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A Laminated Polymer Film Formulation for Enteric Delivery of Live Vaccine and Probiotic Bacteria

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ABSTRACT. Live bacterial cells (LBC) are administered orally as attenuated vaccines, to deliver biopharmaceutical agents, and as probiotics to improve gastrointestinal health. However, LBC present unique formulation challenges and must survive gastrointestinal antimicrobial defenses including gastric acid after administration. We present a simple new formulation concept, termed Polymer Film Laminate (PFL). LBC are ambient dried onto cast acid-resistant enteric polymer films that are then laminated together to produce a solid oral dosage form. LBC of a model live bacterial vaccine and a probiotic were dried directly onto a cast film of enteric polymer. The effectiveness at protecting dried cells in a simulated gastric fluid (pH 2.0) depended on the composition of enteric polymer film used, with a blend of ethylcellulose plus Eudragit L100 55 providing greater protection from acid than Eudragit alone. However, although PFL made from blended polymers films completely released low molecular weight dye into intestinal conditions (pH 7.0), they failed to release LBC. In contrast, PFL made from Eudragit alone successfully protected dried probiotic or vaccine LBC from simulated gastric fluid for 2h, and subsequently released all viable cells within 60min of transfer into simulated intestinal fluid. Release kinetics could be controlled by modifying the lamination method.

KEYWORDS

Oral delivery; Enteric delivery; oral vaccines; probiotics; polymer film

ABBREVIATIONS

TPY broth, Tryptone-Phytone-Yeast broth; LBC, live bacterial cells; SIF, simulated intestinal fluid (pH 7.0); SGF, simulated gastric fluid (pH 2.0); CFU, colony forming unit; MLF, multi-layer laminated film formulation; TF, thick laminated film formulation; EC, ethylcellulose; PFL, polymer film laminate; DoE, design of experiments.

Introduction

Recently there has been a growing need to formulate and deliver increasingly complex biological therapeutics, from peptides, recombinant proteins and monoclonal antibodies to *in vitro* cultured eukaryotic cells, such as stem cells, and therapeutic live bacterial cells (LBC). Therapeutic LBC are being explored for various applications, each of which requires a tailored formulation in order to be delivered effectively. Attenuated live bacterial vaccines either injected (Bacillus Calmette-Guerin) or oral (Ty21a, Vivotif) can closely mimic natural infection and typically promote potent, long-lasting protective immune responses [1].Genetically engineered LBC vaccines can deliver heterologous antigens and induce an immune response to both the attenuated strain and the vector, thus protecting against a wide range of infections [2]. Some orally administered therapeutic LBC are classed as probiotics, and are currently under intense development to modulate the gut microbiota in health and disease [3,4]. Commensal enteric bacteria have also been genetically engineered to deliver biopharmaceutical agents such as IL-10 to treat inflammatory bowel disease [5] or insulinotrophic proteins such as GLP-1 [6]. For all of these fields to advance, oral formulations are needed of LBC that offer the potential for controlled delivery of known doses of viable organisms, maintain stability for long-term storage preferably without refrigeration, and allow cost-effective manufacture.

For LBC to be therapeutically active, cells must be kept alive during formulation and delivery. For example, dead bacteria are less immunogenic than live cells [7,8], and although some health benefits have been suggested for dead probiotic cells, only live cells can replicate within and colonize the colon [9]. Mammals have evolved a highly antimicrobial gastrointestinal (GI) tract as a defense against food- and water-borne microbial enteric pathogens, and LBC administered by the oral route confront multiple challenges after ingestion, including gastric acid, enzymatic degradation, antimicrobial peptides, bile acids, and secreted innate and adaptive immunoglobulins. A successful oral formulation of LBC typically requires enteric delivery to protect from the strong acidic environment in the stomach and target release into the small intestine or beyond, depending on the therapeutic target site. Thus LBC vaccines may be targeted to the ileum rich in Peyers patches, whereas some probiotics are targeted to the large intestine to modulate the intestinal microbiota. Enteric coatings are typically acid-insoluble films that stay intact during transit of solid oral doses through the stomach and subsequently dissolve and release contents as pH increases in the intestine. Tablets and granules are the most common solid oral enteric formulations but capsules can also be effectively enterically coated [10]. Enteric coatings can be used for three distinct purposes: firstly to protect gastric mucosa from irritant or toxic active pharmaceutical ingredient (API), secondly to deliver an API to a lower GI site, and thirdly to protect acid-labile API from gastric acid (e.g. omeprazole). Protection of gastric mucosa and intestinal delivery are both achieved by the enteric film layer remaining intact in acid preventing dose disintegration, whereas API protection is achieved by the enteric polymer film blocking ingress of acid into the solid formulation. Although most enteric polymers are acid insoluble, they do typically swell in acid with the degree of swelling and permeability of enteric polymer films varying dependent on polymer composition [11,12]. LBC formulations must achieve

