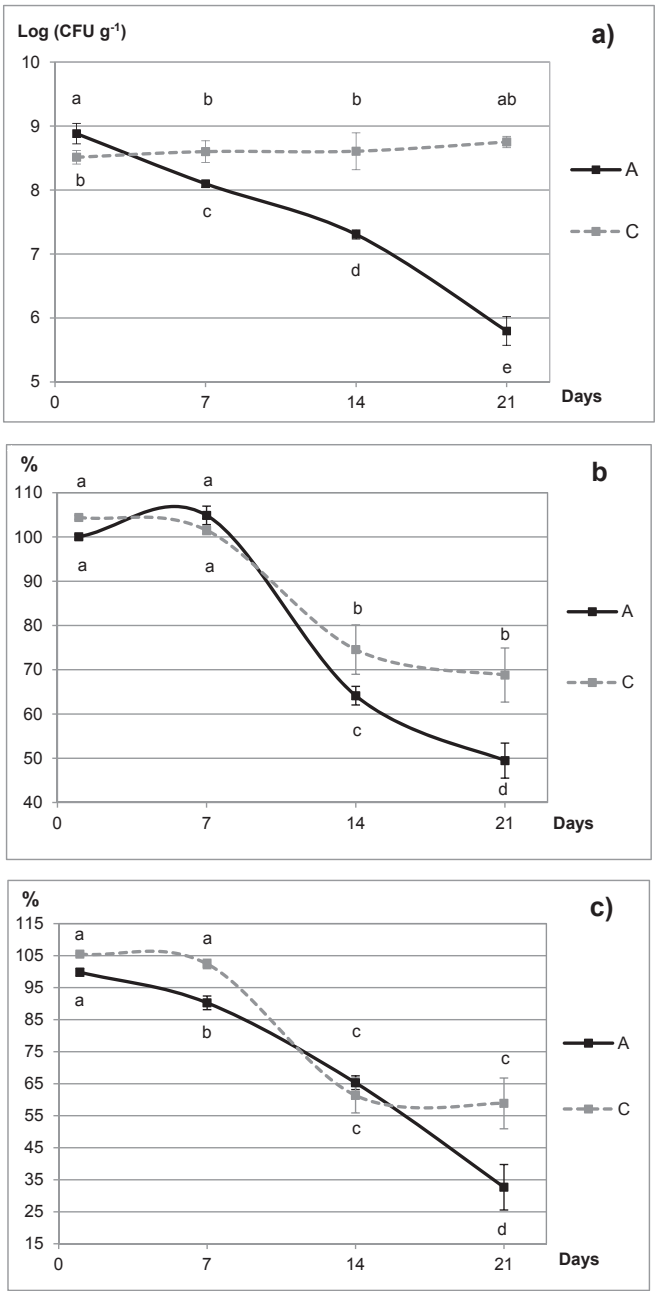

Fig. 3. *L. casei* count in the final product of system A and C stored for 21 days **(a)**, *L. casei* survival to *in vitro* simulated gastric conditions **(b)**, and *L. casei* survival to *in vitro* simulated intestinal conditions **(c)**.
Pumpkin fortified with iron and coated with HPMC based EC containing *L. casei* (system A, ■) and pumpkin without iron fortification coated with HPMC based EC containing *L. casei* (system C, In grey ■).
Values represent the average of two ($n = 2$) independent samples ± SD. Cell count expressed as log (CFU g^{-1} pumpkin) (dry basis). Survival expressed as percentage.
Different letters denote significant ($p \leq 0.05$) difference between the systems.

at break (σ_b) (27 ± 3 MPa) and the strain at break (ε_b) (46 ± 7%) when *L. casei* was absent (control system). When the probiotic was incorporated into the biopolymer matrix the σ_b was 20 ± 3 MPa and the ε_b was 31 ± 5%. Therefore, the incorporation of probiotic into the film resulted in a significant ($p < 0.05$) reduction of stress and strain at break. These changes in the mechanical properties could explain, in part, that B system presented the highest firmness at 21 storage days.

These results suggest that the combined presence of iron and probiotic could delay the textural change during storage in the final product, and that the iron presence or *L. casei* vehiculization separately in the vegetal tissue exerted the main effect on the texture parameters.

