Probiotic Protection by whey-protein based networks

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Introduction

The emergence of probiotic bacteria, which confer a health benefit on the host when consumed in adequate amounts, offers means beyond basic nutritional functions of improving public health through daily diet. The recent escalation in consumer health consciousness has lead to an exponential growth of functional foods on a global basis (Van Cleef 2002). This world market is highly dynamic; in many ways it may even be characterised as an experimental market due to the stimulation of new product development. Therefore, the incorporation of probiotics into dairy food systems offers a solution for development of novel functional foods. However, from a processing point of view, integration of probiotic bacteria into different food matrices (with novel composition and/or with no cold chain maintenance) poses a technological challenge to the manufacturer (Ross 2005), due to the general concept that optimal probiotic functionality is only accomplished with viable cultures. Since *Lactobacillus* spp. lack the ability to survive the harsh acidity and bile concentration commonly encountered in the gastrointestinal (GI) tract (Gardiner 2000), in addition to the high temperatures of dairy processing, great demands are imposed upon probiotic product quality (Temmerman 2003*;* Hamilton-Miller 1999). As a consequence, feverish activity has ensued within the dairy industry in an attempt to improve technological properties of probiotic bacteria for application into novel and non-traditional products.

A great deal of research has focused on stabilisation of probiotics using different carrier systems. The key functionality of these encapsulation techniques is the controlled release of probiotics at the right place and the right time. Unlike freeze- and spray-drying processes, where the dormant micro-organism is delivered directly into the environment, micro-bead matrices may act as a protective barrier, allowing the probiotic cells to pass unscathed through processing, storage & gastric environments, to elicit their desired effect at the site of action. Although alginate encapsulation has been widely used for probiotic bacteria, there is no uniformity in the literature as to the protective nature of the micro-beads against adverse GI conditions (Chandramouli 2004; Smidsrod 1990).

Kos *et al.* (2000) investigated the influence of whey protein on *in vitro* survival of *Lb. acidophilus* M92 cells in simulated GI conditions. Their observations suggested the addition of whey protein as a protector, in the preparation of *L. acidophilus* M92 for probiotic use. Furthermore, immobilisation of bacteria in large whey protein isolate (WPI) beads (approx. 2.8 ± 0.1 mm in diameter) provided probiotic protection against acidic conditions (Reid *et al*. 2005). This research was aimed at amending the technical problems associated with protein micro-bead size, in addition to the assessment of bacterial survival in *ex vivo* porcine GI contents. The human derived *Lb. rhamnosus* GG was employed as the test strain due to the specific–selective media available, which permitted the differentiation of inoculated bacteria from food and GI flora.

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). Bacteria destined for encapsulation were propagated from 1% (v/v) inoculums for 19 h at 37°C. Stationary phase cells were harvested by centrifugation at 6,800 x *g* for 10 min at 4°C, washed and resuspended to obtain a concentrated cell suspension. This cell concentrate was either employed within the encapsulation process, or utilised (as a control) in a free-cell condition.

Sample preparation and encapsulation: A whey protein formulation with and without polysaccharides, specified below, was rehydrated in distilled water for 18 h at 4°C under slight agitation (150 rpm). The solution was treated and subsequently stored at 4°C following the pH adjustment to 7.0 using 100 mM HCl. The bacterial concentrate and protein suspension were blended, yielding a probiotic population corresponding to the stationary phase concentration (10^9 cfu/mL). Protein micro-beads were prepared aseptically at ambient temperature, using an Inotech Encapsulator® (Inotech AG, Dottikon, Switzerland) with a 150 μm nozzle size. The beads were agitated gently at 100 rpm for 1 h, and subsequently recovered and used immediately.

Survival of encapsulated bacteria in ex vivo porcine gastric contents: Gastric contents collected from 5 porcine stomachs were pooled and filtered through glass wool. Porcine gastric juice was obtained by centrifugation, filtered through Whatman filter paper, and subsqeuently checked for sterility on brain heart infusion agar (Merck). Encapsulated bacteria were incubated in gastric contents (50 mL) for 3 h at 37°C under slight agitation (100rpm). At appropriate time intervals, triplicate samples were withdrawn and viable cell counts were determined as described below.

Enumeration of Lb. rhamnosus GG (LGG)*:* Encapsulated bacteria were dispersed using a previously validated homogenisation method (Ultra-Turrax® T10, IKA® Werke, Germany) and serially diluted in maximum recovery diluent (MRD) (Oxoid). LGG was selectively enumerated on MRS-Vancomycin agar using anerobic incubation at 37°C for 48 h 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, viability of the free cell reference was assessed by flow cytometry using BD Cell Viability assay (BD Biosciences, California).

Micro-bead Coating: Four different coating materials, κ-carrageenan, ι-carrageenan, sodium alginate and xanthan gum were obtained from Cybercolloids Ltd. (Co. Cork, Ireland). These hydrocolloids were tested for their adsorption efficiency to whey protein micro-beads. Each sample was rehydrated in water (0.5% w/v) and autoclaved at 121°C for 15 min. A defined volume of protein micro-beads was immersed in each suspension and agitated for 2 h at ambient temperature. Coated beads were subsequently recovered from the respective suspension, resuspended in sterile water and microscopic analysis was performed.

Microscopy: Bright-field light microscopy measurements were performed using a BX51 light microscope (Olympus, Germany). Samples were also analysed using an Asylum MFP-3D Atomic Force Microscope and images were acquired in intermittent-contact, AC mode.