delivery to an intestinal site but protection from acid toxicity is also vital given the high sensitivity of dried bacteria to acid [13,14]. Research in enteric delivery has developed a spectrum of approved enteric polymers coupled to a broad range of coating methodologies. Further improvement of pharmaceutical polymer properties can be achieved by blending polymers to combine distinct desired properties and provide endlessly tunable coating performance [12]. For example, improved protection of API from acid was achieved by blending enteric polymers with insoluble polymers [11,15,16].

LBC have been formulated using enteric coated tablets [17], capsules [18] and granules [19]. Protection from acid using co-administration with buffer solutions has also been used [18], and microencapsulation has been explored for acid protection and controlled delivery [20,21].In all cases drying is required to preserve cell viability during long term storage. However, the dehydration process can damage cells through osmotic and oxidative stress and also denaturation of biomolecules [22]. This can be prevented by sugar glasses formation, an effective and well understood approach to stabilization of biomacromolecules during drying[23]. Although much work has focused on freeze-drying, many bacteria and related complex biological payloads are more robust when freezing is avoided [24]. A continuous, ambient temperature drying process would also be more cost effective than freeze drying, where major costs are incurred by the slow batch turnaround and high cost of large scale freeze drying equipment. Other drying methods, including spray, fluidized bed, foam, vacuum and air convective drying have been explored for the industrial mass-production of dried microorganisms [25]. Perhaps the simplest ambient temperature drying method is to spread material in a thin film to provide a large surface area for evaporation. Ambient temperature drying on a flexible belt is used for continuous, scaleable drying of sensitive food products [26,27]. Ambient temperature drying onto polymer films has historically been used for preservation and storage of microbial reference samples [28], but sophisticated film drying methods has recently been adopted for advanced biotechnology applications ranging from biocatalysis and biosensing to food technology. Flickinger and co-workers developed a sophisticated and robust method for painting and printing live bacteria, using polymer latex plus nontoxic adhesive films to permanently entrap cells, preserving cell viability and metabolic activity on surfaces at ambient temperatures [29].Others have incorporated live lactic acid bacteria into an edible, flexible casein film, with high cell survival after drying and storage, as an antimicrobial meat packaging material [30].

Here, we propose a novel approach to oral formulation of LBC which exploits the benefits of ambient film drying technology and inspired by a novel yet simple and powerful web formulation technology recently developed for oral delivery of small molecule APIs [31,32]. In the Sticky Web approach, a carefully metered dose of API in dry powder form adheres to an adhesive patch printed on a polymer film web. We propose that a cast film of enteric polymer can be used as a web for drying LBC allowing continuous and scaleable ambient temperature lyophilization and combination with a protective enteric film in a single unit operation (Figure 1a). The web comprising dried cells on enteric polymer film can then be directly laminated using edible adhesives to produce an oral solid dosage form (Fig 1b). The resulting polymer film laminate (PFL) formulation can be made using a variety of lamination methods, polymers and film thicknesses to provide fully customized LBC delivery. Here, we investigate if bacteria can

be directly dried onto enteric polymer films, if this material is sufficient for protecting dried bacteria from gastric acid, and if a prototype oral PFL formulation can be used to deliver live vaccine and probiotic bacteria in simulated gastrointestinal fluids.

Materials and Methods

Materials. Eudragit L100 55 (Eudragit L, methacrylic acid-ethyl acrylate copolymer 1:1), was a kind gift from Evonik, Germany. Triethyl citrate, LB broth and LB agar were from Sigma Aldrich (Gillingham, UK). Dow Methocel E15 LV Hydroxypropyl methylcellulose (HPMC) (28-30% Methoxyl, 7-12% Hydroxypropyl content, 12-18mPAs at 2% in water) was kindly supplied by Colorcon (UK). *Bifidobacterium breve* NCIMB 8807 was obtained from the U.K. National Collection of Industrial and Marine Bacteria (NCIMB). Ethyl cellulose (ethoxyl content 48%, 8 to 11 cPs at 5% w/v in 80:20 toluene/ethanol) was from Acros Organics, Belgium. Wilkins-Chalgreen (WC) anaerobe agar was from Oxoid (Basingstoke, UK). Water Blue dye was purchased from Fluka, UK.

