Influence of L-cysteine, oxygen and relative humidity upon survival throughout storage of probiotic bacteria in whey protein-based microcapsules

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A B S T R A C T

The survival rates of Lactobacillus acidophilus Ki, Lactobacillus paracasei L26 and Bifidobacterium animalis BB-12 were studied after whey protein microencapsulation via spray-drying, with or without L-cysteine-HCl, and storage up to 6 months at 5 °C and 22 °C, with variation in relative air humidity and oxygen levels. Lb. paracasei L26 was the least susceptible to storage conditions; above 10^6 cfu g^-1 were recorded by 180 d at 22 °C, irrespective of relative humidity, and the presence/absence of oxygen and L-cysteine. Higher relative humidity, higher temperature and longer storage periods were deleterious to survival of both B. animalis BB-12 and Lb. acidophilus Ki; the effect of L-cysteine-HCl was dependent on the probiotic strain. The effect of overhead oxygen was not significant upon any probiotic strain studied. Whey protein microcapsules containing L-cysteine-HCl protected probiotic cultures from simulated gastrointestinal conditions.

1. Introduction

Probiotic microorganisms can bring about beneficial effects to the health of the human host, provided they are consumed at appropriate levels and as part of a balanced diet. These microorganisms hold great promise for success in the food marketplace; however, a critical issue concerning use of foods as vectors for probiotic bacteria is the fastidious nature of the organisms coupled with the stressful environment of food processing. Since large viable numbers of probiotic strains are required at the moment of consumption (above 10^7 cfu g^-1) for a significant role to be eventually played in the gastrointestinal tract (Adhikari, Mustapha, Grun, & Fernando, 2000; Dolevies & Lacroix, 2005), stability throughout processing and storage are critical issues to be addressed for large-scale production. Therefore, food manufacturers aim to increase viable cell counts, not only during biomass production and food formulation, but also throughout downstream processing and storage prior to ingestion.

Probiotic bacteria, often belonging to the Lactobacillus and Bifidobacterium genera (Weinbreck, Bodnár, & Marco, 2010), are normally added to fresh and cultured dairy foods. These products are characterized by high water activity and shelf-lives of the order of a few days, requiring refrigeration, as is the typical case of yoghurt (Capela, Hay, & Shah, 2006; Gomes & Malcata, 1999; Kailasapathy, 2006; Krasaekoopt, Bhandari, & Deeth, 2003). When drier products containing probiotic strains are tested at room temperature, dramatic losses of their viability over time severely hamper shelf-life (Ross, Desmond, Fitzgerald, & Stanton, 2005; Ubink & Krueger, 2006). High water activity and storage temperature, as well as the presence of oxygen, apparently compromise extended storage (Anal & Singh, 2007; Teixeira, Castro, Malcata, & Kirby, 1995), thus constitute an obvious limitation for diversification of probiotic foods.

Microencapsulation of microorganisms, via tailor-made carriers composed of non-toxic materials, has frequently been used to impart protection against stressful environmental factors. This has allowed several types of food products to become effective carriers of sensitive microorganisms. In particular, delivery of active probiotic cells in a microencapsulated form has received increasing attention in recent years as microencapsulation provides
2. Materials and methods

2.1. Inoculum preparation

The probiotic cultures tested — B. animalis BB-12 (Nu-trish®, Chr.-Hansen, Hørsholm, Denmark) and Lb. paracasei LAFTI® L26 (DELVPRO®, DSM, Sydney, NSW, Australia) — were obtained as freeze-dried cultures, whereas Lb. acidophilus Kf, previously isolated from fermented milk, was obtained from CSK (Leeuwarden, The Netherlands) as a frozen concentrate. The aforementioned microorganisms were reactivated as pre-cultures in de Man—Rogosa—Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France), which were incubated overnight at 37 °C. Except for Lb. paracasei LAFTI® L26, MRS was supplemented with filter-sterilized 0.5 g L⁻¹ L-cysteine-HCl (Fluka, Buchs, Switzerland) to lower the redox potential, and incubated in a plastic anaerobic jar using AnaeroGen sachet (Oxoid, Cambridge, UK).

