On the viability of five probiotic strains when immobilised on various polymers

DINA RODRIGUES,[1] TERESA ROCHA-SANTOS,[1,2] SÉRGIO SOUSA,[3] ANA M GOMES,[3] MARIA M PINTADO,[3] F XAVIER MALCATA,[3†] JOSÉ M SOUSA LOBO,[4] JOSÉ P SILVA,[4] PAULO COSTA,[4] MARIA H AMARAL[4] and ANA C FREITAS[1]*

[1]*ISEIT/Viseu, Instituto Piaget, Estrada do Alto do Gaio, Galifonge, P-3515-776, Lordosa, Viseu, Portugal,* [2]*CESAM and Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal,* [3]*CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal, and* [4]*Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha nº164, P-4050-123 Porto, Portugal*

The viability of probiotic bacteria in six food-grade polymers, at two concentrations, was evaluated in order to predict their feasibility as materials for bacterium immobilisation. Alginate and whey proteins were the most adequate polymers, except for Lactobacillus acidophilus *Ki and* Lactobacillus casei *01 at 2% (m/v) alginate. Xanthan gum appeared to be a potential vector for three strains. L-carrageenan was adequate for both* Bifidobacterium *strains, but not for* Lactobacillus *at 2% (m/v).* Bifidobacterium *strains were not negatively affected by cellulose acetate phthalate, while the opposite held for L.* acidophilus *strains. Chitosan was the poorest polymer for immobilisation of probiotic bacteria.*

Keywords Probiotic bacteria, Viability and survival, Encapsulation materials, Immobilised cultures.

INTRODUCTION

Probiotics are viable micro-organisms that can bring about health benefits to the host, as far as they promote, or at least support a beneficial balance of the autochthonous microbial population in the gastrointestinal tract (Holzapfel *et al.* 1998, 2001). In order to exert this health benefit, probiotic micro-organisms need to be already present at high viable cell numbers in the food product prior to ingestion and sufficient to withstand the natural decrease during gastrointestinal transit; hence, even higher numbers are currently required upstream so as to overcome the detrimental effects of the whole food processing and storage. It has indeed been well established that viable probiotic bacteria should be delivered into the colon at local concentrations not below approximately 10^6 cfu/g or mL, otherwise they will not have a chance to significantly affect the composition and properties of the local intestinal microflora (Puupponen-Pimiä *et al.* 2002; del Piano *et al.* 2006; Lin *et al.* 2008). The large initial biomass required thus adds considerably to the cost of the final food, so alternatives enabling higher concentrations of viable numbers will make probiotic foods more competitive.

Over the last years, research has accordingly focused on alternative food vectors and techniques for probiotic bacteria; microencapsulation is an illustrative example of a technological process aimed at concentrating and protecting probiotic bacteria, and that offers a great potential in delivery of viable cells. Microencapsulation in tailored carriers, based on nontoxic materials and able to impart mechanical protection, indeed allows several types of food products to serve as hosts of sensitive micro-organisms (Lin *et al.* 2008). However, selection of appropriate encapsulating materials is still a challenge (Anal and Singh 2007), because high viable numbers and high individual activities are simultaneously required, which do not in turn impart off-flavours to the final product (Puupponen-Pimiä *et al.* 2002).

Studies of biocompatible materials have encompassed such polymers as alginate, chitosan, xanthan, cellulose acetate phthalate (CAP), whey protein, gelatine and carrageenan, with the specific aim of encapsulating bacteria (Champagne *et al.* 1996; Wenrong and Griffiths 2000; Krasaekoopt *et al.* 2004, 2006; Picot and Lacroix 2004; Capela *et al.* 2007; Muthukumarasamy and Holley 2007; Homayouni *et al.* 2008; Kim *et al.* 2008). Despite

*Author for correspondence. E-mail: afreitas@viseu.ipiaget.org
†Present address: ISMAI – Instituto Superior da Maia, Avenida Carlos Oliveira Campos, P-4475-690, Avioso S. Pedro, Portugal; Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, P-2780-157 Oeiras, Portugal.

all these research studies, which in principle suggest that several polymers may be used to successfully offer protection via microencapsulation of probiotic bacteria, reliable screening of coating materials, in terms of their nature and concentration, is to be conducted with actual probiotic strains.