Preparation of polymer films. Polymer films were cast from solution by solvent evaporation as follows. Eudragit L100 55(12.5% w/v), Ethylcellulose (EC) (5% w/v) or a blend of Eudragit:EC (with Eudragit to ethylcellulose ratio of 25:75, 50:50 or 75:25) were fully dissolved with stirring in ethanol and triethyl citrate plasticizer added to 25% w/w based on polymer mass. HPMC (2% w/v) was dispersed in deionized water heated to 90ºC and then cooled with stirring to ensure complete dissolution following manufacturer recommended protocol. Polymer solutions were cast by solvent evaporation in 90mm diameter Petri dishes with the volumes adjusted (7.5-50mL) to achieve the desired dry film thickness, measured using a micrometer at 5 points on each square of film used to dry LBC, with cast film thickness ranging from 60µm to 200µm. Thicker films (up to 1mm) used to make spacers between laminated dried cell spots (Fig 1b) were cast from higher volumes. Films were dried at room temperature for 3 days or 40 ºC for 2 days. Prior to making bacterial formulations, films were sterilized by UV irradiation for 15 minutes and thereafter handled aseptically. With enteric polymers complete solvent removal was important to prevent toxicity of the ethanol to dried LBC, and thermogravimetric analysis was performed on cast film samples to confirm absence of solvent.

Acid permeability of enteric polymer films. Two assays were used to compare the acid permeability of films cast from Eudragit alone or Eudragit:EC (50:50) blend. Swelling of films in acid was monitored to determine if acid could penetrate into polymer films. Polymer films pieces (1.5 x 1.5 cm) were immersed in 30 mL SGF solution (0.1M HCl) at 37ºC with stirring. To measure film dissolution samples were placed in a SIF solution of phosphate buffer (pH 7.0) at 37ºC with stirring. In both cases, films were weighed before immersion and at the indicated timepoints after removing excess fluid on filter paper. Water gain was calculated as ((wet mass - dry mass)/ dry mass) x 100 and expressed as swelling % w/w. A Franz diffusion cell was also used to determine if acid could diffuse through cast enteric polymer films. Films cast from Eudragit L100 55 or Eudragit:EC (50:50) blend were compared with a control dialysis membrane (12-14000Da MWCO, Medicell International, UK). To prevent film dissolution which will occur with Eudragit at pH greater than 5.5, films were equilibrated in deionized water with the pH adjusted to 4.2 prior to mounting in the diffusion cell, and the acceptor chamber was

filled with deionized water at pH 4.2. The top donor chamber was loaded with 0.1M HCl (pH 2.0) and the lower acceptor chamber was continually stirred and pH monitored at indicated timepoints up to 6h.

Ambient temperature drying of therapeutic bacteria onto polymer films. Two model therapeutic bacterial strains were selected: the anaerobic Gram positive model probiotic *Bifidobacterium breve* (strain NCIMB 8807) and the aerobic Gram negative model mouse live attenuated vaccine *Salmonella* Typhimurium strain SL3261. Single colonies of *B. breve* from Wilkins-Chalgreen anaerobe agar were inoculated into 10mL trypticase-phytone-yeast (TPY) broth, adjusted to optimal starting density and incubated anaerobically at 37˚C for 21-22 hours to obtain cultures with an OD_{600} of 2.0 corresponding to late log phase/initial stationary phase of growth. Single colonies of *S.* Typhimurium SL3261 from LB agar were inoculated into 10mL LB broth, adjusted to optimal starting density and incubated at 37ºC for 18-20 hours to obtain cultures with an OD_{600} of 2.0 corresponding to late log phase/early stationary growth phase. 1 mL aliquots of cells were harvested in microcentrifuge tubes by centrifugation (10000 rpm, 10 min), supernatant removed and cell pellets resuspended in 100 µL sterile lyoprotectant mix of deionized water with 40%w/v trehalose. Measured volumes of the resultant cell slurry were either placed dropwise in spots on cast polymer films, or spread over 1.5 cm^2 films. LBC on films were dried in a desiccator for at least 18 hours; this time was chosen because in kinetic analysis of the drying process after 16h no further water loss was measurable. Residual water content of samples of cells dried onto polymer films was determined using thermogravimetric analysis (TGA), using 5ºC /minute ramp rate from 25ºC to 200ºC in a TA instruments Q500 Thermogravimetric Analyzer. Initial experiments were conducted at room temperature, however it was noted that laboratory temperature varied sufficiently to significantly alter cell recovery post drying and dried cell phenotype; subsequently all drying was completed in environmental chambers with controlled drying temperature. Drying temperature for experiments presented in Figures 2, 5 and 7 was 20ºC and for experiments presented in Figure 4 drying temperature was 26ºC. Temperature and relative humidity during drying were monitored using a USB datalogger (Omega, UK), and stayed within ±1ºC from indicated drying temperature with relative humidity staying between 4-6%.

