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Preservation of functionality of *Bifidobacterium animalis* subsp. *lactis* INL1 after incorporation of freeze-dried cells into different food matrices

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ABSTRACT

The aim of this work was to investigate how production and freeze-drying conditions of *Bifidobacterium animalis* subsp. *lactis* INL1, a probiotic strain isolated from breast milk, affected its survival and resistance to simulated gastric digestion during storage in food matrices. The determination of the resistance of bifidobacteria to simulated gastric digestion was useful for unveiling differences in cell sensitivity to varying conditions during biomass production, freeze-drying and incorporation of the strain into food products. These findings show that bifidobacteria can become sensitive to technological variables (biomass production, freeze-drying and the food matrix) without this fact being evidenced by plate counts.

1. Introduction

Probiotic bacteria are normally commercialized as frozen or lyophilized cultures and are added mainly to fermented milk and cheese, the most used food vehicles for probiotic bacteria (Ranadheera et al., 2010). Much less has been published on survival of probiotics in non-fermented and/or non-dairy food matrices (Saarela et al., 2006). Several factors related to biomass production and incorporation into commercial foods, which affect cell viability and functionality, were described by Ranadheera et al. (2010). The definition of probiotics recognizes the capacity of live microorganisms to exert a beneficial effect on the consumer's health. From the moment they are incorporated into foods, cell viability is routinely used for monitoring functionality during storage by means of plate counts. Thus, scientific criteria (Champagne et al., 1997; Ross et al., 2005) or present legislation in some countries (Amagase, 2008; Degnan, 2008; Saxelin, 2008) require a particular concentration of viable bacteria in a particular product to call it a probiotic food.

Changes in cell functionality have come into focus; it was reported that qualitative and quantitative attributes of interest of a probiotic strain were negatively affected, without changing levels of viable cells accordingly (Saarela et al., 2005). This indicates that the microbiological counts of probiotic bacteria only partially reflect the functional capacity of the strain (Heller, 2001). It is therefore essential to define other microbiological parameters able to detect changes on strain functionality in a particular food matrix. For instance, Saarela et al. (2006) studied the changes of the resistance to simulated gastric acidity in bifidobacteria in fruit juice and low-fat milk during storage. The authors concluded that culture stability data alone did not give an accurate prediction of probiotic functionality in the adverse conditions of a food matrix. Wang et al. (2009) studied the resistance of *Lactobacillus casei* Zhang to simulated gastric juice. The authors showed that during storage at 4 °C for 28 days the acid stress in fermented soymilk negatively affected the resistance to simulated gastric acidity compared to bovine milk used as controls.

The aim of this study was to investigate how production and freeze-drying conditions of *Bifidobacterium animalis* subsp. *lactis* INL1, a probiotic strain isolated from breast milk, affected survival and resistance to simulated gastric digestion along storage of freeze-dried powders and in food matrices.

2. Materials and methods

2.1. Strain

The microorganism used in this study was *B. animalis* subsp. *lactis* INL1, a strain isolated from breast milk and characterized in a previous work (Zacarías et al., 2011). The strain displays the

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capacity to enhance gut mucosal defenses (mediated by IgA) when administered to mice. The strain was kept at −70 °C in MRS (Biokar, Beauvais, France) broth containing 20% (v/v) glycerol (Ciccarelli, Buenos Aires, Argentina) at the INLAIN culture collection. Fresh cultures of the strain (grown overnight) were obtained in MRS broth after incubation at 37 °C during 18 h in an anaerobiosis chamber equipped with Anaerocult A (Merck, Darmstadt, Germany).

2.2. Freeze-drying in different cryoprotectants

Fresh cultures of the strain were centrifuged (8000 × g, 5 °C, 15 min) and washed twice with ¼-strength Ringer's solution (Merck, Darmstadt, Germany). The pellet was resuspended in phosphate-buffered saline (PBS) solution pH 7.4, supplemented with 10% and 15% (w/v) of lactose and sucrose (Merck) solution, respectively, or 12% (w/v) skim milk (Difco, Becton Dickinson, MD, USA). Aliquots (2 ml) of cell suspensions were poured into 10 ml glass vials, frozen with a freezing rate of ca. 1°/min and stored at −70 °C until further processing. Samples were freeze-dried over 22 h in a single chamber freeze-drier (beta 2–16, Christ, Osterode, Germany). The drying vacuum applied was 0.63 mbar for suspensions containing lactose and 0.22 mbar for sucrose assuring product temperatures of −25 °C or −35 °C during primary drying. At the end of the process, vials were sealed under vacuum (crimp vials with rubber stopper and aluminium cap) and stored at 25 °C or −20 °C for further assays. Moisture content was assessed gravimetrically in triplicate (85 °C, ca. 3 h).