During the 21 days of storage, the *L. casei* viability was constant ($p > 0.05$) in the pumpkin without iron fortification (system C), showing an average count of 8.7 ± 0.1 log (CFU g^{-1} pumpkin; db.) (Fig. 3). The probiotic count was reduced 3-log cycles in the system A after 21 days of storage (Fig. 3a) and presented values of 5.8 ± 0.2 log (CFU g^{-1} pumpkin; db.). The international and local regulations establish a probiotic count $>10^6$ CFU g^{-1} (FDA, EFSA and ANMAT). Therefore, the low count of *L. casei* in the pumpkin fortified with iron at 21 days marked the end of the product shelf life.

In order to act as a probiotic in the gastrointestinal tract, the bacteria must be able to survive the acidic conditions in the stomach and to resist bile acids in the small intestine (Monteagudo-Mera et al., 2012). The *L. casei* survived successfully to the simulated gastric acid digestion in both systems till 7 days of storage (Fig. 3b).

After the simulated intestinal digestion, the *L. casei* survival significantly decreased at the 7 days of storage in system A respect to the system B (Fig. 3c). Nevertheless, after 14 days of storage both systems presented similar probiotic survival (~60%). At the end of storage, B system remained stable (59 ± 8%) while system A decreased at 33 ± 7%, representing a total reduction of 3.2 and 6.8 log cycles, respectively.

In line with these results, Vinderola et al. (2011) reported that despite a good stability in cell viability during refrigerated storage, there was a decrease in probiotic tolerance to acid and bile during the storage in commercial fermented milks.

The low value of probiotic survival in the product fortified with iron could be explained with the mineral diffusion from the area with high concentration to the low iron concentration in coating causing a toxic effect on *L. casei* strains (Solioz, Mermod, Abicht, & Mancini, 2011). To our knowledge, no reports on lactic acid bacteria survival in vegetable tissue fortified with iron have been reported hereto. Besides, little knowledge is available about the metal intracellular homeostasis and defence against metal stress by lactic acid bacteria (LAB) (Solioz et al., 2011). The LAB are iron-independent microorganisms; nevertheless under aerobic growth conditions the intracellular ferrous iron is unstable and in the presence of oxygen some reactive species are created, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radicals ($OH^{\bullet}$) which are responsible of cell microorganism toxicity (Solioz et al., 2011).