The cultures were propagated by inoculating fresh MRS media at 10% (v/v), and then incubating under appropriate conditions. The resulting cultures were centrifuged at 1789 × g for 20 min, at 4 °C. The centrifuged growth medium supernatant was then discarded, and the pellet was re-suspended in one tenth of its original volume of aqueous 0.85% (w/v) NaCl (Panreac, Barcelona, Spain) as an isotonic solution to re-suspend cells; salt solution or Ringer solution is commonly used by several authors (Picot & Lacroix, 2004) to prepare cultures.

2.2. Microorganism microencapsulation

Each probiotic suspension was added, at 10% (v/v), to an unsterilized 5% (w/v) whey protein concentrate containing 50% (w/w) protein, WPC50 (Formulab, Maia, Portugal), and delivered by a peristaltic pump to a rotary atomizer (GEA Niro, Sabborg, Denmark) coupled to a drying chamber (Arsopi, Vale da Cambra, Portugal), and spray-dried using 160 and 75 °C as inlet and outlet-air temperatures, respectively, to generate the intended microcapsules. In order to obtain microcapsules with L-cysteine-HCl, a similar procedure was used for each probiotic suspension with WPC50, but supplemented with 0.5 g L⁻¹ L-cysteine-HCl.

2.3. Storage survival

After microencapsulation, microcapsules (1–1.5 g) were stored (in duplicate) in perforated petri dishes (50 mm) at 22 °C up to 6 months, which in turn were maintained in glass sterilized sealed flasks (0.5 L) in the presence (or absence) of oxygen (StabilOx® Oxygen Absorbers, Multisorb, Buffalo, NY, USA), under various relative humidities (12, 32 and 45%) brought about by two-way gels (HumidiPack, Wayzata, MN, USA). Assessment of survival of free cells of each probiotic strain during storage under similar conditions had been carried out previously and revealed that viable cells of Lb. acidophilus Kf, B. animalis BB-12 and Lb. paracasei L26 could not survive longer than approximately 21, 60 and 90 d of storage, respectively (data not shown).

To serve as a comparison reference, WPC50 microcapsules, as well as free cells (suspended in an isotonic solution) of each probiotic strain were also stored at 5 °C during the same timeframe, only in the presence of oxygen and without relative humidity control (i.e., under conditions mimicking regular storage in a domestic refrigerator). In all situations, sampling was made at 0, 7, 14, 21, 30, 45, 60, 90, 120, 150 and 180 d.

2.4. Microbiological analyses

For each sample, 0.6 g of microcapsules was diluted in 6 mL of a sterile aqueous solution of 0.85% (w/v) NaCl at room temperature (22 °C), and homogenized in a Stomacher at 230 rpm for 7 min. Viable cell numbers were enumerated, following the procedure of Miles and Misra (1938), on MRS agar in the case of Lb. paracasei L26, and on MRS agar supplemented with 0.5 g L⁻¹ L-cysteine-HCl for the other two strains. Plates were incubated for 48 h at 37 °C, after several decimal dilutions with sterile 0.1% (m/v) peptone water, in boxes maintained under anaerobiosis with Anaerogen sachets.
(Oxoid) in the case of Lb. acidophilus Ki and B. animalis BB-12, and under regular aerobic conditions in the case of Lb. paracasei L26. Plate count agar, incubated aerobically at 37 °C for 48 h, was used in parallel to monitor putative cross-contamination arising from laboratory manipulation, but the viable counts found were always negligible (data not shown).

2.5. Microscopical observations

To ascertain the morphological features of microcapsules, samples taken at various storage times were placed on a double sided adhesive carbon tape (NEM tape, from Nisshin, Tokyo, Japan), and observed with a low-vacuum scanning electron microscope (JSM-5600LV, from JEOL, Tokyo, Japan) operated under 10 Pa and at an accelerating voltage of 15–20 kV.