2.3. Cell enumeration and determination of the resistance to simulated gastric digestion (SGD)

Freeze-dried cells were reconstituted with distilled water (the same volume lost during freeze-drying) and the cell suspensions were allowed to stand for 15 min at room temperature for rehydration. Serial dilutions were made in ¼-strength Ringer's solution and adequate dilutions were plated in duplicate on the surface of MRS agar. Plates were incubated in an anaerobic chamber for 48 h at 37 °C.

In order to select the most adequate cryoprotectant for further studies, resistance of freeze-dried cells to SGD was assessed as an accelerated storage stability test (Saarela et al., 2005) in fresh overnight cultures and in freeze-dried cells kept at 25 °C for 4 weeks. Cell suspensions were brought to pH 2.0 with 5 N and 0.1 N HCl in the presence of 0.5% (w/v) NaCl and 0.3% (w/v) porcine pepsin (Merck) and incubated at 37 °C in a water bath for 90 min. Cell counts were performed before acidification and after 90 min of exposure to the solution simulating the gastric digestion.

2.4. Growth kinetics in a biofermentor and resistance to SGD

B. animalis subsp. *lactis* INL1 was grown in a 1.5 l biofermentor (Biostat B, Sartorius Stedim Systems, Melsungen, Germany) at pH 6.5 and 5.0, respectively, as controlled by the addition of 8 M NaOH solution. Biomass production was conducted at 37 °C with a CO_2 influx of 0.2 l min^{-1} and a stirring rate of 300 rpm. Cell growth was monitored every 2 h by measuring optical density (at 620 nm) and cell counts on MRS agar. Biomass samples (700 ml) were taken at 12 and 22 h of culture, centrifuged, washed twice with ¼-strength Ringer's solution, suspended in 10% (w/v) lactose and frozen at −70 °C before freeze-drying. The resistance to SGD was assessed in freeze-dried cells grown at different pH and harvested at different incubation times as explained above.

2.5. Scanning electron microscopy studies

Bacterial cultures grown overnight in MRS broth were diluted 1:5 with ¼-strength Ringer's solution, and 1-ml samples were filtered on a Nuclepore track-etch membrane (13 mm diameter, Whatman). Membranes were washed with 1 ml ¼-strength Ringer's solution. For fixation, membranes were transferred into 2% glutaraldehyde (EM-Grade; Plano, Wetzlar, D) (in 0.1 M Sörensen phosphate buffer, 0.1 M KH_2PO_4, 0.1 M $Na_2HPO_4 \times 2H_2O$, pH 7.2) and stored at 4 °C until further use. Before drying, membranes were washed 3-times in 0.1 M phosphate buffer (pH 7.2). Membranes were transferred into graded series of 30%–100% ethanol and subsequently into graded series with 30%–100% acetone (ethanol and acetone: water-free, SeccoSolv-quality; Merk, Darmstadt, D). Critical point drying with liquid CO_2 was done in a CPD030 apparatus (BAL-TEC, Lichtenstein). Double sided adhesive carbon discs (Leit-Tabs, 12 mm diameter, Plano, Wetzlar, D) were used for mounting the samples on standard aluminium pin stubs (Plano, Wetzlar, D). The fixation-, dehydration- and critical point drying steps were omitted for freeze-dried samples, which were directly mounted on stubs. The specimens were coated with a 20-nm gold layer in a sputter coater (MED020, Bal-Tec, Lichtenstein). Samples were viewed in an XL30 scanning electron microscope (FEI Company, Eindhoven) in high vacuum mode with a secondary electron detector at an acceleration voltage of 10 kV.

2.6. Addition of freeze-dried bifidobacteria to commercial foods and study of the resistance to SGD after the refrigerated storage

Bifidobacteria cultures freeze-dried in 10% (w/v) lactose were added to food products with a special focus on non-dairy baby foods. 100 ml aliquots of foods (banana-carrot juice, apple-banana puree, peach-apple puree, fruits-cereal puree, vanilla-flavoured milk drink and orange juice, brands were not disclosed due to confidential reasons) were inoculated with bifidobacteria cultures grown in the biofermentor at pH 5.0 for 22 h and freeze-dried in 10% (w/v) lactose. No air was left in the head-space of the aliquoted samples. Cell counts were performed immediately after addition and after 4 weeks of storage at 5 °C. Resistance to SGD was assessed in the samples at the end of the refrigerated storage period. For this, 10 ml of the food product were added to 90 ml of a solution containing 0.5% (w/v) NaCl and 0.3% (w/v) porcine pepsin and adjusted to pH 2 with HCl solution. After mixture, pH was readjusted to 2.0 by the addition of 0.1 N HCl whenever needed.