Viable cell counting. Live cell recovery was evaluated using serial dilution of samples and agar plate colony counting at all steps: before and after drying onto polymer films, and during acid resistance and dissolution tests. Serial 3-fold dilutions were made in TPY or LB broth in sterile 96-well plates with a multichannel pipettor. In detail, 10 µL spots dried onto polymer film were rehydrated in 10 mL of rehydration medium, of which 50 µL samples were transferred into 100 µL of broth, to complete 16 serial 3-fold dilutions and 5 µL samples of each dilution were then spotted onto square plates of Wilkins-Chalgreen anaerobe or LB agar to give dilution factors of 10^5 to 10^{10}. To determine lowest detection limit of 10^3 CFU/mL 10µL samples was also plated directly from rehydration medium. Samples were dried for 10-15 minutes prior to incubation. *B. breve* plates were incubated anaerobically at 37 ºC for at least 48 hours and *S.* Typhimurium plates were incubated aerobically at 37 ºC for at least 24 hours. Colonies were counted and final viability expressed as colony forming unit per mL (CFU/mL) calculated relative to the initial volume cell slurry. To follow viable cell recovery after drying and exposure

to simulated GI conditions viable cell counts were expressed relative to the initial volume of cell slurry before drying.

Acid protection after direct drying onto enteric polymer films. Cells were dried onto the enteric polymer Eudragit or a blend of Eudragit and ethylcellulose as above, and resistance to acid tested by pipetting 40µL of SGF (0.1M HCl) on the film surface on the opposite side to the dried cells; as a control the same volume of SGF was pipetted directly onto the cell spot on the film (see diagram in Figure 4). After 2 hours of exposure to acid the viability was determined by complete dissolution into buffered peptone water and compared to the viability of replicate samples directly released into buffered peptone water.

Preparation of polymer film laminate oral formulations. To produce encapsulated polymer film laminate (PFL) formulations, cells were dried onto 1.5cm^2 Eudragit or Blend polymer film squares and two complete squares were laminated either side of a single layer of thick film or multiple layers of film containing a central hole of an equal size to the dried cell spot (Figure 1b), thereby encapsulating the dried cells. Prototype formulations were produced using either, double sided adhesive tape or rapid setting, non-toxic cyanoacrylate adhesive to seal the multiple polymer film layers around the dried cells or dye. To produce thick film (TF) formulations (Figure 1b), 10µL spots of LBC slurry were dried onto Eudragit polymer film pieces of 1.5 cm^2 as described above. A square of thicker cast polymer film (1 mm) was prepared with a central hole punched of an equal size to the dried cell spot (4mm diameter), and cyanoacrylate adhesive used to seal cell-bearing polymer film squares face to face on either side of the thick film, enclosing the dried cells. Multi-Laminate film (MLF) formulations (Figure 1b) were produced from two film squares with dried cell spots separated face-to-face around 10 layers of thin (100 µm) polymer film with punched holes; the 10 thin layers with punched holes were laminated first with cyanoacrylate adhesive, and after curing for 1h the outer two film squares were sealed with cyanoacrylate adhesive. In all cases, during sealing laminates were compressed with moderate pressure for 1h to allow the adhesive to cure fully prior to in vitro dissolution tests. In some tests, Water Blue dye was incorporated instead of dried cells to study small molecule release from laminates; 1.5 mg of dye powder was encapsulated within the laminate formulation.

Protection and live cell release from polymer film laminate formulation in simulated gastrointestinal conditions. Prototype PFL formulations were immersed in simulated gastric fluid (SGF) (pH 2.0, 20 mL of 0.1 M HCl) at 37ºC for 2 hours with orbital shaking at 100 rpm. Samples were taken at 0, 1 and 2 hours and live cell release counted, although PFL formulations remained intact in SGF with no dissolution or cell release detected by visual inspection. After 2 hours in SGF, formulations were transferred into 10 mL simulated intestinal fluid (SIF) (0.68% w/v monobasic potassium phosphate and 1.5% w/v peptone at pH 7.0) and incubated at 37ºC with orbital shaking at 100 rpm. Samples were taken and live cell release determined at 0, 1, 2 and 3 hours after transfer from SGF. For each experiment identical samples of LBC dried onto films but not laminated were incubated for 20 min in SIF and live cell recovery counted to determine the post-drying viable cell number prior to making and testing PFL formulations.