Therefore, the main objective of this work was to establish a protocol to study the viability of several probiotic strains, viz. *Lactobacillus casei* 01, *Lactobacillus acidophilus* La-5®, *L. acidophilus* Ki, *Bifidobacterium animalis* BB-12® and *Bifidobacterium lactis* Bo, regarding several immobilisation materials, viz. sodium alginate, xanthan, *L*-carrageenan, CAP, chitosan in sodium alginate and whey protein concentrate, at various concentrations. If the compatibility of each said polymer with each said strain is known in advance, a higher chance for successful encapsulation afterwards will result.

MATERIALS AND METHODS

Source of micro-organisms and preparation of inocula

The Nu-trish® probiotic cultures of *B. animalis* BB-12®, *L. acidophilus* La-5® and *L. casei* 01 were obtained as freeze-dried cultures from CHR-Hansen (Horsholm, Denmark), whereas *L. acidophilus* Ki and *B. lactis* Bo were obtained as frozen concentrates from CSK (Leeuwarden, The Netherlands).

All probiotic bacteria were individually inoculated (at 2%) into 50 mL of MRS broth (Himedia, Mumbai, India), supplemented with 0.5 g/L *L*-cysteine-HCl (Panreac, Barcelona, Spain), in flat-bottomed glass flasks entirely filled so as to exclude oxygen and thus assure anaerobic conditions, and incubated at 37°C for 48 h. Upon growth, the probiotic cells were washed twice with sterile saline solution (0.85%, m/v NaCl), centrifuged at 2,236 × g for 10 min, and suspended in 4 mL of the aforementioned saline solution before inoculation.

Viability of micro-organisms exposed to immobilisation materials

The pre-selection of polymers, and associated concentrations, for immobilisation was based on published work (Klein and Vorlop 1985; Wenrong and Griffiths 2000; Krasaekoopt *et al.* 2006; Mandal *et al.* 2006), as well as preliminary work developed within our group (D Rodrigues, AM Gomes, MM Pintado, JP Silva and AC Freitas, unpublished observation). The six polymers eventually selected, as well as the two concentrations to be tested and their preparation methods are described in Table 1.

Each flask, containing 21 mL of immobilisation material prepared according to Table 1, was inoculated with 4 mL of suspended probiotic cells dispersed in sterile saline water with a mean concentration of 1.4×10^7 cfu/mL. Each of a total of 60 assays, corresponding to a regular factorial combination of six polymers, two concentrations and five probiotic strains, was performed, in triplicate, at 37°C for 180 min. These incubation conditions were chosen as a compromise between the optimum temperature of the micro-organisms and as long as possible a period of contact with the pre-polymer during microencapsulation.

Samples of probiotic bacteria were collected right upon inoculation, and at 30, 90 and 180 min upon incubation. Enumeration was performed via the Miles and Misra (1938) method in MRS agar with 0.5 g/L *L*-cysteine-HCl, with incubation at 37°C for 48 h–under aerobic conditions for *L. casei*-01, and under anaerobic conditions (GENbox anaer; Biomérieux, Craponne, France) for the other probiotic strains. Microbiological counts performed on plate count agar, incubated aerobically at 37°C for 48 h, were used in parallel to monitor putative cross-contamination arising from manipulation in the laboratory.

The viable cell profiles, reported as the ratio of mean log cfu(t)/mean log cfu(t_0), where t and t_0 denote current time and initial time, respectively, throughout a 180-min period at 37°C, and pertaining to each combination of probiotic bacteria and polymer, at two different concentrations, are displayed in Figures 1 and 2, respectively.

Statistical analyses of experimental data

Data were presented as means of three replicates. For each probiotic bacterium, statistical comparison between groups was made using a three-way analysis of variance (ANOVA), to assess whether each processing factor, viz. immobilisation material and concentration and incubation time, was a significant source of variation. Statistical analysis was performed using SigmaStat™ software (Systat Software, USA), which resorted to the Holm-Sidak method for pair-wise comparisons, at the 0.05 level of significance.