2.7. Statistical analysis

Experiments were replicated at least twice in independent assays. Data were analyzed using the one-way ANOVA procedure of SPSS software (SPSS Inc., Chicago, IL, USA). The differences between means were detected by the Duncan's Multiple Range Test. Data were considered significantly different when $P < 0.05$.

3. Results

3.1. Freeze-drying in different cryoprotectants

Fresh cultures of *B. animalis* subsp *lactis* INL1 were freeze-dried in different cryoprotectants and submitted to an accelerated viability test (25 °C, 4 weeks) in order to detect differences in survival and/or in resistance to SGD in relation to the cryoprotectant used. Table 1 shows that regardless the cryoprotectant used, high levels (ca. 10 log orders) of viable bacteria were obtained after freeze-drying and after 4 weeks of storage at 25 °C. When resistance to SGD was assessed in overnight fresh cultures of *B. animalis* subsp *lactis* INL1,

Table 1
Survival of *B. animalis* subsp. *lactis* INL1 to freeze-drying (FD), to storage at 25 °C for 30 days and to simulated gastric digestion (pH 2, 90 min, 37 °C).

Cryoprotectant	Cell counts (Log CFU/mL ± standard deviation)				
	Before FD	After FD[a]	After storage[b]	Simulated gastric digestion[c]	
				$t = 0$ min	$t = 90$ min
PBS	10.40 ± 0.01^{A}	10.29 ± 0.05^{A}	10.11 ± 0.12^{A}	8.11 ± 0.12^{B}	$< 4^{C}$
10% Lactose	10.31 ± 0.04^{A}	10.23 ± 0.06^{A}	10.38 ± 0.15^{A}	8.38 ± 0.15^{B}	7.72 ± 0.17^{C}
15% Lactose	10.31 ± 0.03^{A}	10.15 ± 0.09^{A}	10.23 ± 0.18^{A}	8.23 ± 0.18^{B}	7.67 ± 0.15^{C}
10% Sucrose	10.41 ± 0.01^{A}	10.32 ± 0.13^{A}	10.38 ± 0.48^{A}	8.37 ± 0.47^{B}	5.30 ± 0.16^{C}
15% Sucrose	10.53 ± 0.08^{A}	10.28 ± 0.06^{A}	10.60 ± 0.30^{A}	8.85 ± 0.18^{B}	5.09 ± 0.29^{C}
12% Skim milk	10.28 ± 0.13^{A}	10.18 ± 0.06^{A}	10.03 ± 0.22^{A}	8.03 ± 0.22^{B}	7.66 ± 0.13^{C}

[A]Values in the same line, in the first three columns, with different superscript capital letters are significantly different ($P < 0.05$).
[B,C]Values in the same line, in the last two columns, with different superscript capital letters are significantly different ($P < 0.05$).
[a] Freeze-dried cells were reconstituted with the same amount of water lost during freeze-drying and the cell suspension was allowed to stand for 15 min at room temperature before viable cell enumeration.
[b] Freeze-dried cells were stored under vacuum at 25 °C for 30 days.
[c] Freeze-dried cells were resuspended in distilled water, diluted in Ringer's solution and brought to pH 2 with HCl in the presence of a final concentration of 0.3% (w/v) porcine pepsin. Cell suspensions at pH 2 were incubated at 37 °C for 90 min for simulated gastric digestion.

no significant differences were observed in cell counts before and after exposure to low pH (data not shown). In freeze-dried cells, the use of PBS solution as cryoprotectant resulted in a reduction of viable cell counts of more than 4 log orders when cells were exposed to low pH (Table 1). When sucrose was used as cryoprotectant, cell viability was reduced by ca. 3 log orders. Lactose and skim milk were equally effective in protecting bifidobacteria against the harsh conditions of the simulated digestion. These differences in the viability of freeze-dried cells under simulated SGD conditions are in line with the scanning electron micrographs of freeze-dried cells: Fig. 2a–b is illustrating that lyophilized INL1 cells are tightly aggregating in large cell clusters, when PBS was used as sole cryoprotectant. However, in the presence of 12% skim milk (Fig. 2c–d) or 10% lactose (Fig. 2e–f) as cryoprotectants, cells are smoothly embedded and separated in an amorphous matrix of these protecting substances. 10% (w/v) lactose was chosen as the cryoprotectant for further studies, in relation to skim milk, in order not to carry over dairy flavours when used in non-dairy food matrices.

