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Compositional and physicochemical factors governing the viability of *Lactobacillus rhamnosus* GG embedded in starch-protein based edible films

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ABSTRACT

Probiotic incorporation in edible films and coatings has been shown recently to be an efficient strategy for the delivery of probiotics in foods. In the present work, the impact of the compositional, physicochemical and structural properties of binary starch-protein edible films on *Lactobacillus rhamnosus* GG viability and stability was evaluated. Native rice and corn starch, as well as bovine skin gelatine, sodium caseinate and soy protein concentrate were used for the fabrication of the probiotic edible films. Starch and protein type both impacted the structural, mechanical, optical and thermal properties of the films, and the process loss of *L. rhamnosus* GG during evaporation-dehydration was significantly lower in the presence of proteins (0.91–1.07 log CFU/g) compared to solely starch based systems (1.71 log CFU/g). A synergistic action between rice starch and proteins was detected when monitoring the viability of *L. rhamnosus* GG over four weeks at fridge and room temperature conditions. In particular, a 3- to 7-fold increase in the viability of *L. rhamnosus* GG was observed in the presence of proteins, with sodium caseinate – rice starch based films offering the most enhanced stability. The film's shelf-life (as calculated using the FAO/WHO (2011) basis of 6 log viable CFU/g) ranged between 27-96 and 15–24 days for systems stored at fridge or room temperature conditions respectively.

1. Introduction

The term probiotics refers to live organisms, which when administered in adequate amounts (FAO/WHO, 2011), confer a health benefit on the host (FAO/WHO, 2002). Probiotics exert a broad spectrum of beneficial health effects including reduction of the relapse frequency of *Clostridium dificile* or Rotavirus associated diarrhoea, reduction in the symptoms of irritable bowel syndrome and inflammatory bowel disease, modulation of the immune system, reduction of lactose intolerance symptoms and prevention of atopic allergies (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). Delivery of sufficient viable cells can be quite restrictive for food manufacturers as a considerable amount of living cells are inactivated during food processing (heat, mechanical and osmotic stress), storage (exposure to acute toxic factors such as oxygen, hydrogen peroxide and water vapour) or during interaction with the matrix (Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010). In addition, disintegration and passage of the ingested food matrix through the gastrointestinal tract can also critically impact the colonisation ability and the composition of the probiotic intestinal microbiota (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012).

Encapsulation is a physicochemical or mechanical process that has been successfully implemented to retain cell viability under sub-lethal environmental conditions. It can also be used to delay release of the encapsulated living cells during gastro-intestinal transit (Burgain, Gaiani, Linder, & Scher, 2011; Cook et al., 2012). To date technologies based on cell entrapment in dehydrated

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matrices (using spray, freeze or fluidised bed drying) and cross-linked biopolymer based micro-beads are the most common routes to maintain probiotic efficacy (Burgain et al., 2011; Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, & Fisk, 2014b; Soukoulis et al., 2014). Immobilisation of living cells either by physical entrapment in biopolymer networks (e.g. cross-linked or entangled polysaccharide hydrogel systems) or by absorption/attachment in pre-formed carriers and membranes is a well-established strategy for microbial stability in other industries. Examples include biomass production (lactic acid and probiotic starters), fermentation (wine, milk) and metabolite production such as lactic, citric acid, bacteriocins and exopolysaccharides (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). In addition, immobilisation of probiotic bacteria in edible films or coatings has been recently introduced as a novel method for the encapsulation of probiotics (Altamirano-Fortoul, Moreno-Terrazas, Quezada-Gallo, & Rosell, 2012; Kanmani & Lim, 2013; López de Lacey, López-Caballero, & Montero, 2014; Soukoulis et al., 2014b). López de Lacey, López-Caballero, Gómez-Estaca, Gómez-Guillén, and Montero (2012) reported that *Lactobacillus acidophilus* and *Bifidobacterium bifidum* entrapped in gelatine based coatings stored for 10 days at 2 °C showed extended shelf life and prolonged viability. In their study, Kanmani and Lim (2013) reported that the viability of multiple probiotic strains e.g. *L. reuteri* ATCC 55730, L. plantarum GG ATCC 53103 and L. acidophilus DSM 20079 in starch-pullulan based edible films was strongly influenced by the pullulan to starch ratio and storage temperature. Similarly, in a series of studies we have found that the viability of *Lactobacillus rhamnosus* GG in edible films is strictly dependent on the composition of the matrix, with whey proteins and prebiotic soluble fibres promoting the stability of *L. rhamnosus* GG during air drying (37 °C for 15 h) and storage (4 and 25 °C at 54% RH) (Soukoulis et al., 2014; Soukoulis et al., 2014b). We have also demonstrated the feasibility of polysaccharides - whey protein concentrate based edible films as effective carriers of probiotics in pan bread (Soukoulis et al., 2014). The coating of bread crusts with a probiotic containing film enabled the production of probiotic bakery products which can deliver live probiotic cells under simulated gastrointestinal conditions without any major changes to the physicochemical, texture or appearance of bread (Soukoulis et al., 2014).