Kinetics of dissolution and dye release from polymer film laminates. Prototype PFL formulations containing Water Blue dye were immersed in SGF (pH 2.0, 20 mL) for 2 hours at

37ºC, with shaking. Samples were taken from this solution at 0, 1 and 2 hours and dye concentration measured spectrophotometrically at λ=580nm. After 2 hours, formulations were transferred into SIF (pH 7.0, 10 mL) and incubated at 37ºC with orbital shaking at 100 rpm. Dye concentration was measured at the indicated times and percentage of dye released calculated.

Statistical analysis

Data and results are reported as mean with error bars indicating standard deviation. Statistical evaluation of comparing the significance of the difference in viability between the means of two groups was performed using a two-tailed unpaired Student's *t*-test; a value of $p < 0.05$ was accepted as significant.

Results and Discussion

Drying bacteria onto enteric polymer films. Although we envisage that cells can be continuously dried onto a polymer web (Figure. 1a), our focus in this study was to determine the feasibility of successfully drying bacteria onto a cast enteric polymer film, and protecting from acid. Initial experiments therefore investigated the conditions needed for effective ambient temperature drying of vaccine and probiotic LBC onto films of pharmaceutical polymers acceptable for oral delivery. Cells of a model probiotic strain of *B. breve* were mixed with a protectant mix containing the lyoprotectant trehalose and dried at ambient temperature onto films cast from pharmaceutical polymers. A drying time of 16 - 20 hours was established by completing detailed analysis of the kinetics of drying by monitoring sample mass at hourly intervals. These studies showed that > 95% water loss was achieved by 16h, with no significant further water loss at 20h and beyond. After drying, the residual water content of 3.32 ± 0.53%, (n=3) of the dried cells was determined by thermogravimetric analysis. Two methods were directly compared to ensure controlled and even distribution of cells onto cast films prior to drying: either a measured volume of cell slurry was spread over a marked area of polymer film, or drops of the same cell slurry in identical excipient were dried without spreading to produce a 'cell spot' of known cell dose. Although the surface area after spreading was 3 times higher than cell spots (2.25 vs 0.8 cm^2) which would likely result in a faster drying rate, both samples of dried cells were tested for viable cell recovery at the same timepoint and no difference in viability on HPMC films after drying was observed between these methods (Figure 2a). Subsequent experiments were therefore performed using dried cell spots of fixed volume and thus known input LBC number. Acceptable viability post-drying was achieved onto enteric polymer films cast from Eudragit alone or a blend of Eudragit:EC (50:50 w/w), as well as HPMC films (Figure 2b). The highest live cell recovery was achieved onto HPMC films with a cell loss of 0.5 log, with drying onto enteric polymer films giving slightly greater loss of viability of up to 1 log (Figure 2b).

Previous work has already established that drying LBC can reduce cell viability to an extent that depends on the drying process, cell strain and growth stage, and lyoprotectant excipients [25,33]. In pilot experiments with *B. breve*, the loss in cell viability observed after drying onto cast polymer films ranged from 0.5 log loss (e.g. Fig 2) to >3 log loss. The excipient in which cells

were resuspended prior to drying and the drying temperature were the variables which had the greatest influence over post-drying viability. The excipient trehalose was chosen for experiments performed here as when compared to other excipient mixes (e.g. buffer or medium alone) it provided the most consistent live cell recovery in a range of different drying conditions. Increasing the drying temperature from 20ºC to 35ºC resulted in lower post-drying viability. For example, when dried at 20ºC between 1.2-1.5 log loss of viable cell count was observed; in contrast, at 35ºC from 2.6 to 3.4 log loss was observed. The surface onto which LBC were dried also had a moderate but significant influence over loss during drying, with drying onto Eudragit and Eudragit:EC blend films resulting in greater loss of viability than drying onto HPMC (Figure 2b). Similar viable cell recovery was observed after LBC drying onto films cast from other pharmaceutical polymers including alginate and Aqoat™ HPMC-AS (data not shown). Further investigations are required to understand how the polymer film surface influences LBC loss during drying.

One main aim of drying therapeutic LBC is to improve product stability, since in general dried microorganisms can retain viability for longer than liquid cultures. Although many factors affect the stability of dried LBC and the focus of this study was not to achieve thermostability, it is important that dried cells had residual moisture below 10%,[34,35] as observed by TGA. However, the optimum residual moisture content for stability varies with the composition of the fluid in which organisms are dried, with the storage atmosphere, the species and strain, and the physiological state of the organisms,[36] and so optimum water content for longest product stability will need to be individually determined for each LBC formulated.