RESULTS AND DISCUSSION

Lactobacillus acidophilus La5®

Regarding *L. acidophilus* La5®, both concentrations of sodium alginate and WPC_{50} studied did

Table 1 Detailed list of polymers, concentrations and preparation methods

Immobilisation material	*Concentration*	*Preparation*
Alginic acid sodium salt from brown algae (Fluka, Oslo, Norway)	2–4%	0.5 or 1.0 g of sodium alginate was dissolved in 21 mL of deionised water, homogenised at 40°C, left to stabilise and hydrate overnight at room temperature, and eventually sterilised (121°C for 15 min)
Xanthan gum from *Xanthomonas campestris* (Fluka, Lyon, France)	1–3%	0.25 or 0.75 g of xanthan was dissolved in 21 mL of deionised water, heated at 80°C for 1 h followed by 10 min at 90°C, left to stabilise overnight at room temperature and eventually sterilised (121°C for 15 min)
L-carrageenan (Fluka, Copenhagen, Denmark)	1–2%	0.25 or 0.5 g of *L*-carrageenan was dissolved in 21 mL of hot (40°C) deionised water, homogenised and heated at 70°C for 30 min, left to stabilise overnight at room temperature and eventually sterilised (121°C for 15 min)
Cellulose acetate phthalate[a] (Fluka, St. Louis, USA)	0.3–0.6%	0.375 or 0.75 g of CAP was dissolved in 21 mL of a sterile disodium phosphate solution (1.48 g Na_2HPO_4/100 mL H_2O) and heated at 60°C until complete solubilisation, left to stabilise and hydrate overnight at room temperature, and then pH was adjusted to 6.5 with sterile 1 M HCl
Chitosan[a], low molecular weight, (Aldrich, St. Louis, USA) in sodium alginate 2% (m/v)	0.3–0.6%	0.075 or 0.15 g of chitosan was dissolved in 21 mL of sterilised 1% (m/v) acetic acid followed by the addition of 0.5 g of sodium alginate, left to stabilise and hydrate overnight at room temperature, and then pH was adjusted to 5.7–6.0 with sterile 5 M NaOH
Whey protein concentrate[a] at 50% (WPC_{50})	5.0–10.0%	1.25 or 2.5 g of WPC_{50} was dissolved in 21 mL of sterilised deionised water and homogenised until complete solubilisation, and left to stabilise and hydrate overnight at room temperature

[a] Not resistant to sterilisation (absence of contamination was checked otherwise).

not cause any decrease in the numbers of viable cells throughout incubation time; hence, these appeared to be the most adequate polymers for their eventual immobilisation. Note that alginate is the most widely used encapsulating material, owing to its low cost, ease of handling and biocompatibility (Krasaekoopt *et al.* 2004). Furthermore, several authors have demonstrated that microencapsulation of probiotic bacteria, viz. *L. acidophilus*, *L. casei* and *B. bifidum*, with sodium alginate improved their survival, especially against such adverse conditions as low pH, high bile salt concentration and heat processing (Krasaekoopt *et al.* 2004; Mandal *et al.* 2006; Kim *et al.* 2008). The direct contact with WPC_{50} even increased the number of viable cells of *L. acidophilus* La5® throughout the incubation period; this may unfold a potential prebiotic effect, as whey protein has been found elsewhere to promote probiotic growth and preservation (Pimentel-González *et al.* 2009), probably because of the functional properties of whey proteins in hydration, gelling and surface-action. Whey proteins are indeed among the bioactive substances with a best potential for use as coating agents, as they are entirely biodegradable and already used with success in the formulation of many types of food (Gbassi *et al.* 2009).

On the other hand, survival was hampered when cells of *L. acidophilus* La5® were inoculated in xanthan gum, *L*-carrageenan, CAP and chitosan in alginate. These results of biocompatibility are consistent with those by Champagne *et al.* (1996), according to whom xanthan gum did not demonstrate a positive effect on survival and stability of freeze-dried lactic acid bacteria assessed for up to 12 months of storage. Carrageenan has been used to encapsulate bacteria (Adhikari *et al.* 2000), whereas CAP is considered to be physiologically inert when administered *in vivo,* thus being widely used as an enteric coating material for release of core substances in intestine-targeted delivery systems (Anal and Singh 2007). However, a comprehensive study encompassing different concentrations of polymers in contact specifically with probiotic strains, under conditions that simulate

