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

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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).
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* La5®, *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* La5® 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 $1.4 \times 10^7$ 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(t0), where t and t0 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 SigmaStatTM 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
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 WPC50 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

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Table 1 Detailed list of polymers, concentrations and preparation methods

| Immobilisation material                  | Concentration | Preparation                                                                 |
|-----------------------------------------|---------------|----------------------------------------------------------------------------|
| Alginate sodium salt from brown algae   | 2–4%          | 0.5 or 1.0 g of sodium alginate was dissolved in 21 mL of deionised water,  |
| (Fluka, Oslo, Norway)                    |               | 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  | 1–3%          | 0.25 or 0.75 g of xanthan was dissolved in 21 mL of deionised water, heated  |
| (Fluka, Lyon, France)                    |               | 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® (Fluka, St. | 0.3–0.6%      | 0.375 or 0.75 g of CAP was dissolved in 21 mL of a sterile disodium         |
| Louis, USA)                             |               | phosphate solution (1.48 g Na2HPO₄/100 mL H₂O) 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 N HCl          |
| Chitosan®, low molecular weight, (Aldrich, St. | 0.3–0.6%      | 0.075 or 0.15 g of chitosan was dissolved in 21 mL of sterilised 1% (m/v)     |
| Louis, USA) in sodium alginate 2% (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 N NaOH                                   |
| Whey protein concentrate® at 50% (WPC50) | 5.0–10.0%     | 1.25 or 2.5 g of WPC50 was dissolved in 21 mL of sterilised                  |
|                                         |               | deionised water and homogenised until complete solubilisation, and left to  |
|                                         |               | stabilise and hydrate overnight at room temperature                         |

*Not resistant to sterilisation (absence of contamination was checked otherwise).
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...
Holm-Sidak criterion. cally equivalent to each other, according to the ble cell numbers of

La5\textsuperscript{®} than was the case with the negative effect of chitosan was less pronounced under both concentrations of sodium alginate, 01 remained approximately constant.

The initial viable cell numbers recorded for La5\textsuperscript{®} La5\textsuperscript{®} were observed with L. acidophilus, CAP and WPC based microcapsules increased their tolerance to high acid environments. Furthermore, studies pertaining to immobilisation of B. infantis in \( \kappa \)-carrageenan, reported by Ouellette et al. (1994) and Doleyrès et al. (2002, 2004), indicated no problems of biocompatibility.

Xanthan gum clearly interfered with the stability of B. animalis BB-12\textsuperscript{®} 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 La5\textsuperscript{®} viable cell numbers, promoted only a slight decrease in their B. animalis BB-12\textsuperscript{®} 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\textsuperscript{®}; comparisons between L-carrageenan and chitosan (\( P = 0.484 \)), between 0 and 30 min (\( P = 0.099 \)) 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\textsubscript{50} (\( P = 0.741 \)), and between 30 and 90 min (\( P = 0.703 \)).

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\textsuperscript{®}; 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\textsuperscript{®}; and even larger decreases were observed with L-carrageenan and CAP. No negative interactions were observed with xanthan gum or WPC\textsubscript{50}, at both concentrations, whereas the negative effect of chitosan was less pronounced than was the case with L. acidophilus La5\textsuperscript{®}. 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\textsubscript{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 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.

Bifidobacterium animalis BB-12\textsuperscript{®}
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\textsuperscript{®}, good survival rates were observed in sodium alginate and WPC\textsubscript{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\textsubscript{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.
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 WPC50, 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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**REFERENCES**

Adhikari K, Mustapha A, Grun I U and Fernando L (2000) Viability of microencapsulated bifidobacteria in set yoghurt during refrigerated storage. *Journal of Dairy Science* **83** 1946–1951.

Anal A K and Singh H (2007) Recent advantages in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science and Technology* **18** 240–251.

Capela P, Hay T K C and Shah N P (2007) Effect of homogenisation on bead size and survival of encapsulated probiotic bacteria. *Food Research International* **40** 1261–1269.

Champagne C P, Mondou F, Raymond Y and Roy D (1996) Effect of polymers and storage temperature on the stability of freeze-dried lactic acid bacteria. *Food Research International* **29** 555–562.

Doyley R, Fliss I and Lacroix C (2002) Quantitative determination of the spatial distribution of pure- and mixed-strain immobilized cells in gel beads by immunofluorescence. *Applied Microbiology and Biotechnology* **59** 297–302.

Doyley R, Fliss I and Lacroix C (2004) Continuous production of mixed lactic starters containing probiotics using immobilized cell technology. *Biotechnology Progress* **20** 145–150.