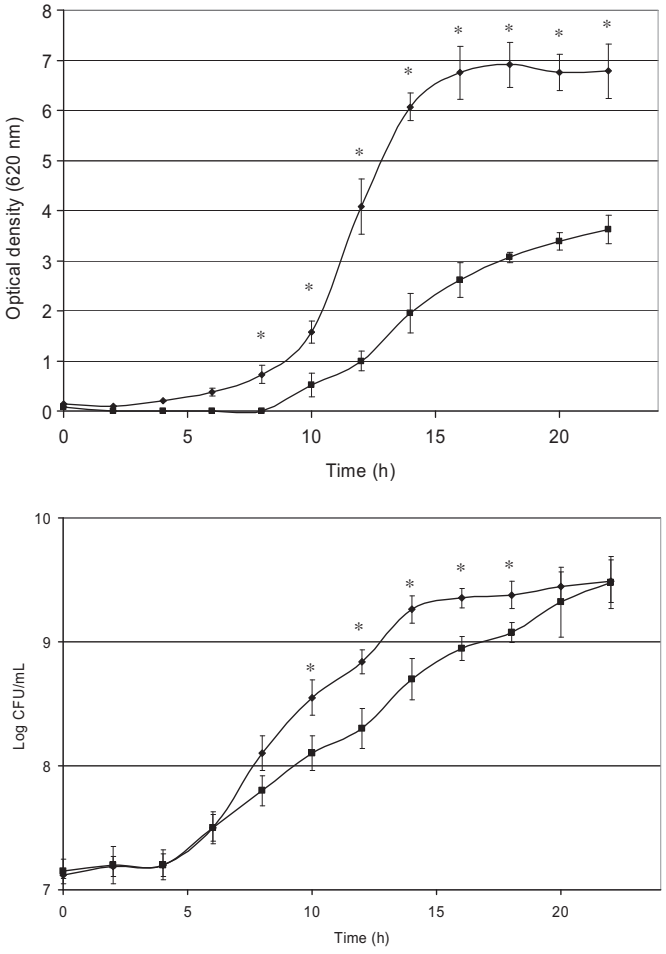

Fig. 1. Growth kinetics of *Bifidobacterium animalis* subsp. *lactis* INL1 at pH 6.5 (◆) and 5.0 (■) as determined by optical density at 620 nm (upper graph) and colony forming units (lower graph). * Values, significantly different ($P < 0.05$) between pH 6.5 and 5.0 for the same sampling time.

3.2. Growth kinetics in a biofermentor and resistance to SGD

Biomass of *B. animalis* subsp. *lactis* INL1 was produced in a biofermentor at controlled pH-values of 6.5 and 5.0. Growth kinetics are shown in Fig. 1. Although growth at pH 5.0 was slower compared to growth at pH 6.5, similar viable cell counts (ca. 10^9 CFU ml^{-1}) were obtained at both pH after 22 h of fermentation. However, the optical density of cells grown at pH 6.5 was significantly higher than at pH 5.0. Scanning electron microscopy showed significant differences in cell morphology of cells grown either under uncontrolled pH (rod-like cells with a low tendency of pleomorphism, Fig. 3a) or under controlled pH-values of 5 (Fig. 3b) or 6.5 (Fig. 3c). Under controlled pH conditions, cells revealed typically branched pleomorphic morphologies (Fig. 3b–c). Maximum cell branching occurred in the culture grown at controlled pH 5.0 where cells showed a tendency for clumping (and supposingly aggregation in liquid culture), which is correlating with the lower adsorption readings shown in Fig. 1. It should be noted that the cells are also coated be a higher quantity of extracellular material (possibly exopolysaccharides) when grown at uncontrolled pH conditions as stress stimulus.