2.6. Gastrointestinal simulation

Free cells of each probiotic strain, as well as WPC50 microcapsules containing either of the three strains, were exposed to simulated gastrointestinal conditions after 15, 60 and 120 d of storage at 5 °C, according to the procedure described by Madureira, Amorim, Pintado, Gomes, and Malcata (2011), with slight modifications. The typical conditions prevailing in the mouth, oesophagus—stomach, duodenum and ileum were sequentially applied as follows: the mouth micro-environment (including mastication) was paralleled following the method of Höld, de Boer, Zuidema, and Maes (2000) and Choi, Chung, Lee, Shin, and Sung (2007), using a synthetic saliva solution prepared with 100 U mL−1 α-amylase in 1 mM CaCl2, and pH was adjusted to 6 using 1 M NaHCO3; this simulated saliva was added at a rate of 0.6 mL min−1. For the oesophagus—stomach step, 25 mg mL−1 pepsin was prepared in 0.1 M HCl; this solution was added in equal-sized aliquots during the gastric phase, at a rate of 0.05 mL per mL or g of sample and pH was gradually decreased to pH 2 using 1 M HCl (Aura et al., 2005). Duodenum conditions were simulated with 2 g L−1 pancreatin and 12 g L−1 bile salts dissolved in 0.1 M NaHCO3; this solution was added at a rate of 0.25 mL per mL or g of sample (Laurent, Besançon, & Caporiccio, 2007). The increase of pH that takes place in the ileum was simulated by gradually adding 0.1 M NaHCO3.

All enzyme solutions were freshly prepared for each experiment and filter-sterilized through a 0.22 μm-membrane filter (Millipore, Billerica, MA, USA). A rotary water bath at 37 °C was used to simulate the temperature and peristaltic movements that prevail during human gastrointestinal transit.

2.7. Statistical analyses

For each probiotic bacterium, a two-way analysis of variance (ANOVA) was carried out with SigmaStat™ (Systat Software, Chicago, IL, USA), to assess whether each factor (viz. the presence or absence of l-cysteine-HCl in the microcapsule composition, as well as presence or absence of oxygen, and several relative humidities during storage) was a significant source of variation throughout storage time, at a significance level of P = 0.05. Two-way ANOVA was also applied to yield data presented in Table 1. ANOVA is valid provided that the experimental errors are independently and normally distributed, and possess a constant variance, hence, the original data had to be transformed via λ-power forms, so as to achieve normality (Box, Hunter, & Hunter, 1978; Freitas & Malcata, 1996; Freitas, Sousa, & Malcata, 1995). The Holm-Sidak method was finally used for pairwise comparisons, also at the P = 0.05 level of significance.

3. Results

3.1. Storage at 22 °C

The microencapsulation yield (defined as the mass of microcapsules formed normalized by the amount of protein prevailing right before microencapsulation) was dependent on the presence or absence of l-cysteine-HCl (Table 1). However, yield values did not differ substantially for microcapsules containing l-cysteine-HCl (P > 0.05). Values of 39–46% were recorded for the three probiotic strains under assessment, but such values dropped to 23–31% for B. animalis BB-12 or Lb. paracasei L26 microcapsules without l-cysteine-HCl. Despite the differences observed, probiotic strains, as well as the presence or absence of l-cysteine-HCl, did not appear as significant factors (P > 0.05) for microencapsulation yield. A higher density of viable cells was observed when microencapsulation included l-cysteine-HCl, especially in the case of Lb. acidophilus Ki; however, such an effect was negligible for Lb. paracasei L26, since 108 cfu g−1 was observed in both types of microcapsules. For Lb. acidophilus Ki and B. animalis BB-12, absence of l-cysteine-HCl led to substantial losses in viability in comparison to their initial viable numbers prior to microencapsulation (Table 1).

The evolution in time of viable cells of each probiotic strain, stored under various conditions, is displayed in Fig. 1. Lb. paracasei L26 appeared to be the strain least susceptible to the parameters under scrutiny; highest numbers of viable cells (i.e., above 109 cfu g−1) were recorded by 180 d of storage at 22 °C, irrespective of relative humidity, the presence or absence of oxygen or l-cysteine-HCl in the microcapsules. Besides storage time, only relative humidity appeared to be significant (P < 0.05) towards Lb. paracasei L26 viability.

