Improved viability of probiotic bacteria by whey protein products

A. Brodkorb[1*], S. Doherty[1], L. Z. Wang[1], P. R. Ross[1,2], G. F. Fitzgerald[2,3] and C. Stanton[1,2]

[1] Moorepark Food Research Centre, Teagasc , Fermoy, Co. Cork
[2] Alimentary Pharmabiotic Centre, Cork [3] Department of Microbiology, University College Cork, Cork, Ireland
*Corresponding author: andre.brodkorb@teagasc.ie

Introduction

Probiotic strains are predominantly selected by their health benefits, while their technological performance e.g. tolerance to heat, oxygen, acid and bile are also important traits (Ross *et al.* 2005). Their viability is generally regarded as an essential requirement for their clinical efficacy. In recent years, encapsulation and entrapment of probiotic bacteria have been studied for their potential use in fermented and other dairy product (Krasaekoopt *et al.* 2003). Whey proteins or gels thereof, usually in conjunction with complex polysaccharides, have been used as matrices for protection of nutraceuticals (Rosenberg *et al.* 2004; Chen *et al.* 2007) and bacteria (Guérin *et al.* 2003; Picot *et al.* 2004; Ainsley Reid *et al.* 2005), mainly in freeze- or spray-dried form. In this paper a bacterial suspension of *Lactobacillus rhamnosus GG* (LGG) was treated with whey protein or peptide products at pH5.2 and stored in aqueous media for up to 14 days at different temperatures. The study was therefore aimed at overcoming some of the technological challenges involved in the development of high viability probiotic ingredients, which can be used for the development of functional foods based on stable probiotics.

Material and Methods

Sample preparation and encapsulation: BiPro whey protein isolate (WPI) purchased from Davisco Foods International, Inc[®], with a protein content of 98% was used as the native whey protein product. The native β-lactoglobulin and α-lactalbumin content was analysed by reverse phase-HPLC and estimated at 82% and 16%, respectively. A 5% (w/v) solution was prepared in Milli-Q[®] water and remained stirring overnight at 4ºC. A portion of this mixture was heat treated (78ºC for 30 minutes) in sealed glass bottles and immediately cooled on ice. Whey protein hydrolysate was produced from a 10% (w/v) protein, using the protease/peptidase complex Flavourzyme[®] (Novozymes France SA) with an enzyme to substrate ratio of 1/100 at 50ºC in a temperature-controlled reaction vessel. Upon completion of the enzymatic hydrolysis, thermal inactivation of the enzyme was performed and the hydrolysate was freeze dried and stored at 4ºC.

For the encapsulation treatments, a concentrated *Lactobacillus rhamnosus GG* (LGG) bacterial suspension (washed twice and re-suspended in citrate-phosphate buffer at pH 5.2) was added to the native, heat-denatured and hydrolysed whey protein solutions at room temperature resulting in a final protein concentration of 2% (w/v). Concentrated HCl was added under agitation up to the isoelectric point of whey proteins, pH 5.2 and kept on ice until further use. Temperature storage and heat-stress were performed in a temperature-controlled water bath and in the case of 25 ºC and 3 7ºC, transferred to a thermostatic room after temperature equilibration. At appropriate time intervals, samples were removed and dispersed by a previously validated homogenisation method (Ultra-Turrax[®] T10 or T18, IKA[®] Werke, Germany), serially diluted in maximum recovery diluent (MRD) (Oxoid) and pour-plated on MRS agar. Survivors were enumerated after 3 days of anaerobic incubation at 37ºC. Tests were conducted in triplicate and mean log survivor counts were expressed.

Bacterial strain and culture conditions: The probiotic strain *Lactobacillus rhamnosus GG* (ATCC 53103, (LGG, Valio Ltd., Finland) was previously isolated from the human gastrointestinal tract (Ross *et al.* 2005), and obtained from University College Cork, Ireland, under a restricted materials

transfer agreement. Harvested cells of this strain were stored as stock solutions in 50% (v/v) aqueous glycerol at -20 °C. *LGG* was sub-cultured at 1% (v/v) in MRS (de Man *et al.* 1960) Oxoid broth (Oxoid Ltd., Hampshire, UK), for 18 h (stationary phase) at 37°C under anaerobic conditions, obtained by using an activated Anaerocult A gas pack (Merck, Darmstadt, Germany). Colony forming units (CFU) were determined by homogenisation, serial dilution and pour plating as previously described. Plates were also incubated under identical conditions. In addition to plate counts, viability of the cells was measured by flow cytometry using BD Cell Viability assay (BD Biosciences, San Jose, California). Samples were diluted in orthophosphate staining buffer (pH 7, 0.1M) to an approximate concentration range of 10^7 CFU/mL. Proteinase K (Sigma-Aldrich, Ireland Ltd.) was applied to each sample (10% v/v) and treated samples were incubated at 37°C for 30 minutes. Each sample was stained immediately with Thiazole Orange (TO) and Propidium Iodide (PI) according to the manufacturer's instructions (Becton Dickinson, San Jose, California) and incubated in the dark at room temperature for 20 minutes. Liquid counting beads (BD Biosciences, San Jose, California) at room temperature and an aliquot (10% v/v) was added to each sample using the reverse pipetting technique. Prepared samples were acquired on a BD FACS ¥ Canto II flow cytometer, equipped with 488-nm laser excitation and BD FACS Diva software using a side scatter (SSC) threshold. Samples were assessed by fluorescence microscopy to confirm that the target organisms were stained.