When cells grown at pH 6.5 and 5.0 for 12 and 22 h, respectively, were freeze-dried in 10% (w/v) lactose and submitted to SGD, cells grown at pH 5.0, regardless the harvesting time, were more resistant to the adverse conditions mimicking the gastric transit (Table 2). No differences in cell counts were observed before and after the exposure of cells to low pH. This may be explained by the protective role of pre-exposure to sub-lethal stress. On the other hand, cells grown at pH 6.5 were sensitive to the gastric acidity, presenting a cell decay of approximately 2 log orders when exposed to simulated gastric conditions (Table 2), regardless of the fermentation time. When freeze-dried cells grown at controlled pH of 5.0 and 6.5 were analyzed by scanning electron microscopy, cell integrity was much better preserved at pH 5 (Fig. 2g–h) than at pH 6.5 (Fig. 2i–j).

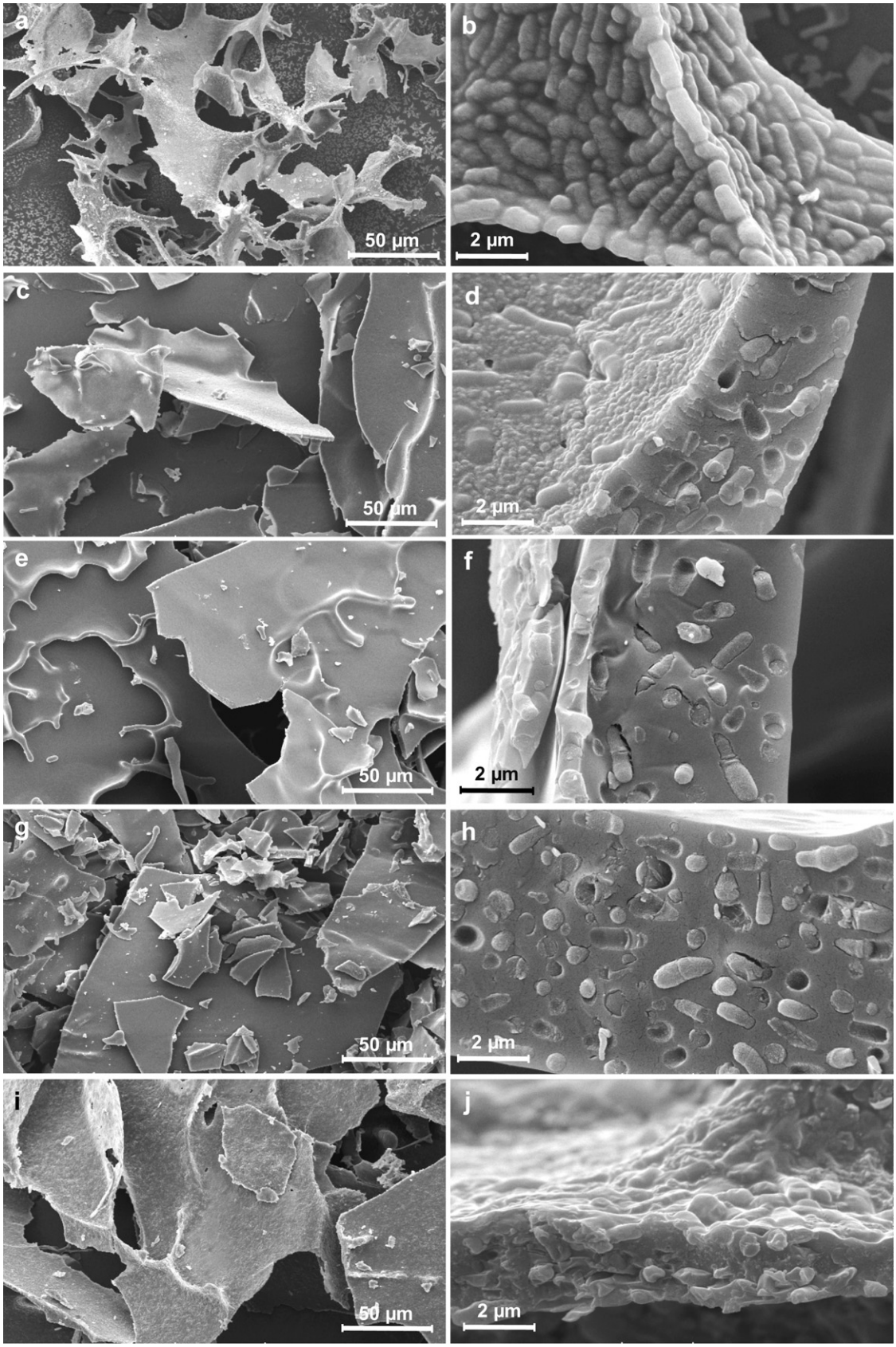

Fig. 2. Scanning electron micrographs of *B. animalis* subsp. *lactis* INL1 grown without pH control for 22 h and freeze-dried in PBS buffer (a–b), in 12% skim milk (c–d) and in 10% (w/v) lactose (e–f) as cryoprotectant. Cultures were also grown for 22 h at controlled pH of 5.0 (g–h) or a controlled pH of 6.5 (i–j) and freeze-dried in 10% (w/v) lactose as cryoprotectant. Specimens are shown at low (left column: 500× primary magnification) and at high magnification (right column: 10,000× primary magnification).