As expected, higher relative humidity and longer storage periods also led to lower numbers of viable cells; such decreases were more significant in the case of B. animalis BB-12 and Lb. acidophilus Ki, however, the variation in viability as storage time elapsed was not uniform among probiotic strains. In the case of Lb. paracasei L26, a slower overall decrease over time was observed (with a maximum reduction of 3 log cycles at 45% relative humidity, RH), but such decrease was faster in the first 60 d of storage followed by a much smoother pattern up to 180 d; Lb. acidophilus Ki

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**Table 1**

Variation in the number of viable cells (average ± standard deviation) of Lactobacillus acidophilus Ki, Bifidobacterium animalis BB-12 and Lactobacillus paracasei L26 prior to, and after microencapsulation in WPC50 by spray-drying, and the corresponding microcapsule (MC) yield.

<table>
<thead>
<tr>
<th>Probiotic strain</th>
<th>WPC50 microcapsules</th>
<th>Viable cells prior to microencapsulation (cfu mL−1)</th>
<th>Viable cells after microencapsulation (cfu g−1)</th>
<th>MC yield (%)</th>
<th>w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lb. acidophilus Ki</td>
<td>With l-cysteine-HCl (0.5 g L−1)</td>
<td>(8.25 ± 0.35) × 10⁶</td>
<td>(5.28 ± 0.35) × 10⁶</td>
<td>41.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without l-cysteine-HCl</td>
<td>(6.50 ± 0.71) × 10⁶</td>
<td>(6.50 ± 1.41) × 10⁶</td>
<td>44.0</td>
<td></td>
</tr>
<tr>
<td>B. animalis BB-12*</td>
<td>With l-cysteine-HCl (0.5 g L−1)</td>
<td>(9.75 ± 0.35) × 10⁶</td>
<td>(1.48 ± 0.03) × 10⁶</td>
<td>39.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without l-cysteine-HCl</td>
<td>(1.83 ± 0.18) × 10⁶</td>
<td>(1.28 ± 0.03) × 10⁶</td>
<td>22.7</td>
<td></td>
</tr>
<tr>
<td>Lb. paracasei L26</td>
<td>With l-cysteine-HCl (0.5 g L−1)</td>
<td>(4.13 ± 0.38) × 10⁵</td>
<td>(5.88 ± 0.63) × 10⁵</td>
<td>45.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without l-cysteine-HCl</td>
<td>(6.38 ± 1.49) × 10⁵</td>
<td>(7.50 ± 2.80) × 10⁵</td>
<td>30.7</td>
<td></td>
</tr>
</tbody>
</table>

* Based on the theoretical maximum weight of microcapsules produced from 6 L of 5% (w/v) WPC50 (300 g).
and *B. animalis* BB-12 underwent decreases in their viable cells, with the former exhibiting a higher loss of viability rate, revealing it to be the most susceptible, with reductions of $4 \times 10^5$ log cycles by 180 d of storage at 45% RH. Simpson et al. (2005) reported similar magnitude of viable cell reduction for *B. animalis* BB-12 in skimmed milk powders obtained by spray-drying, but after 90 days of storage at 25°C.

The presence of oxygen throughout storage did not have significant effect ($P > 0.05$) for any of the probiotic strains studied; only a slight negative effect was observed for *Lb. acidophilus* Ki microencapsulated with l-cysteine-HCl by 120 d of storage at 22°C. Conversely, the presence or absence of l-cysteine-HCl proved to be a significant effect ($P < 0.05$) for survival of microencapsulated *Lb. acidophilus* Ki and *B. animalis* BB-12. Surprisingly, inclusion of l-cysteine-HCl in microcapsule formulation negatively affected ($P < 0.05$) the viability of *B. animalis* BB-12 throughout time, for all relative humidities tested. On the other hand, the presence of l-cysteine-HCl turned to be a protection factor upon *L. acidophilus* Ki, as made apparent by the lower decreases in its viable cells.

To assess the morphological features of the microcapsules, scanning electron microphotographs are displayed in Fig. 2. No effects of either relative humidity or storage time upon microcapsule structure and dimension can be detected. In fact, the microcapsules did not lose their initial morphology, and in particular maintained their original shape and no swelling was observed at higher humidities. It should be emphasized that the microcapsules were characterized by irregular geometrical shapes similar to spheres from which air had been removed, with variable dimensions (5–50 μm), owing to the nature of the spray-drying process. Similar shapes were reported by Lian, Hsiao, and Chou (2002) for spray-dried starch microcapsules. The presence or absence of l-cysteine-HCl or oxygen was not reflected upon visual differences in morphology (data not shown) of the microcapsules.