Acid permeability of enteric polymer films. Published studies [11,12] have established that a hydrophobic polymer such as EC can be blended with enteric polymers such as Eudragit to increase the protection of an API from acid. Protecting dried bacteria from gastric acid is especially important here because of their increased acid sensitivity compared to cells in culture [13], combined with the direct contact between dried cells and the enteric film. We thus compared films cast from both Eudragit and a Eudragit:EC (50:50) blend for acid permeability. The methacrylic acid : ethyl acrylate copolymer Eudragit L was used, which dissolves above pH 5.5. When enteric polymer films were immersed in SGF, they remained intact but swelling and mass gain was observed indicating a degree of acid permeability. As expected [11,15,16] the degree of swelling in SGF was lower and slower with Eudragit:EC than Eudragit alone (Figure 3a).

Although swelling was observed with both Eudragit and Eudragit:EC blend films in SGF indicating penetration of acid into the polymer, this assay did not indicate whether acid can penetrate fully through the films. The penetration of acid through films was therefore tested using a Franz diffusion cell. To prevent rapid dissolution of the film, the acceptor chamber was filled with water acidified to pH 4.2. The donor chamber was filled with SGF (pH 2.0), and as expected a control cellulose dialysis membrane allowed acid to rapidly permeate, with the acceptor chamber pH equilibrating to pH 2.5 by 4 hours (Figure 3b). In contrast, the acceptor pH remained at pH4.2 with either Eudragit or Eudragit:EC films (Figure 3b), demonstrating the acid impermeability of these films. No acid permeation was observed through Eudragit and Eudragit:EC blend films ranging in thickness from 75 – 350 μm (data not shown). Having

measured acid permeability, films were then tested at pH 7.0 to check dissolution and release in intestinal conditions. Eudragit films fully dissolved within 30 min immersion in SIF pH 7.2 (Figure 3c),but Eudragit:EC blend films gained weight and swelled, reaching 5x starting weight by 6 hours (Figure 3c), gaining characteristics of a hydrogel. This indicated that the Eudragit had leached out of the film leaving a porous network of insoluble EC, and raised the possibility that blended films with increased acid impermeability may not be able to release LBC.

Protecting dried LBC from acid with enteric polymer films. Having established that Eudragit:EC blend films were less acid permeable than Eudragit alone, but that both films were equally able to block acid diffusion, the ability of the two enteric films to protect dried LBC from direct acid exposure was compared. In these experiments, cells were dried onto enteric films at an increased temperature of 26ºC, resulting in greater cell loss on drying than that observed in previous experiments where drying was conducted at 20ºC (compare Figures 2 vs 4). To avoid this increased loss of viability on drying, in subsequent experiments (see Figures 5 and 7 below) a drying temperature of 20ºC was used.

When dried cells were exposed directly to SGF, viability rapidly dropped below the limit of detection of $<10^3$ CFU/mL representing a $>10^5$-fold loss. In contrast, when acid was placed on the opposite side of films for 2 hours, followed by determination of remaining viable cell counts, significant numbers of viable cells were recovered (Figure 4), confirming that enteric polymer films are sufficient to provide a degree of protection from acid. As expected from the Franz diffusion cell study, both enteric films protected dried cells when SGF was placed onto the film surface opposite the dried cells for 2 hours (Figure 4). However, the cell recovery seen after 2 hours acid exposure was greater with the Eudragit:EC blend than Eudragit alone, indicating that the blended polymer film provided improved protection from acid in agreement with the swelling results (Figure 3a) and prior work [11,15,16]. Indeed, with the Eudragit:EC blend less than 0.5 log reduction in viable cell recovery was observed after 2 hours exposure of the film to SGF. In contrast, a nearly 2 log reduction in viable cell recovery was seen with pure Eudragit films, suggesting that the observed swelling in acid (Figure 3a) may have also allowed sufficient acid to permeate through films to adversely affect the viability of cells dried onto Eudragit films. It is possible that the increased viable cell recovery with Eudragit:EC blend over Eudragit alone films could be attributable to increased release of viable cells, rather than simply increased protection from acid. However, both films released similar viable cell numbers when directly exposed to SIF, with the small apparent increase in cell release from Eudragit:EC blend films not being found to be statistically significant (figure 4; $p>0.05$).

We conclude that cells dried directly onto enteric polymer films can be protected from acid. Significant cell recovery after two hours acid exposure was achieved, with an Eudragit:EC blend offering better acid protection than Eudragit alone.