Gbassi G K, Vandamme T, Ennahar S and Marchioni E (2009) Microencapsulation of *Lactobacillus plantarum* spp. in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology* **129** 103–105.

Holzapfel W H, Haberer P, Snel J, Schillinger U and Huis in’t Veld J H J (1998) Overview of gut flora and probiotics. *International Journal of Food Microbiology* **41** 85–101.

Holzapfel W H, Haberer P, Geison R, Björkroth J and Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *American Journal of Clinical Nutrition* **73** 365S–373S.

Homayouni A, Azizi A, Ehsani M R, Yarmand M S and Ravassi S H (2008) Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of symbiotic ice cream. *Food Chemistry* **111** 50–55.

Jeon Y J, Park P J and Kim S K (2001) Antimicrobial effect of chitoooligosaccharides produced by bioreactor. *Carbohydrate Polymers* **44** 71–76.

Jeon Y I, Kamil J Y V A and Shahidi F (2002) Chitosan as an edible invisible film for quality preservation of herring and Atlantic cod. *Journal of Agricultural and Food Chemistry* **50** 5167–5178.

Kim S, Cho S Y, Kim S H, Song O, Shin I, Cha D S and Park H J (2008) Effect of microencapsulation on viability and other characteristics in *Lactobacillus acidophilus* ATCC 43221. *LWT – Food Science and Technology* **41** 493–500.

Klein J and Vorlop D K (1985) Immobilisation techniques: cells. In *Comprehensive Biotechnology*, pp 542–550. Cooney C L and Humphrey A E, eds. Oxford: Pergamon Press.

Krasaekoopt W, Bhandari B and Deeth H (2004) The influence of coating materials on some properties of alginite beads and survivability of microencapsulated probiotic bacteria. *International Dairy Journal* **14** 737–743.

Krasaekoopt W, Bhandari B and Deeth H (2006) Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage. *LWT – Food Science and Technology* **30** 177–183.

Lee J S, Cha D S and Park H J (2004) Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles. *Journal of Agricultural and Food Chemistry* **52** 7300–7305.

Lin J, Yu W, Liu X, Xie H, Wang W and Ma X (2008) *In vitro* and *in vivo* characterization of alginate-chitosan-alginite
artificial microcapsules for therapeutic oral delivery of live bacterial cells. *Journal of Bioscience and Bioengineering* **105** 660–665.

Mandal S, Puniya A K and Singh K (2006) Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. *International Dairy Journal* **16** 1190–1195.

Miles O and Misra S S (1938) The estimation of the bactericidal power of the blood. *Journal of Hygiene* **45** 41–45.

Mortazavian A, Ravazi S H, Ehsani M R and Sohrabvandi S (2007) Principles and methods of microencapsulation of probiotic microorganisms. *Iranian Journal of Biotechnology* **5** 1–18.

Muthukumarasamy P and Holley R A (2007) Survival of *Escherichia coli* O157:H7 in dry fermented sausages containing micro-encapsulated probiotic lactic acid bacteria. *Food Microbiology* **24** 82–88.

Ouellette V, Chevalier P and Lacroix C (1994) Continuous fermentation of a supplemented milk with immobilized *Bifidobacterium infantis*. *Biotechnology Techniques* **8** 45–50.

del Piano M, Morelli L, Strozzi G P et al. (2006) Probiotics: from research to consumer. *Digestive and Liver Disease* **38** S248–S255.

Picot A and Lacroix C (2004) Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yogurt. *International Dairy Journal* **14** 505–515.

Pimentel-González D J, Campos-Montiel R G, Lobato-Calle-ros C, Pedroza-Islas R and Vernon-Carter E J (2009) Encapsulation of *Lactobacillus rhamnosus* in double emulsions formulated with sweet whey as emulsifier and survival in simulated gastrointestinal conditions. *Food Research International* **42** 292–297.

Puupponen-Pimiä R, Aura A M, Oksman-Caldentey K M, Myllärinen P, Saarela M, Mattila-Sandholm T and Poutanen K (2002) Development of functional ingredients for gut health. *Trends in Food Science and Technology* **13** 3–11.

Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P and Kailasapathy K (2000) Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and yogurt. *International Journal of Food Microbiology* **62** 47–55.

Wenrong S and Griffiths M W (2000) Survival of bifidobacteria in yogurt and simulated gastric juice following immobilisation in gellan-xanthan beads. *International Journal of Food Microbiology* **61** 17–25.