### 3.2. Storage at 5°C

Microcapsules containing each probiotic strain, with or without l-cysteine-HCl, were also stored for up to 6 months at 5°C, but only in the presence of oxygen and without relative humidity control in an attempt to assess survival under refrigeration conditions; the resulting data are depicted in Fig. 3. As expected, this low storage temperature proved beneficial to all microencapsulated strains, in particular, to *Lactobacillus* strains (Fig. 3a,c). The lowest decrease in microencapsulated viable cell numbers was observed for *Lb. paracasei* L26, with some degree of variability especially within the first 60 d, but the numbers of viable cells were always above $10^9$ cfu g$^{-1}$. As happened with storage of *Lb. paracasei* L26 at 22°C, l-cysteine did not play a role in the survival of viable cells in the microcapsules. The protective effect by whey protein microencapsulation, in itself, is particularly evident by 60 d of storage at 5°C; the number of viable cells in free form decreased continuously throughout storage time, with a sharper decrease in the last 30 d against a relatively stable protective effect for microencapsulated cells.
Survival rates in microencapsulated form of both *Lb. acidophilus* Ki and *B. animalis* BB-12 were also higher at 5 °C than at 22 °C as storage time elapsed; by 180 d, viable numbers above 10^6 cfu g⁻¹ were observed for *B. animalis* BB-12, in the presence or absence of l-cysteine. Once again, the effect of l-cysteine was significant (P < 0.05), and responsible for higher rates of survival of *Lb. acidophilus* Ki: by 180 d, 10^7 cfu g⁻¹ was recorded for l-cysteine-containing microcapsules, whereas absence of l-cysteine-HCl promoted a sharper decrease of viable cell numbers, especially after 60 d to ca. 10^5 cfu g⁻¹. The protective effect promoted by whey protein microencapsulation is remarkable in the case of *Lb. acidophilus* Ki, since those cells could not survive longer than 60 d when in free form; for *B. animalis* BB-12 the onset of this protective effect is less visible. Lower values were reported by Goderska and Czarnecki (2008) for *Lb. acidophilus* and *Bifidobacterium bifidum* after 120 d of storage at 4 °C in starch capsules obtained by spray-drying.

3.3. Gastrointestinal transit

Free cells and WPC₅₀ microcapsules, containing either of the three strains and previously stored at typical refrigeration conditions for 15, 60 and 120 d, were exposed to simulated gastrointestinal tract. (Due to constraints pertaining to the amounts of sample available, it was not possible to carry out assays of microcapsules without l-cysteine-HCl in the case of *Lb. acidophilus* Ki and *B. animalis* BB-12 stored at 5 °C as well on microcapsules stored at 22 °C). The resulting data are shown in Table 2. Higher rates of survival were exhibited by the three probiotic strains, when microencapsulated in WPC₅₀ containing l-cysteine-HCl, than by their free cell counterparts after exposure to the simulated gastrointestinal conditions. Specifically, *Lb. acidophilus* Ki could not survive in free cell form upon passage through simulated duodenum and ileum, but its resistance improved dramatically when microencapsulated; storage at 5 °C did not decrease *Lb.
**Table 2**

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Free cells</th>
<th>Microencapsulated cells</th>
<th>% B. animalis BB-12 (log cfu g⁻¹)</th>
<th>% L. acidophilus Ki (log cfu g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouth (pH 6, 37°C)</td>
<td>15</td>
<td>60</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Stomach (pH 6 to 2, 37°C)</td>
<td>10</td>
<td>60</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Duodenum (ΔpH 2 to 5, 37°C, 45 rpm)</td>
<td>10</td>
<td>60</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Ileum (ΔpH 5 to 6.5, 37°C, 45 rpm)</td>
<td>10</td>
<td>60</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Jejunum (ΔpH 13, 100 mL portions)</td>
<td>10</td>
<td>60</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>

Values are the average ± standard deviation. Different letters in the same row and for each probiotic strain indicate significant differences (P < 0.05) between free cells and microencapsulated cells. ND, not detected.