Prototype Polymer Film Laminate oral formulations. Live bacterial cells were dried onto enteric polymer films and prototype solid oral formulations illustrating the Polymer Film Laminate concept (Figure 1) were made and tested. To evaluate the suitability of PFL made from enteric polymer films for protecting live dried LBC from gastric acid and subsequently releasing viable cells into the intestine, prototypes were tested in simulated gastrointestinal conditions (2

hours in SGF pH 2.0 and 3 hours in SIF pH 7.0) and cell viability sampled at regular timepoints. Prototype PFL containing a single 10µL spot of dried cells with a viable cell density equivalent to 10^9 CFU/mL (i.e. approximately 10^7 CFU *B. breve* per dose) were made by laminating either Eudragit or Eudragit:EC blend films together using layers of adhesive tape with a central hole cut matching the dried cell spot (Figure 1b). After 2 hours of exposure in SGF pH 2.0, both Eudragit or Eudragit:EC blend formulations were intact and no cells released. Importantly, 1 hour after transfer into SIF pH 7.0, the Eudragit PFL formulations had fully dissolved and cell recovery equivalent to the starting cell density of 10^9 CFU/mL was observed, indicating no loss in cell viability after 2h in SGF at pH 2.0 (Figure 5). In contrast, after 3 hours in SIF pH 7.0 the Eudragit:EC blend PFL became highly swollen but intact with no disruption of the polymer matrix, and no viable cells were recovered in SIF pH 7.0 (Figure 5). This was surprising considering the improved protection from acid seen with film alone (Figure 4). Indeed, when swollen Eudragit:EC blend PFL samples were mechanically disrupted significant numbers (>$2x10^7$ CFU/mL) of viable cells were recovered (data not shown) suggesting that the absence of viable cell recovery was not due to loss of cell viability but instead represented a failure to release LBC.

To determine if the Eudragit:EC films were capable of releasing low molecular weight molecules into SIF, dye release studies were completed with PFL made from Eudragit or Eudragit:EC blend loaded with Water Blue dye instead of dried LBC, and exposed to SGF followed by SIF. In contrast to the lack of release of LBC from the Eudragit:EC PFL prototype, complete release (>90%) of Water Blue dye was seen from both enteric polymer formulations within 1 hour of transfer from SGF into SIF (Figure 6) confirming that although the Eudragit:EC PFL does not fully dissolve at pH 7.0, it can still release low molecular weight contents. In previous work where Eudragit:EC film coatings were applied to tablets, rapid release was achieved, possibly due to disintegration of the tablet following swelling at elevated pH [37].

We conclude that although blending enteric polymers with ethylcellulose can increase protection from acid and is suitable for film coating tablets and low molecular weight APIs, the blended film is unsuitable for LBC delivery in PFL. This observed poor release from PFL could be attributed to a number of reasons, including the large size of bacterial cells or interactions between bacterial cells and EC. Although electrostatic interactions between neutral (EC) and negatively charged (Eudragit) polymers with negatively charged bacterial cells is unlikely, but undissolved EC could bind cells through hydrophobic interactions. Further studies of the porosity of Eudragit:EC films are required to fully understand the range of APIs that could be successfully released from formulations formed from this polymer blend.

Surprisingly, although in acid protection experiments significant cell loss – of up to 100-fold compared to controls samples not exposed to acid – was observed with Eudragit films (Figure 4), complete recovery of identical viable cell counts to control samples was seen from Eudragit PFL (Figure 5) after 2 hours exposure to SGF in both experiments. There are two explanations of the improved protection from acid seen with prototype PFL compared to the previous experiments where acid was simply pipetted onto the opposite side of Eudragit films to dried cells. Firstly, it is possible that dried cells within PFL are better sealed from acid than the film pieces exposed to

acid without lamination. Secondly, in acid protection studies (Figure 4) cells were dried at 26ºC whereas in contrast prototype PFL formulations were made from cells dried at a lower temperature of 20ºC (Figure 5). This temperature was chosen to reduce cell loss during drying, as when drying temperatures were directly compared it was discovered that drying at the an elevated temperature of 35ºC gave 2.6 to 3.4 log loss of viability, in contrast to drying at 20ºC where loss was 1.2 to 1.5 log. Further preliminary studies suggested that increased drying temperatures increase the sensitivity of dried cells to acid as well as reducing viability after drying (Figures 4 vs 5 and unpublished observations).

In conclusion, the Eudragit PFL is capable of efficiently protecting dried live probiotic bacteria from 2h immersion in SGF. Although the drying process always gave a loss close to 1-log reduction in viable cell number, the overall delivery efficiency considering all process steps is adequate and compares well with other formulations of probiotic cells [20,38].

