Evaluation of protein matrices as structural safeguards for controlled delivery of probiotic bacteria

S. Doherty[1], P. R. Ross[1,2], C. Stanton[1,2], G. F. Fitzgerald[2,3] and A. Brodkorb[1*]
[1] Moorepark Food Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork
[2] Alimentary Pharmabiotic Centre, Cork [3] Department of Microbiology, University College Cork, Cork, Ireland
*Corresponding author: andre.brodkorb@teagasc.ie

INTRODUCTION

There is no doubt that functional foods generate one of the most dynamically developing segments in the food industry. Modern consumers believe that food and health are symbiotically related which ultimately catalysed the inflow of functional foods on a global basis. The exponential growth of gut-health products, especially probiotic dairy products, has escalated the ongoing controversy as to whether cultures must be viable for efficacy in all cases (Charalampopoulos *et al*., 2003; Stanton *et al*., 2005).

As more scientific evidence accrues, the dairy industry have been quick to recognise the market potential resonating from the health benefits associated with probiotic bacteria. Lactic acid bacteria and bifidobacteria are the most widely studied bacteria in the probiotic field and are permanent residence of the intestinal microbiota (Siro *et al*., 2008). However, from a processing point of view, integration of probiotic bacteria into dairy-based food systems represents a difficult challenge to a food manufacturer (Ross *et al*. 2005). Thus, probiotics should be technologically suitable for integration into different food systems so that they retain viability and efficacy throughout storage and following consumption. The wide use of dairy proteins, in a variety of foods, opens interesting opportunities for milk proteins as cost-effective delivery systems for bioactive compounds such as probiotic bacteria. Therefore, this study is aimed at addressing the technical problems associated with viability losses during processing, storage and gastric transit through the application of cell entrapment technology for probiotic stabilisation. This study serves as proof of principle for the development of protein–based carrier systems with inherent protective characteristics for probiotic bacteria.

MATERIAL AND METHODS

Bacterial strain and culture conditions: The probiotic strain *Lactobacillus rhamnosus* GG (ATCC 53103, Valio Ltd., Finland), was procured from University College Cork, under a restricted materials transfer agreement. Harvested cells were stored as stock solutions in MRS broth (Oxoid Ltd., Hampshire, U.K.) containing 50% (v/v) aqueous glycerol at -20°C. The frozen culture was grown in MRS broth at 37°C under anaerobic conditions; achieved using activated Anaerocult A gas packs (Merck, Darmstadt, Germany). Stationary phase cells destined for encapsulation were propagated from 1% (v/v) inoculums for 19 hours at 37°C. Cells were harvested by centrifugation, washed and resuspended to obtain a concentrated cell suspension. This cell concentrate was either employed within the encapsulation process, or utilized in a free-cell condition.

Sample preparation and encapsulation: A milk protein formulation with and without polysaccharides, was rehydrated in distilled water for 16 hours at 4°C under slight agitation (150

rpm). The solution was treated and subsequently stored at 4°C following neutral pH adjustment using 100 mM HCl. The bacterial concentrate and protein suspension were blended, yielding a probiotic population corresponding to the stationary phase concentration (10^9cfu/mL). Monodisperse protein micro-beads were prepared aseptically using an encapsulation device (Inotech Encapsulator®, Dottikon, Swtizerland) with a 150 µm nozzle size. The beads were agitated gently for a pre-determined time period, subsequently recovered and used immediately for 1) single or double coating or 2) *ex vivo* porcine incubation.

Micro-bead Coating: Six different polysaccharide coating materials were kindly donated by Cybercolloids Ltd. (Cork, Ireland) and assays were developed for testing the adsorption efficiency of each coating biopolymer to the protein micro-bead surface. Stock solutions of each biopolymer were prepared within the concentration range 0.5 – 1.2% (w/v) and autoclaved at 121°C for 15 minutes. The optimum addition ratio of probiotic-loaded micro-beads to coating solution was established for each biopolymer solution to facilitate electrostatic deposition of the coating material onto the micro-bead surface. Coated micro-beads were subsequently recovered from the respective suspension and assayed during *ex vivo* gastro-intestinal (GI) incubation.

Zeta Potential (ζ) Assessment: The electrical properties of *Lb. rhamnosus* GG cell surface were evaluated by microelectrophoresis. The electrophoretic mobility (EM) was determined in the pH range 2-7 and stationary phase cells were harvested and resuspended in 10 mM KNO_3. Following pH adjustment, ξ was derived from the velocity of the cell suspension under an applied electric field of 150 V using a Zetasizer (Malvern, Worchester, UK). In addition to this, protein micro-beads, formulated at various pH values, were homogenized and their zeta potential was subsequently evaluated using similar conditions.

Microscopy: Probiotic cell distribution and viability in micro-bead matrices was visually examined under a Leica TCS SP5 confocal scanning laser microscope (CSLM) (Leica Microsystems, Wetzler, Germany). Micro-bead structures were stained using a method involving LIVE/DEAD *Bac*Light cell viability stain (Gardiner *et al*., 2000).

Enumeration of Lb. rhamnosus GG (LGG)*:* Encapsulated bacteria were dispersed using a previously validated homogenisation technique and serially diluted in maximum recovery diluent (MRD) (Oxoid). *Lb. rhamnosus* GG was selectively enumerated on MRS-Vancomycin agar using anaerobic incubation at 37°C for 48 hours and total lactobacilli counts were also enumerated on LBS agar using identical incubation conditions. Tests were conducted in triplicate and mean log survivor counts were plotted as a function of incubation time. In addition to plate counts, cell viability was assessed by flow cytometry using BD Cell Viability assay (BD Biosciences, California).