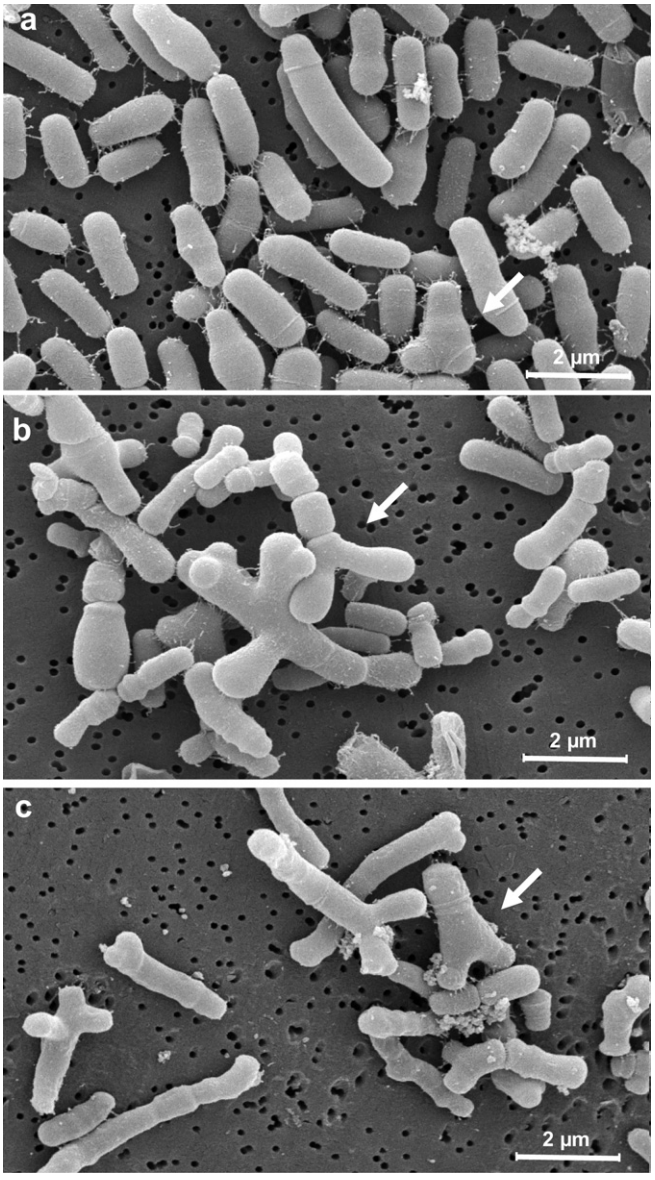

Fig. 3. Scanning electron micrographs of *B. animalis* subsp. *lactis* INL1 grown for 22 h at uncontrolled pH (a) or at a controlled pH of 5 (b) and 6.5 (c). The white arrows indicate a similar typical bifidobacteria morphology of cells.

3.3. Addition of freeze-dried bifidobacteria to commercial foods and study of the resistance to SGD after refrigerated storage

Cells grown at pH 5.0 for 22 h were freeze-dried in 10% (w/v) lactose and added to commercial foods products, mainly those of non-dairy nature devoted to babies and children (fruit juice, fruit puree). No differences in cell counts after 4 weeks of storage at 5 °C were observed for food products A, C, D, E and F. However, a significant ($P < 0.05$) reduction in cell viability ranging from 0.4 to 0.8 log cycles was observed in products B and C (Table 3). Interestingly, major differences in cell sensitivity with respect to the food matrix became evident when resistance to SGD was assessed. A negligible lost in cell viability (smaller than 0.3 log orders) was observed in products A and E. For products B, C and D, the cell decay was of ca. 1 log order after exposure to low pH. The highest reduction in cell counts (ca. 3 log orders) after exposure to simulated gastric juice was observed in product F. This last product, in particular, did not induce significant changes in cell counts during refrigerated storage. However, the bifidobacteria cells became very sensitive to the simulated adverse conditions of the stomach.