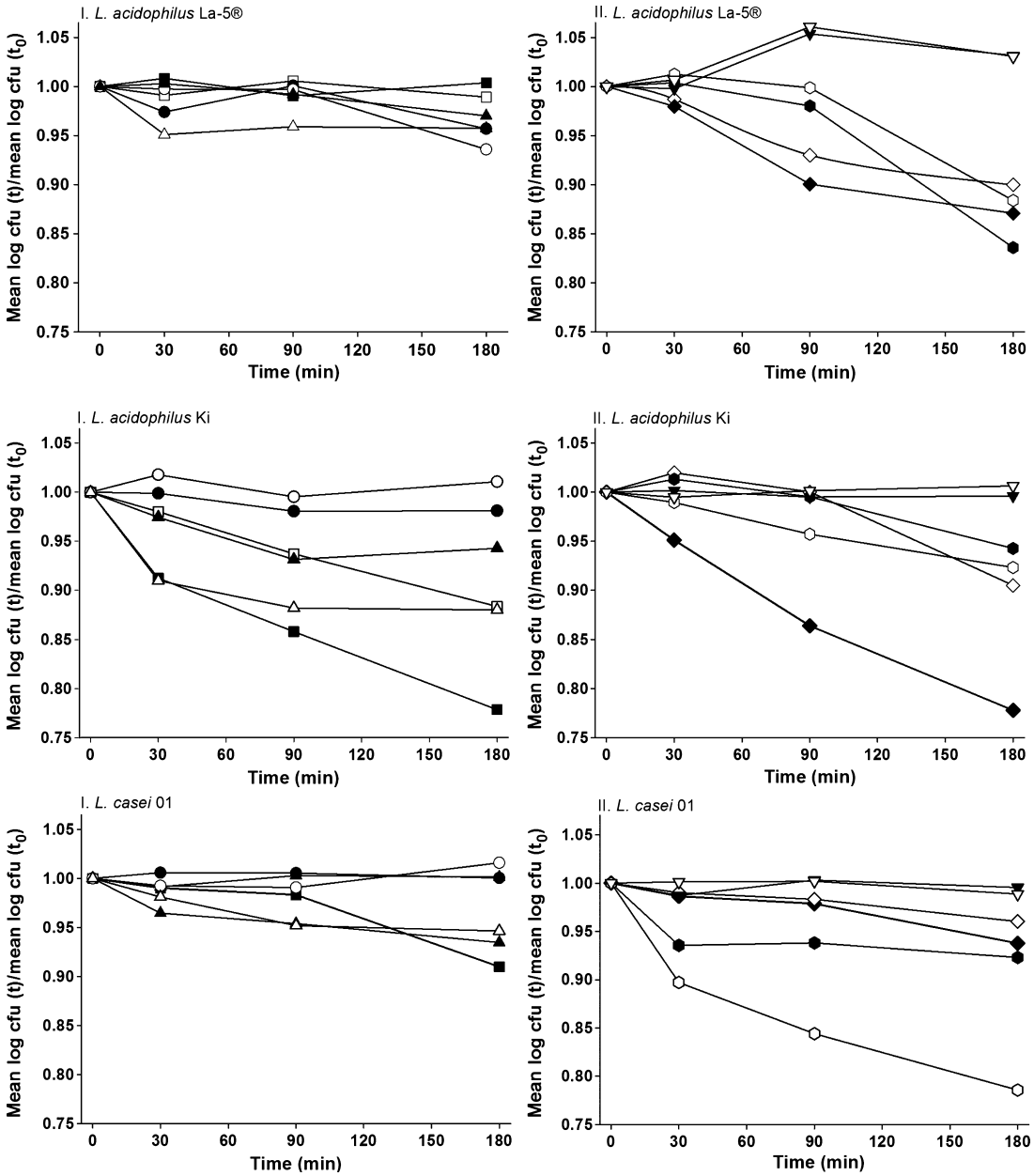

Figure 1 Viable cell profiles of *Lactobacillus acidophilus* La-5®, *L. acidophilus* Ki and *L. casei*-01 throughout time of incubation (*t*) in: (I) 2.0% (■) or 4.0% (□) sodium alginate, 1.0% (●) or 3.0% (○) xanthan gum and in 1.0% (▲) or 2.0% (△) of *L*-carrageenan at 37°C; and (II) 1.5% (◆) or 3.0% (◇)cellulose acetate phthalate, 0.3% (⬢) or 0.6% (⬡) chitosan in 2% of sodium alginate, and in 5.0% (▼) or 10.0% (▽) whey protein concentrate at 50% at 37°C.