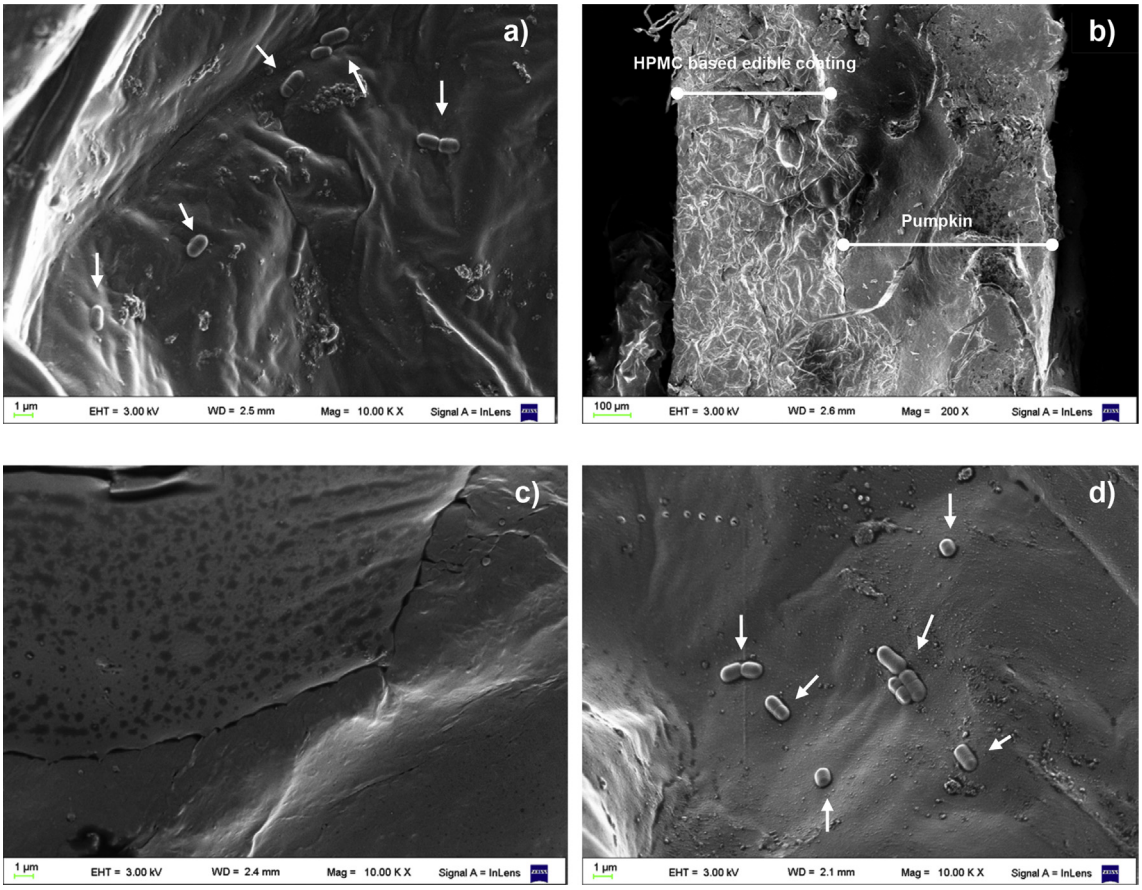

Fig. 4. Scanning electron microscopy of snacks based on pumpkin at different magnifications.
a) Surface and **b)** cross section of pumpkin snacks fortified with iron and coated with HPMC based EC containing *L. casei*. **c)** Surface of pumpkin snacks fortified with iron and coated with HPMC based EC. **d)** Surface of pumpkin snacks without iron fortification and coated with HPMC based EC containing *L. casei*.
Arrows show *L. casei* cells.

3.5. Microstructure analysis

In Fig. 4a it is possible to observe the surface of pumpkin fortified with iron and coated with HPMC based EC containing *L. casei* (system A). The arrows indicate the probiotic cells presence into the biopolymeric matrix. A good adherence of HPMC based EC to the vegetal matrix is manifested on Fig. 4b. Fig. 4c shows the surface of the pumpkin fortified with iron and coated with HPMC based EC (system B) in absence of probiotic cells. The surface of the pumpkin without iron fortification and covered with EC HPMC based containing *L. casei* (system C) is presented in Fig. 4d, where probiotic cells adherence to the biopolymeric matrix and some cellulose fragments were detected.

3.6. Sensory evaluation

Iron fortification of beverages or foods are commonly associated to undesirable changes in colour and the metallic taste which makes difficult the consumer's acceptance (Gahruie, Eskandari, Mesbahi, & Hanifpour, 2015). Therefore, the sensory evaluation was performed in the pumpkin with (systems A) and without (system C) iron fortification; both covered with HPMC based EC containing *L. casei*.

According to ISO 4120:2004 for a Triangle Test, a minimum of forty-one correct responses are required in a number of ninety-six assessors to assume that differences exists between samples. Since the number of correct answer (thirty-nine panellists) was lower than theoretical value, no sensory differences were perceivable due to iron fortification ($p > 0.05$).

As regards to the Affective Test, both samples were accepted well by the consumers. No significant differences ($p > 0.05$) were established for the overall acceptability between system A and B (data not shown), receiving a punctuation above 5 ± 1 on a seven point scale being rated as “like slightly” (ISO 4121:2003).

4. Conclusions

The ingestion of a normal pumpkin size serving of about 25 g would provide a content of iron above 30% of the RNI, and a probiotic concentration above that is required by the international organizations (EFSA and FDA) for considering as probiotic product at the moment of consumption. The probiotic concentration remained $>10^7$ CFU g^{-1} for 14 days and the viability was affected by the mineral fortification. The presence of *L. casei* tends to improve the iron bioaccessibility by reducing insoluble iron content in simulated lumen conditions.

The combined presence of iron and probiotic delayed the textural change. The rate constants of colour degradation were not affected due to iron fortification in the pumpkin, or due to *L. casei* presence. The sensory evaluation showed that the pumpkin fortified with iron was not perceivable as different by the untrained panel, and the product received above 5- on a 7-point scale being rated as “like slightly”.

The pumpkin tissue as well as the EC application represent interesting strategies to formulate non-dairy products fortified with iron salts and probiotics.

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