**Discussion**

Probiotic microorganisms can bring about health benefits to the host only upon consumption to adequate viable levels in order to influence the profile of the intestinal microflora in a sustained manner (Lourens-Hattingh & Viljoen, 2001); usually, the recommended number of viable cells by the time of consumption is at least 10⁷ cfu mL⁻¹ (or cfu g⁻¹; equivalent to 10⁹ cfu per 100 g or 100 mL portions), hence, the spray-drying microencapsulation process itself, and the subsequent storage at 22°C appeared feasible in attempts to find a dry vector to deliver probiotic bacteria at room temperature throughout extended storage periods. The actual performance in terms of viability appears to be strain-dependent, as well as greatly influenced by the presence of l-cysteine-HCl, and independent of the presence of oxygen, but affected by the relative humidity prevailing during storage. Commercial powders of freeze-dried probiotics have well above 10¹⁰ cfu g⁻¹, therefore the viable counts in this study appear low and its commercial interest is debatable. There are two reasons for the low numbers. First, no attempt was made to have the highest density possible in the cell suspension before spray-drying. A tenfold improvement could be done at that level. Secondly, viability losses during spray-drying led to only a partial recovery of the powder, resulting in a yield of viable biomass lower than 10%. Improvements to the spray-drying process may allow technology to be applied to all strains besides those that better survive the high temperatures used in the drying process.

The effect of overhead oxygen was not significant (P > 0.05) for any probiotic strain studied; these results seem to contradict the usual heuristic rule, that the presence of oxygen compromises cell survival during extended storage periods (Anal & Singh, 2007; Teixeira et al., 1995) probably due to detrimental oxidative changes of the cell membrane (Hsiao, Lian, & Chou, 2004). Although oxygen tolerance varies among *Bifidobacterium* species, it is still unclear how oxygen sensitivity plays a role in determining survival following spray-drying (Simpson et al., 2005; Talwalkar, Kailasapathy, Peiris, & Arumugasamy, 2001). It is known that proteins are good barriers to oxygen and moisture (Gennadios, 2002); this may account for the protection provided by microcapsules made from WPC50 that contain 50% protein. The lactose content of WPC50 can also limit the diffusion of substances through the whey protein wall and thus lead to high microencapsulation efficiency values.

As expected, higher relative humidities showed an unfavourable effect upon the three probiotic bacteria; similar results were recently reported by Heidebach, Forst, and Kulczik (2010) for *Lactobacillus acidophilus* Ki resistance to low pH and bile salt, irrespective of storage time, except when storage took place for 120 d, which led to a faster decline under ileum conditions.

In the case of *B. animalis* BB-12 and *L. paracasei* L26, microencapsulation did improve their resistance to the simulated gastrointestinal tract. Decreases of 2 log cycles and above were indeed recorded for free cells, whereas a maximum of only 1 log cycle occurred for the microencapsulated counterparts. The assay carried out with *L. paracasei* L26 microencapsulated with or without l-cysteine-HCl, and previously stored for 120 d, did not show any significant protective effect (P > 0.05) by l-cysteine-HCl during exposure to the gastrointestinal environment, consistent with the survival results obtained for storage at 5 or 22°C.

The effect of storage conditions on the survival of probiotic strains under gastrointestinal conditions caused slight reductions of viable cells in the microcapsules as storage time elapsed in some cases, but mostly were not significant. A more pronounced effect was observed for *Lb. acidophilus* Ki and *B. animalis* BB-12.
storage of Bifidobacterium BB-12 at 25 °C in freeze-dried casein-based microcapsules. In the present study, storage at 22 °C and under 12% RH promoted the highest survival rates throughout storage: i) 180 d for L. paracasei L26, with viable numbers above 10^7 cfu g^-1, irrespective of presence of l-cysteine-HCl; ii) 150 d for B. animalis BB-12, with viable numbers also above 10^7 cfu g^-1 without l-cysteine-HCl, or above 10^6 cfu g^-1 with l-cysteine-HCl; and iii) 120–150 d for L. acidophilus K1, with viable numbers above 10^6 cfu g^-1 regardless of presence or absence of l-cysteine-HCl. All these values demonstrate a significant protective effect by whey protein microencapsulation relative to non-encapsulated cells, since free cells of L. acidophilus K1, B. animalis BB-12 and L. paracasei L26 could survive for no longer than 21, 60 and 90 d of storage, respectively.