Microscopy: Bright-field light microscopy measurements were carried out using a BX51 light microscope (Olympus, Germany). Samples were deposited on glass slides and immediately analysed. For AFM analysis, samples were appropriately diluted and deposited onto mica and dried at room temperature. Images were acquired in intermittent-contact, AC mode using an Asylum MFP-3D Atomic Force Microscope.

Results and discussion

Heat-induced denaturation of whey proteins at low ionic strength can form stable 10 to 100nm sized particles, which can further aggregate and subsequently gel upon acidification or increase in ionic strength. Both approaches have been used for encapsulation, though under different conditions than presented in this paper. When acid is added rapidly under agitation, micron-sized particles (10 to 200µm, depending on conditions) can be formed. These porous particles can host bacteria when added before the acidification. Three treatments were carried out using: (I) native WPI, (II) heat-treated WPI and (III) hydrolysed WPI at pH 5.2.

All treatments significantly improved (P~0.05) probiotic viability at 25, 37 and 57°C compared to the bacterial references in phosphate/citrate buffer at pH 5.2. At 4°C, there was no difference between treatments and reference even after 14 days storage and the bacterial viability decreased by less than one log units (results not shown). Hydrolysed whey protein induced the highest protective effect, followed by denatured and native whey proteins, see Figure 2 for storage at different temperatures. Closer examination of the protein matrices at pH 5.2 revealed major microscopic differences. There was some protein precipitation observed in treatment I, which was caused by residual denatured material in commercially available WPI. Particle size increased upon acidification, also visible in the image (I) in Figure 1. However, bacteria were evenly distributed in serum and precipitate and spatial enrichment was not evident. Denatured whey protein (approx. 95% of the protein denatured), formed irregular gel particles. Size and shape strongly depended on length and speed of agitation as well as length and temperature of storage. Figure 1 (II) shows a typical gel particle, that resulted from slow agitation during acidification. Bacteria are embedded or entrapped within the gel particle and the serum appeared depleted of any bacteria. Microscopic images of samples from treatment III, where a crude mixture of hydrolysed whey protein peptides was acidified in the presence of bacteria, revealed small, irregular shaped protein particles. These were formed by aggregation of non- or weakly-charged peptides. However, in contrast to

treatment II, peptides are also expected in the soluble phase. Bacteria were enriched in both aggregated phase and on the aggregate/serum interface, see Figure 1 (III).

Treatment I, using native WPI, showed that the presence of whey proteins in solution remarkably improved viability of bacteria, whereas entrapment in a protein gel matrix further enhanced survival, likely due to restriction in spatial movement. The optimum conditions for increased survival appeared to be provided by a sol/gel interface.

Atomic Force Microscopy (AFM) images (Figures 3 and 4) of the bacteria in heat-denatured whey protein gel particles showed bacteria and protein in close vicinity of each other. However, a micro-phase separation is apparent and aggregation or encapsulation of bacteria by protein coating is not supported by these images.
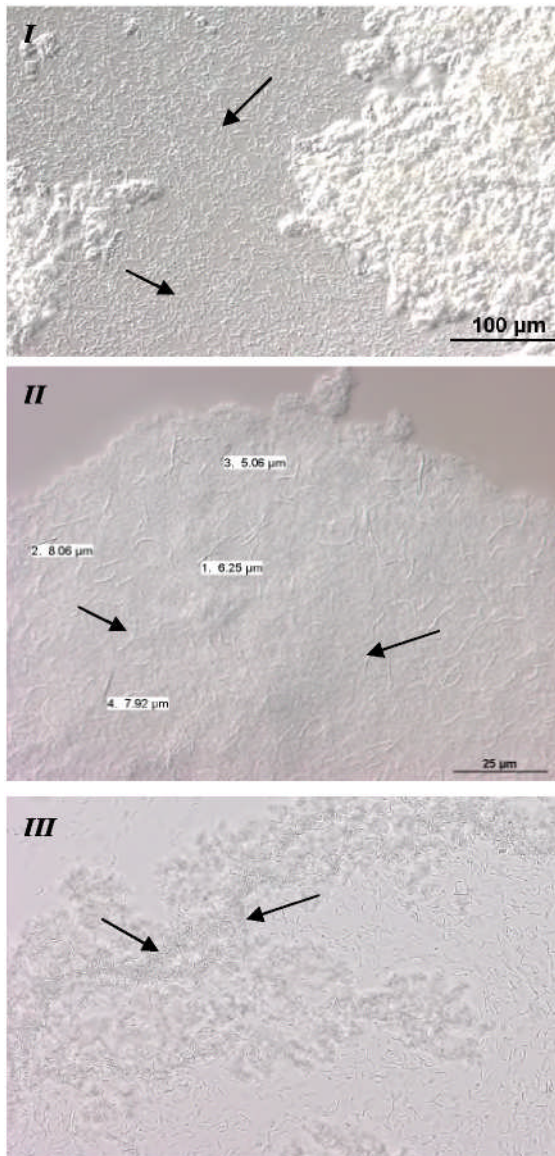