Table 2
Survival of *B. animalis* subsp. *lactis* INL1 to simulated gastric digestion (SGD) in cultures grown at pH 6.5 and 5.0 and freeze-dried in 10% (w/v) lactose.

Cells grown at pH-constant	Cell harvested at time (h)	Cell counts (Log CFU/mL ± standard deviation) during SGD	
		0 min	90 min
6.5	12	8.39 ± 0.14^{A}	6.61 ± 0.43^{B}
	22	8.42 ± 0.27^{A}	6.45 ± 0.21^{B}
5.0	12	8.46 ± 0.23^{A}	8.22 ± 0.17^{A}
	22	8.49 ± 0.07^{A}	8.29 ± 0.16^{A}

[A,B]Values in the same line with different superscript capital letters are significantly different ($P < 0.05$).

4. Discussion

In this study we investigated the resistance of *B. animalis* subsp. *lactis* INL1 to simulated gastric digestion (SGD) in order to identify conditions/products that confer the strain the ability to better survive the adverse conditions of the stomach. Cells of *B. animalis* subsp. *lactis* INL1 were freeze-dried in the presence of different cryoprotectants (PBS, lactose, sucrose or skim milk), followed by a storage period at 25 °C for 4 weeks, as an accelerated storage stability test previously proposed for bifidobacteria (Saarela et al., 2005). No changes in cell counts were observed for any of the cryoprotectants used when cell counts, after freeze-drying and after storage at 25 °C for 4 weeks, were compared. However, the application of a simple test for evaluation of resistance to SGD allowed to discriminate among cryoprotectants that induce more or less cell death during exposure to low pH. In this respect, 10% and 15% (w/v) lactose and 12% skim milk were equally effective for minimizing cell death during gastric digestion. These results are in accordance with those of Saarela et al. (2009), who reported different survival of *Lactobacillus rhamnosus* E800 exposed to SGD depending on whether cells had been freeze-dried in carriers such as sucrose, polydextrose or oat fibre. On the contrary, it was reported that different freeze-drying conditions (sucrose or reconstituted skim milk or fermentation time) had no significant effect on acid tolerance of *B. animalis* subsp. *lactis* E−012010 (Saarela et al., 2005), emphasizing the strain-dependency of this kind of results.

B. animalis subsp. *lactis* INL1 was grown in a biofermentor at pH 6.5 and 5.0 and acid tolerance was assessed in cells harvested at 12 and 22 h of fermentation and freeze-dried in 10% (w/v) lactose. The

Table 3
Survival of *B. animalis* subsp. *lactis* INL1 in different commercial foods during refrigerated storage and to simulated gastric digestion (SGD).

Food product[a]	At time 0		After 4 weeks of storage at 5 °C		
	pH	Cell count[b]	pH	Cell count[b]	Cell death in SGD[c]
A	4.16	8.40 ± 0.25^{A}	4.25	8.29 ± 0.11^{A}	-0.24 ± 0.12
B	3.94	8.24 ± 0.09^{A}	3.99	7.59 ± 0.15^{B}	-1.33 ± 0.24
C	3.81	8.28 ± 0.12^{A}	3.86	7.84 ± 0.26^{B}	-1.03 ± 0.59
D	3.97	8.40 ± 0.08^{A}	4.14	8.06 ± 0.07^{A}	-1.05 ± 0.16
E	6.23	8.34 ± 0.17^{A}	6.04	8.42 ± 0.10^{A}	-0.18 ± 0.12
F	3.77	8.38 ± 0.14^{A}	3.78	8.14 ± 0.06^{A}	-2.97 ± 0.26

[A,B]Values of cell counts in the same line with different superscript capital letters are significantly different ($P < 0.05$).
[a] The food products used were: banana-carrot juice (A), apple-banana puree (B), peach-apple puree (C), fruits-cereal puree (D), vanilla-flavoured milk drink (E) and orange juice (F).
[b] Log CFU/mL ± SD.
[c] Difference ± SD of the Log CFU/mL before and after simulated gastric digestion.

strain achieved similar numbers of viable cells at both pH, although reached those values at a slower rate when grown at pH 5.0. Major differences were observed in the absorbance of the cultures, noticing higher values when cells were grown at pH 6.5. This might be explained by the higher branching and pleomorphic characteristics of the cells at pH 5.0 that might cause a higher preference for cell aggregation and sedimentation in liquid culture. We have also documented the capacity of production of exopolysaccharides by strain INL1 during growth at uncontrolled pH conditions (Fig. 3a). Production of EPS by bifidobacteria has been reported recently (Alp et al., 2010; Salazar et al., 2011), and Ruas-Madiedo et al. (2009) reported that stress conditions caused by bile did also result in an increase of EPS production in *B. animalis*.