immobilisation, had not been made available until now. As regards the chitosan and the alginate combination, a clear negative effect throughout time was perceived upon *L. acidophilus* La5®, at both its concentrations, as shown in Figure 1. In fact, chitosan has been considered for applications in food preservation because of its antimicrobial features (Jeon *et al.* 2001, 2002), especially at concentrations above 0.5%, so the negative interactions between chitosan in 2% of sodium alginate and *L. acidophilus* La5® were somehow expected.

From the ANOVA analysis, all main effects, viz. type of polymer and concentration, and incubation time, were statistically significant, as well as the interactions between polymer type and its concentration, and between polymer type and incubation time, concerning viability of *L. acidophilus* La5® ($P < 0.001$). However, xanthan and *L*-carrageenan

($P = 0.740$), xanthan and CAP ($P = 0.740$), and *L*-carragenan and CAP ($P = 0.161$) were statistically equivalent to each other, according to the Holm-Sidak criterion.

Lactobacillus acidophilus Ki

Survival of *L. acidophilus* Ki upon contact with the various immobilisation materials under scrutiny did not parallel that of *L. acidophilus* La5®: sodium alginate caused a significant decrease in viable cells throughout time, at both concentrations assayed, as shown in Figure 1, unlike that observed with *L. acidophilus* La5®; and even larger decreases were observed with *L*-carrageenan and CAP. No negative interactions were observed with xanthan gum or WPC_{50}, at both concentrations, whereas the negative effect of chitosan was less pronounced than was the case with *L. acidophilus* La5®. For *L. acidophilus* Ki, the concentration of polymer was not a statistically significant factor ($P = 0.062$), but the type of polymer and incubation time were ($P < 0.001$); all multiple pair-wise comparisons indeed appeared as statistically significant.

The differences observed between the two strains of *L. acidophilus* are somewhat surprising, and emphasise the importance of performing biocompatibility studies in advance when one intends to immobilise probiotic strains even if data exist for another strain of the same species. To the best of our knowledge, such a study had not been previously undertaken and such a strain dependence has not apparently been reported so far.

Lactobacillus casei 01

The initial viable cell numbers recorded for *L. casei* 01 remained approximately constant under both concentrations of sodium alginate, xanthan gum, CAP and WPC_{50} tested, except for sodium alginate at 4% (m/v) by 180 min at 37°C. These results indicate that *L. casei* 01 is the most resistant species of *Lactobacillus* studied. Nevertheless, and similar to that observed with the other *Lactobacillus* strains, a clear negative effect was perceived for *L*-carrageenan and chitosan in 2% (m/v) sodium alginate, especially at the higher concentration of each such polymer; e.g. the viable cell numbers of *L. casei* 01 decreased by 2 log cycles after 180 min of contact with 0.6% (m/v) of chitosan in 2% (m/v) sodium alginate, as apparent in Figure 1. According to Mortazavian *et al.* (2007), the efficiency of chitosan towards maintaining the viability of probiotic cells is not adequate; hence, it is often used only as an outer coating.

ANOVA confirmed those experimental observations, as all main effects were statistically significant regarding viable cells of *L. casei* 01, as well as all interactions except the one of polymer concentration with time ($P = 0.115$). All comparisons based on the Holm-Sidak method also proved statistically significant, except between CAP and WPC_{50} ($P = 0.741$), and between 30 and 90 min ($P = 0.703$).