The aim of the present work was to investigate the impact of the compositional, physicochemical and structural properties of binary starch-protein edible films on *L. rhamnosus* GG viability and stability. Binary films were chosen to offer greater processing flexibility to the films and enhance *L. rhamnosus* GG viability and stability. A series of edible films comprising native starch (either rice or corn) and a protein, either sodium caseinate, soy protein concentrate or bovine gelatine type II, were prepared with *L. rhamnosus* GG and subsequently evaluated for their ability to entrap and stabilise *L. rhamnosus* GG. The resulting physical, structural, optical and thermal properties of the probiotic films were characterised.

2. Materials and methods

2.1. Materials

A *L. rhamnosus* GG strain with established probiotic activity was used (E-96666, VTT Culture collection, Espoo, Finland). Native starch isolated from rice or corn and bovine skin gelatine Type II was obtained from Sigma–Aldrich (Gillingham, UK). Soy protein concentrate (SPC) and sodium caseinate were purchased from Acron Chemicals (Birmingham, UK). Glycerol (purity >99%) was used as plasticising agent (Sigma–Aldrich, Gillingham, UK).

2.2. Stock culture preparation and growth conditions of *L. rhamnosus* GG

One mL of sterile phosphate buffer saline pH 7.0 (Dulbecco A PBS, Oxoid Ltd., Basingstoke, UK) was added to the lyophilised culture of *L. rhamnosus* GG and after adequate mixing, the bacterial aliquot was streaked onto MRS-agar medium (MRS Agar, Oxoid Ltd., Basingstoke, UK). The samples were cultured under anaerobic conditions in hermetically sealed plastic containers containing Anaerogen® (Oxoid Ltd., Basingstoke, UK) at 37 °C for 48 h. A small amount of the colonies was collected with a sterilised loop and suspended in the cryo-medium of the Microbank systems (Pro-Lab Diagnostics UK, Merseyside, UK). The plastic bead cultures were stored in a freezer at −80 °C (Behboudi-Jobbehdar, Soukoulis, Yonekura, & Fisk, 2013).

One bead of the deep frozen cultures was placed in MRS broth (Oxoid Ltd., Basingstoke, UK). Aliquots were incubated for 48 h at 37 °C under anaerobic conditions in plastic jars. Cell pellets were collected by centrifugation (3000 g for 5 min). The supernatant was discarded and cells were washed twice using phosphate buffer saline pH 7.0.

2.3. Preparation of the film forming solutions

Two individual starch and six binary starch: protein (1:1) film forming solutions containing 4% w/w biopolymer total solids were prepared by dispersing the dry materials (native starch and protein) in distilled water at 50 °C under agitation for 1 h. After the addition of the plasticiser at a level of 30% (i.e. 1.2% w/w) of the total biopolymer solids, the aqueous dispersions were adjusted to pH 7.00 ± 0.05 using sodium hydroxide (0.1 M). Samples were then heated to 90 °C for 20 min to complete starch gelatinisation and protein denaturation and destroy any pathogens. The film forming solutions were then cooled to 40 °C until inoculation with *L. rhamnosus* GG pellets.

2.4. Preparation and storage of the edible films

One hundred mL of each film forming solution was inoculated with *L. rhamnosus* GG (6 pellets) and degassed (40 °C for 10 min). Thirty mL of each solution was aseptically transferred to sterile petri dishes (inner diameter 15.6 cm; Sarstedt Ltd., Leicester, UK) and the films were cast (37 °C for 15 h) in a ventilated incubator (Sanyo Ltd., Japan). Dry films were peeled intact and conditioned at room (25 ± 1 °C; ca. 54% RH) or fridge temperature (4 ± 1 °C; ca. 59% RH) in desiccators containing saturated magnesium nitrate solution. Separate films (10 × 10 cm^2 individual squares, stored and conditioned at 25 °C; 54% RH, 3 d), were made for the characterisation of the physicochemical, mechanical and structural properties of the probiotic edible films.

2.5. Enumeration of *L. rhamnosus* GG

One mL of the probiotic film forming solution was suspended in 9 mL of sterile PBS and vortexed for 30 s to ensure adequate mixing. The method described by López de Lacey et al., (2012) with minor modifications was adopted for the recovery of *L. rhamnosus* GG from the bread crust. More specifically, 1 g of edible film containing *L. rhamnosus* GG was transferred to 9 mL of sterile PBS and left to hydrate and dissolve under constant agitation in an orbital incubator at 37 °C for 1 h. The resulting solutions were subjected to serial dilutions in PBS. Each dilution was plated on a de Man, Rogosa and Sharpe (MRS) agar (Oxoid Ltd., Basingstoke, UK) and the plates were stored at 37 °C for 72 h under anaerobic conditions to allow colonies to grow. Enumeration of the bacteria was performed in triplicate,