Figure 1: Images of probiotic treatments: (I) native WPI, (I) denatured WPI and (I) WP hydrolysate. Arrows indicate regions of high cell density
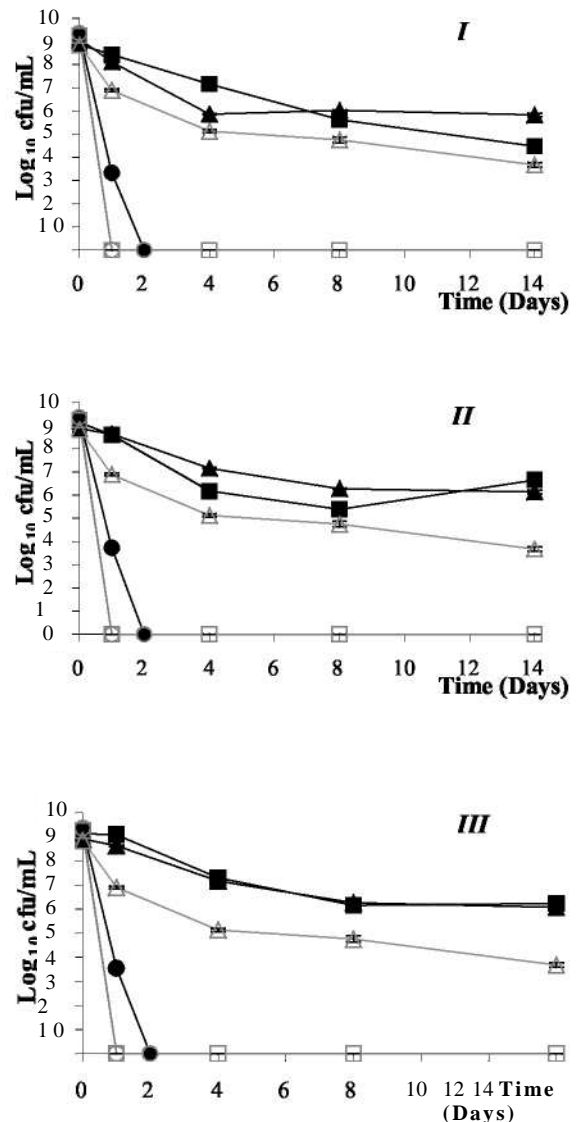
Figure 2: Mean log survival counts of as a function of storage time of probiotic treatments:(I, top) native WPI, (I, middle) denatured WPI and (I , bottom) WP hydrolysate. at 25$^{°}$C ($^{°}$C(■) and57$^{°}$C()). Full markers correspond to treated samples, empty markers to bacterial reference in citrate-phosphate atpH 5.2.
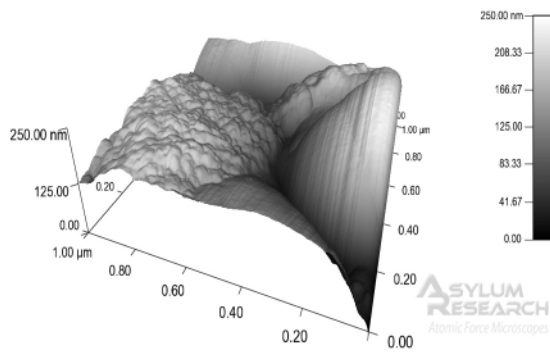
Figure 3: AFM image of heat-denatured whey
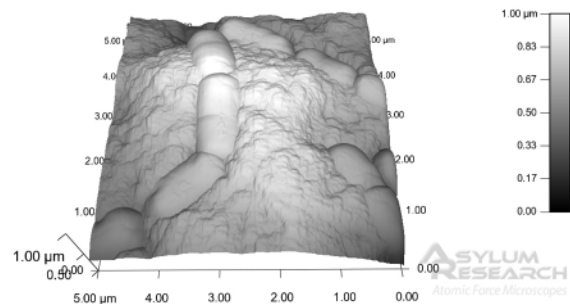proteins embedded between three bacteria.

Figure 4: AFM image of probiotic bacteria embedded
in heat-denatured whey proteins

Conclusion

Storage survival of the probiotic bacteria LGG in aqueous phase could be improved by treatment with whey protein or peptide products compared to storage in phosphate/citrate buffer under the same conditions. Results indicate an enrichment of bacteria in aggregated or gelled material or on the interface to the serum phase. However, closer examination revealed a micro-phase separation between bacteria and whey protein/peptide matrix. More detailed analysis of the interaction is necessary to fully understand the mode of protection

Acknowledgements

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