Bifidobacterium animalis BB-12®

In what pertains to the behaviour of strains from the *Bifidobacterium* genus, a strain-dependent trait was once again found. In the case of *B. animalis* BB-12®, good survival rates were observed in sodium alginate and WPC_{50} at both concentrations, in *L*-carrageenan at 1.0% (m/v) and in CAP at 3.0% (m/v). For both concentrations of WPC_{50}, a significant ($P < 0.05$) increase in viable cells occurred especially in the first 30 min at 37°C, as seen in Figure 2. Likewise, a slight increase during the first 30 min of contact was promoted by sodium alginate. Sultana *et al.* (2000) reported that the encapsulation of *Bifidobacterium* spp. with alginate-starch produced a low decline of their viable counts in yoghurt throughout 8 weeks of storage, whereas Picot and Lacroix (2004) found that immobilisation of *Bifidobacterium* spp. in water-insoluble whey protein-based microcapsules increased their tolerance to high acid environments. Furthermore, studies pertaining to immobilisation of *B. infantis* in κ-carrageenan, reported by Ouellette *et al.* (1994) and Doleyres *et al.* (2002, 2004), indicated no problems of biocompatibility.

Xanthan gum clearly interfered with the stability of *B. animalis* BB-12® cells: by 90 min of contact at 37°C, a decrease by 1 log cycle in viable cell numbers took place at both concentrations of xanthan gum assessed. Chitosan was also responsible for a decrease in viable cell numbers throughout time, especially at the higher concentration tested. However, the lower concentration of this polymer, i.e. 0.3% (m/v), which caused significant decreases in *L. acidophilus* La-5® viable cell numbers, promoted only a slight decrease in their *B. animalis* BB-12® counterparts.

According to ANOVA, all main factors under study, viz. type of polymer and incubation time ($P < 0.001$), as well as polymer concentration ($P = 0.005$), appeared as statistically significant factors towards viable cells of *B. animalis* BB-12®; comparisons between *L*-carrageenan and chitosan ($P = 0.484$), between 0 and 30 min ($P = 0.099$)