Storage at high relative humidities increases the water content of the microcapsules, which may be detrimental to bacterial survival due to acceleration of oxidation processes (Teixeira et al., 1995; 5–11% RH has indeed been indicated as optimal values for bacteria survival (Castro, Teixeira, & Kirby, 1995). However, it is interesting to note that cell inactivation in the microencapsulated form does not follow simple first order kinetics (as reported by Higl et al., 2007; for glassy sugar matrices as microencapsulation matrix), but rather a two-stage behaviour (see Fig. 1), especially at higher humidities. A higher inactivation rate seems to prevail in the first 50 d and a lower inactivation rate afterwards, for both Lb. paracasei L26 and B. animalis BB-12. The existence of a critical range of water content where the survival rate decreases drastically may partially account for this observation.

The effect of l-cysteine-HCl was dependent on the probiotic strain but consistent at both storage temperatures for a given strain. No effect was actually detected for Lb. paracasei L26 survival, probably because lactobacilli are often facultative anaerobes (Talwalkar & Kailasapathy, 2003); a protective effect was observed for L. acidophilus K1 survival, whereas a negative effect was recorded in the case of B. animalis BB-12, especially at 22 °C. The results pertaining to this bifidobacteria strain were somewhat unexpected since several authors have reported that milk supplemented with amino acids (e.g., cysteine) improved survival of bifidobacteria (Dave & Shah, 1997, 1998; Comes, Vieira, & Malcata, 1998; Ravula & Shah, 1998); according to Güler-Akin and Akin (2007), specific addition of cysteine to bio-yogurts led to higher counts of B. bifidum, Lb. acidophilus K1 and Lb. paracasei L26 throughout storage for 14 d. Maybe an unfavourable interaction of that amino acid with the strain in microencapsulated form, or with the microencapsulation process itself, played a role since the data available in the literature pertain exclusively to free cells.

Storage at 5 °C produced a lower decrease in viable cell numbers throughout the 180 d-period of all probiotic strains but with a decreasing pattern somewhat resembling that obtained at 22 °C. Higher rates of survival of the three microencapsulated probiotic strains in the presence of l-cysteine-HCl were recorded, as compared with the free cells, after passage through the simulated gastrointestinal tract; this clearly demonstrated its protective effect, chiefly against acids and bile salts. In general, the storage conditions did not significantly affect the stability throughout the simulated gastrointestinal tract, since the proportion of reduction from mouth to ileum was almost the same for the three storage times considered. Due to their buffering capacity, proteinaceous matrices have been claimed to convey a protective effect, especially under low pH-gastric conditions (Heidebach, Forst, & Kolozik, 2009); a few studies have further corroborated the potential of microencapsulation to improve probiotic survival during gastrointestinal transit (Anal & Singh, 2007; Champagne & Fustier, 2007).

5. Conclusions

Microencapsulation allowed dry microcapsules to be employed as vectors for probiotic strains that are intended for storage under regular room conditions. The exact benefits of WPSCβ-lactoglobulin microencapsulation depend on the specific strain and the environmental conditions provided. Lb. paracasei L26 was the least susceptible to storage conditions whereas higher relative humidity, temperature and longer storage periods were harmful to B. animalis BB-12 and L. acidophilus K1. The impact of l-cysteine-HCl was a strain-specific trait, yet overall oxygen did not significantly affect any probiotic strain studied. Besides permitting expansion of the range of probiotic foods to those characterized by lower water activity, the need for a cold storage network may essentially be waived with obvious benefits for economic feasibility of the overall process.

Acknowledgements

The authors gratefully acknowledge Formulab, Multisorb, DSM and Chr. Hansen for providing the whey protein concentrate, oxygen absorbers and probiotic strains, respectively. This work was funded by FEDER under the Operational Program for Competitiveness Factors – COMPETE and by National funds via FCT – Fundação para a Ciência e a Tecnologia within the framework of project PROBIOCAPS – references PTDC/AGR-ALI/71051/2006 and FCOMP-01-0124-FEDER-008792.

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