Freeze-dried cells, previously harvested at 12 and 22 h of fermentation, were submitted to SGD to assess their tolerance to adverse conditions. No cell death after exposure to low pH was observed for cells grown at pH 5.0. However, cells grown at pH 6.5 experienced ca. 2 log orders of decrease in cell counts, irrespective of the harvesting time (12 or 22 h). These results are in agreement with those of Saarela et al. (2009), who reported a higher gastric acidity tolerance of *L. rhamnosus* E800 when grown at pH 5.0 compared to pH 5.8. Other parameters of functionality are dependent on the pH at which probiotic cells are produced. Sashihara et al. (2007) demonstrated that cultures of *Lactobacillus gasseri* OLL2809 grown at lower pH were more effective for the induction of IL-12 on eukaryotic cells. In our study, no differences in tolerance to SGD were observed for bifidobacteria grown at pH 6.5 and harvested at 12 or 22 h. However, other studies showed differences in the functionality of probiotic cells when harvested at different time points along the growth curve. Sashihara et al. (2007) demonstrated that *L. gasseri* OLL2809 cells harvested in the stationary phase stimulated IL-12 by murine splenocytes to a greater extent than logarithmic phase cells. Haller et al. (1999) observed that lactic acid bacteria in the stationary phase induced a higher quantity of TNF-α by human peripheral blood mononuclear cells than cultures in the logarithmic growth phase. Maassen et al. (2003) observed *in vivo* differences in the immunostimulatory activity of *L. casei* depending on the growth phase. In this case, the ratio between IgG1 and IgG2a antibodies in blood serum of mice was higher when animals received *L. casei* cells in the stationary phase. Finally, a higher efficiency of hydrolysis of glycochenodeoxycholic acid by resting cells of *B. animalis* DN-173 010 was observed in cells collected at 7 h (exponential growth phase) compared to cells harvested at 14 h of culture (stationary phase) (Lepercq et al., 2004).

Finally, the influence of the food matrix on the resistance to SGD was assessed in freeze-dried cultures of *B. animalis* subsp. *lactis* INL1 incorporated in food products, with a special emphasis on non-dairy baby foods. There is scarce information about the storage stability of bifidobacteria in fruit juices (Saarela et al., 2006) and, to the best of our knowledge, there is also very limited information about the fate of bifidobacteria in baby food like fruit purees or juices. No changes in cell viability were observed for the majority of the products assessed after a storage period of 4 weeks at 5 °C. For all of the products, a decrease in cell counts lower than 1 log order was observed. However, major differences were observed in the tolerance to the adverse conditions of the SGD. Two products induced reductions in cell viability lower than 0.3 log orders, 3 products induced a reduction in cell counts of ca. 1 log order whereas in one product, where no changes in cell viability had been observed during storage, the exposure to low pH resulted a reduction in cell counts of ca. 3 log orders. These results are in good agreement with those of Saarela et al. (2006) who found significant differences in acid tolerance of freeze-dried *B. animalis* subsp. *lactis* E2010 cells harvested from milk, fruit juice or phosphate-buffered saline solution held at 4 °C during two weeks. Pure cultures of freeze-dried *B. animalis* subsp. *lactis* E2010 also experienced changes in the acid tolerance during storage at 5 °C (Mättö et al., 2006). Wang et al. (2009) reported no significant changes in the viable counts of *L. casei* Zhang in bovine or soymilk fermented milk. However, *L. casei* Zhang in the fermented bovine milk samples showed higher survival rate in simulated gastric juice at pH 2.0 than in the fermented soymilk samples along storage for 28 days.

5. Conclusions

Taken together, our results confirm the statement made by Saarela et al. (2006) that viability or stability of cultures alone is not an adequate enough predictor for the strain functionality in adverse conditions such as low pH. Certain parameters of cell functionality such as gastric acidity and bile tolerance, adherence to gastrointestinal epithelium and the final effect of the probiotic bacteria on the host might be affected by the food ingredients used in probiotic delivery (Tuomola et al., 2000; Sashihara et al., 2007; Ranadheera et al., 2010). This work demonstrated the usefulness of determining resistance of bacteria to SGD for evaluating differences in cell sensitivity to varying conditions during biomass production, freeze-drying and incorporation of probiotic bacteria into food products.

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