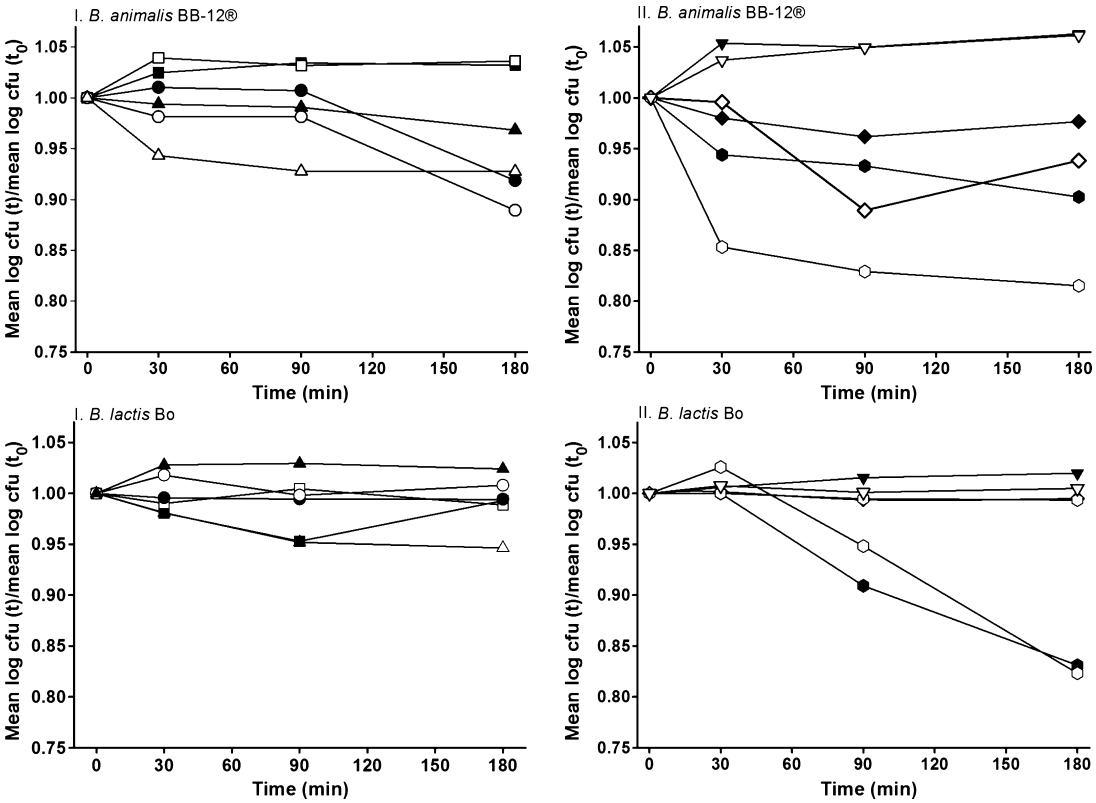

Figure 2 Viable cell profiles of *B. animalis* BB-12® and *Bifidobacterium lactis* Bo throughout time of incubation (t) in: (I) 2.0% (■) or 4.0% (□) sodium alginate, 1.0% (●) or 3.0% (○) xanthan gum and in 1.0% (▲) or 2.0% (△) *L*-carrageenan at 37°C; and (II) 1.5% (◆) or 3.0% (◇) of cellulose acetate phthalate, 0.3% (⬢) or 0.6% (⬡) chitosan in 2% of sodium alginate, and in 5.0% (▼) or 10.0% (▽) whey protein concentrate at 50% at 37°C.

and between 90 and 180 min ($P = 0.065$), yielded statistically nonsignificant results.

Bifidobacterium lactis Bo

Survival of *B. lactis* Bo, when exposed to every polymer, was essentially identical, except when in the presence of chitosan with sodium alginate; this is shown in Figure 2, where a sharp decline was observed by 30 min of contact, leading to a decrease in 1 log cycle at 0.6% (m/v); for xanthan gum, this *Bifidobacterium* strain exhibited a higher tolerance, irrespective of its concentration. According to Wenrong and Griffiths (2000), bifidobacteria immobilised in gellan-xanthan beads survived significantly better than free cells in pasteurised yoghurt, and their tolerance to high acid environments was likewise increased. As with other probiotic strains, all main effects were found to be statistically significant for this *Bifidobacterium* strain ($P < 0.001$). The effect of concentration was apparently more intense for *L*-carrageenan and for chitosan than for the other polymers. On the other hand, comparisons between alginate and xanthan ($P = 0.311$) and between 0 and 30 min ($P = 0.231$) yielded nonsignificant results.

Immobilisation polymers

Based on the experimental data produced, the conclusion may be drawn that alginate, and especially WPC_{50}, are the most adequate vectors for immobilisation on probiotic strains, as they show the highest levels of noninhibition and biocompatibility at both concentrations studied. However, exceptions were noted for sodium alginate at 2% (m/v) and *L. acidophilus* Ki, as well as for *L. casei* 01 upon a contact period of 180 min. For the remaining polymers, no consistent trend could be seen.

Xanthan gum, at both concentrations, appeared as a potential vector for three of the five probiotic strains under study; it presented some detrimental effect only upon *L. acidophilus* La5® and *B. animalis* BB-12®. *L*-carrageenan was, in turn, shown to be a potential vector for both *Bifidobacterium* strains, especially at 1% (m/v) for *B. lactis* Bo; at 2% (m/v), a somewhat negative effect was

observed, mainly with the *Lactobacillus* strains. Likewise, CAP did not cause a pronounced negative effect upon *Bifidobacterium* strains, yet the opposite was observed with both strains of *L. acidophilus*.

Finally, chitosan in 2% (m/v) alginate was in general the polymer with the poorest performance for eventual immobilisation of probiotic bacteria, especially at 0.6% (m/v); its antimicrobial characteristics make it inadequate for direct contact with probiotic bacteria, even though Lee *et al.* (2004) claimed good results when this polymer was used to spray coat alginate microcapsules, aimed at effectively delivering viable bacterial cells to the colon.

CONCLUSIONS

This research effort provided a first contribution to the systematic immobilisation of probiotic bacteria; indeed, it described a simple, practical and reliable screening procedure, aimed at demonstrating that biocompatibility between encapsulation polymers and bacteria is strain-dependent. A similar screening method is recommended whenever optimum microbial viability and stability are intended during an immobilisation process of probiotic bacteria. However, further studies are warranted to fully rationalise the nature of the polymer–bacterium interactions.

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