Survival of encapsulated bacteria in ex vivo porcine gastro-intestinal (GI) contents: Gastric and small intestinal contents collected from 5 porcine GI tracts were pooled and filtered through glass wool. Following a series of purification steps, sterility assessment and enzyme characterization were performed on the respective GI regional contents. Probiotic-loaded micro-beads were incubated in gastric contents (pH 2) for 3 hours at 37°C under slight agitation (100 rpm). At appropriate time intervals, triplicate samples were withdrawn and viable cell counts were determined as described above. In addition to this, the enhanced acid tolerance of coated micro-beads was investigated in amplified acidic environments (pH 1.8) using similar incubation conditions.

Liberation of Encapsulated LGG in ex Vivo Porcine Gastro-intestinal Contents: Aliquots of protein encapsulated *Lb. rhamnosus* GG were incubated in porcine contents from different sections of the GI tract (stomach, anterior small intestine) anaerobically for 12 hours. Various methods of analysis were performed (in triplicate) to determine probiotic cell release from the protein micro-beads, with concomitant evaluation of micro-bead integrity as a function of incubation time at various sections in the GI tract. Following this, the release characteristics of *Lb. rhamnosus* GG from coated micro-beads was investigated in different regions of ex vivo GI contents.

RESULTS AND DISCUSSION

Figure 1 illustrates the homogenous distribution of probiotic cells within the protein micro-bead matrix. This encapsulation procedure generated micro-beads with high encapsulation and loading efficiencies of 96.3% ± 0.9% and 10^9 CFU/mL, respectively**.** Furthermore, their uniform and spherical morphology permitted the determination of true micro-bead diameter**.** Figure 2 illustrates the electrostatic deposition of charged biopolymer coatings onto the micro-bead structure. Zeta potential analysis of micro-beads illustrated a controlled charge oscillation as a function of micro-bead coating layers. Moreover, Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR) determined distinct molecular changes in the presence of single or double coating layers (data not shown).

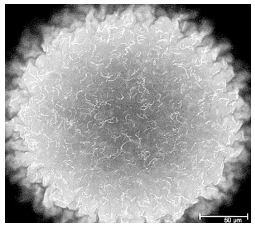

Figure 1: Confocal Laser Scanning Microscope (CSLM) image of an uncoated probiotic-loaded micro-bead

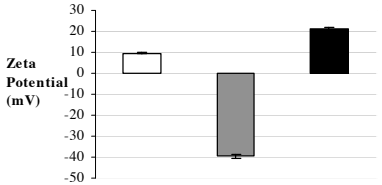

Figure 2: Microelectrophoresis of non-coated (□), single-coated (▩) and double-coated (■) micro-beads

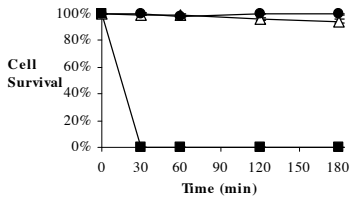

Figure 3: Survival of free (■) and encapsulated (Δ) *Lb. rhamnosus GG* exposed to *ex vivo* porcine gastric contents at pH 2 in addition to encapsulated cells in the presence of a double coating (●) at pH 1.8 at 37°C.

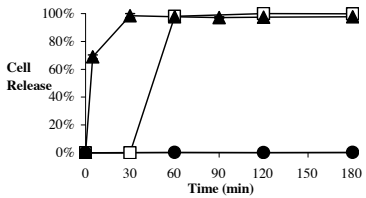

Figure 4: Release profile of encapsulated *Lb. rhamnosus GG* exposed to *ex vivo* porcine intestinal contents in the presence (□) and absence (▲) of a double coating, in addition to cell release from double coated micro-beads (●) in gastric contents at 37°C.

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The survival of free and encapsulated bacteria in *ex vivo* gastric conditions is shown in Figure 3. There was a 94.5% ±1.5% cell survival for encapsulated *Lb. rhamnosus* GG after 3-hour incubation in gastric contents (pH 2). However, cell enumeration and flow cytometry also confirmed that non-encapsulated free cells experienced complete viability loss after only 30-minute incubation. These contrasting results demonstrate the detrimental effect of porcine gastric conditions upon the survival of *Lb. rhamnosus* GG while simultaneously revealing the ability of microencapsulation to promote probiotic acid tolerance during acidic conditions. Furthermore, double-coated micro-beads expressed enhanced probiotic survival (99.9% ± 0.4%) following 3-hour incubation at pH 1.8, which illustrates improved probiotic viability in low pH environments as a function of coating layer deposition.

In addition to this, cell enumeration, chromatography and microscope analysis demonstrated controlled cell release from the protein matrix. Double and non-coated micro-beads achieved complete cell release following 60 and 30-minute residence time in small intestinal contents (jejunum; pH 6.6), respectively. This is a significant finding since the biological activity attributed to probiotic bacteria is reliant upon their survival during gastric transit and subsequent presence within the small intestine of the host. It is clear from the data presented that encapsulated bacterial cells survived well compared to non-encapsulated free cells. Also, the addition of coating material further enhanced probiotic survival during gastric transit, without significantly hampering cell release at the target site.

CONCLUSION

In summary, these results demonstrate that protein micro-beads have an excellent capacity to encapsulate bioactive organisms that are sensitive to stomach circumstances, with concomitant controlled release at a defined location. Thus, this encapsulation technique may act as a platform technology for promoting targeted delivery of a range of sensitive ingredients with potential applications within the food and pharmaceutical industries.

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