Results & Discussion

Figure 1 illustrates the homogeneity of micro-bead shape and size. Encapsulation produced perfect

spheres, thus allowing true micro-bead diameter to be estimated (335 μm ± 0.37 μm)**.** The survival of free and encapsulated bacteria in *ex vivo* gastric conditions is shown in Figure 2. There was a 0.78 log reduction in viable cells of encapsulated LGG after 3 h incubation in gastric contents (pH 2.8 – 3.4), while non-encapsulated cells experienced complete viability loss after only 30 min incubation. Flow cytometric analysis confirmed these findings (figure 4). However, no cell loss was experienced for free cells suspended at pH 7 (phosphate buffer 200 mM). Thus, these contrasting results demonstrate that the harsh conditions of the porcine gastric environment had a detrimental effect upon survival of LGG.

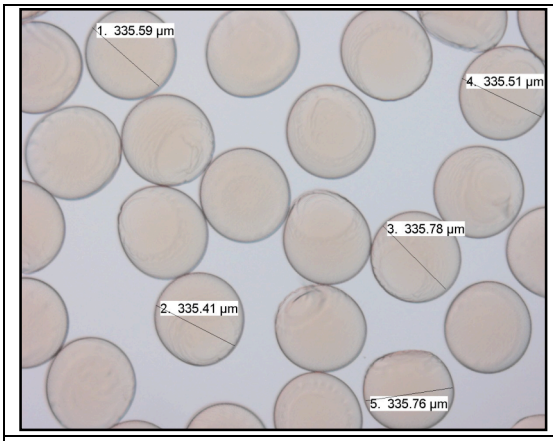

Figure 1: Morphology of micro-beads

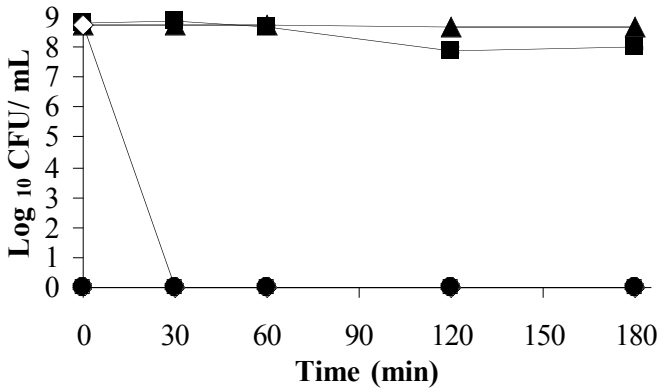

Figure 2: Survival of free and encapsulated LGG in ex vivo porcine gastric juice (PGJ). Symbols: ▲— free LGG at pH 7, ■— encapsulated LGG in PGJ, ◊ — free cells in PGJ, ●— total lactobacilli count in PGJ

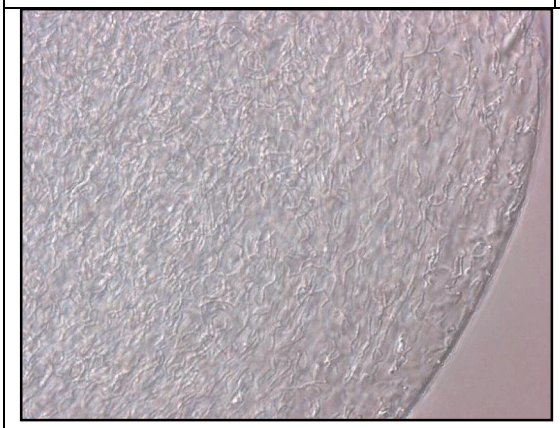

Figure 3: Image of encapsulated LGG in micro-beads following 3 h incubation in PGJ at 37°C

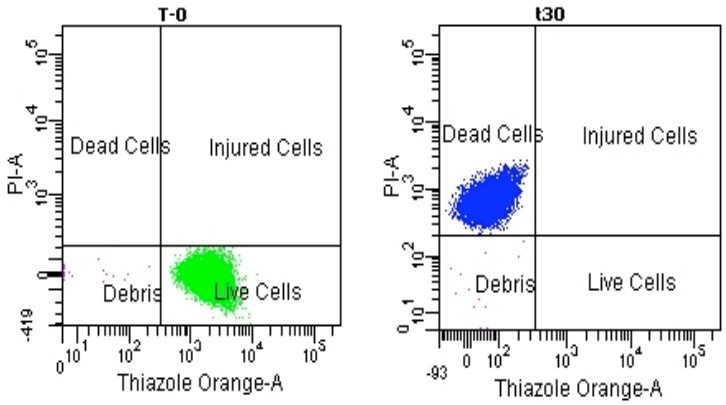

Figure 4: Flow cytometric enumeration of reference (free-cell) samples in PGJ at 37°C after 0 min (T-0) and 30 min (T-30) incubation

Additional microscope analysis (Figure 3), illustrates that micro-bead integrity was retained following 3 h incubation in the porcine gastric juice (PGJ). Cell enumeration also confirmed the absence of cell release from micro-beads into the surrounding PGJ, due to the fact that total cell counts in the gastric contents remained undetected throughout the incubation period. This is a significant finding, due to the fact that probiotics are reliant upon their survival through the stomach, to elicit their health benefits. Although some authors have reported the effect of whey proteins on the survival of probiotic bacteria in simulated gastric conditions (Reid 2005), there is no evidence supporting probiotic survival in *ex vivo* gastric contents. It is clear from the data presented that encapsulated bacterial cells survived well compared to non-encapsulated free cells. Preliminary tests involving polysaccharides coating of whey protein beads yielded promising results, making it

possible to envision a whey protein - hydrocolloid micro-bead formulation.

Conclusion

Whey protein encapsulation can improve gastric transit tolerance of *Lb. rhamnosus* GG. The method presented in this study may be useful for ensuring selection of potential probiotic bacteria capable to survive gastric passage.

Acknowledgements

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