Controlled probiotic and vaccine release from enteric PFL formulations. Building on the observation that live cells are protected from acid and rapidly released from Eudragit PFL formulations, further PFL were made with varying bacterial payload, film thickness, type of adhesive and method of lamination. Firstly, the effect of PFL construction on protection from SGF and release in SIF was investigated. PFL containing *B. breve* dried onto Eudragit films were laminated using adhesive tape or non-toxic cyanoacrylate adhesive. The use of multiple thin spacers laminated between cell-loaded films was compared to a single thick spacer layer. In all cases, endpoint cell recovery was excellent, with cell recovery 3 hours post transfer into SIF identical to that seen when replicated samples of cells dried onto films were directly incubated for 20 minutes in SIF without lamination, indicating no loss of viability after 2 hours immersion in SGF (Figure 7a). However, different release kinetics were observed depending on the lamination method and adhesive used. Thus although complete cell release was seen after only 60 minutes in SIF with PFL produced from multiple layers of thin film laminated with adhesive tape, or when thick Eudragit films were laminated using edible adhesive, in contrast complete cell recovery was delayed until 3 hours after transfer into SIF when multiple layers of thin films were laminated with edible adhesive (Figure 7a). This delay in release is most likely to be caused by the increased amount of adhesive present with the MLF formulations that appeared to slow down the film disintegration in SIF. This excess of adhesive with prototype MLF formulations was caused by the manual lamination method and could be overcome by accurately metering the quantity of edible adhesive used to assemble PFL.

Although experiments with the model probiotic *B. breve* demonstrated the concept of direct enteric formulation of dried cells, bacteria vary significantly in their response to drying and resistance to gastrointestinal microbicides. Therefore similar PFL formulations were produced using the model live bacterial vaccine *Salmonella enterica* subspecies Typhimurium strain SL3261. The viability of *S.* Typhimurium after drying onto Eudragit films was excellent when measured after direct release in SIF, with less than 0.5 log loss post-drying (Figure 7b). In contrast, when directly exposed to SGF pH 2.0 no viable cells were recovered, as expected given the known sensitivity of this strain to pH <3 (data not shown and [13]). Importantly, when PFL were made from *S.* Typhimurium dried onto Eudragit films following 2 hours immersion in SGF,

followed by transfer to SIF, complete viable cell release was achieved (Figure 7b).When Eudragit film thickness was varied, the kinetics of vaccine cell release could be modified, with 100µm films releasing total LBC load within 60 min of transfer into SIF in contrast to 165µm films which delayed release until 2-3 hours after transfer (Figure 7b).

By varying film thickness and PFL lamination method, controlled enteric release of viable LBC from a solid oral formulation is feasible. The recovered relative cell density of 10^{10} CFU/mL (expressed relative to the initial cell volume) from prototype PFL produced containing a single spot dried from 10µL cells indicates a live cell dose of 10^8 viable cells per formulation, and confirms the feasibility of producing PFL with standard doses of LBC, where typical oral doses of both live bacterial vaccines and probiotics range from 10^8 to 10^{10} viable bacteria [18,39,40]. Increasing LBC dose beyond 10^8 can be achieved simply by increasing the number of dried cell spots in the PFL, or by increasing the cell density in the slurry, the volume of slurry and the area of the dried cell spot. The post-drying viability, cell dose delivered, and product stability of formulated dried LBC is already known to depend upon a wide range of variables, including cell species and strain, culture conditions, drying conditions and excipients used[33], for this reason it will be essential for systematic multivariate DoE optimization to be used to develop specific formulations using this new process.

PFL represents an alternative to other oral formulations explored for LBC delivery including tablets, capsules, and microcapsules [21]. Potential benefits include low-cost scaleable continuous drying, for example utilizing Refractance Window drying [26,27]. The PFL concept has similarities to orodispersable films used for delivery of small molecule APIs such as rasagiline mesylate or tadalafil, used as a substrate for metered drug printing [41].

Although these results indicated that live cell delivery is feasible using PFL, the live cell dose achieved, cell phenotype after release, and exact location of live cell release cannot be accurately predicted from the simple *in vitro* dissolution conditions used here. Further *in vitro* survival and release studies are planned using more complex simulated gastrointestinal fluids, for example including physiological concentrations of intestinal microbicides such as bile acids [14,42]. Ultimately however, the performance of PFL for therapeutic LBC delivery must be tested by determining live cell release in animal models that mimic human GI transit (e.g. using large monogastric mammal such as pigs), and therapeutic benefits such as vaccine immunogenicity or modulation of GI microbiota using probiotics can only be determined in human clinical studies.

Conclusions These studies demonstrate the feasibility of a new oral formulation concept for simple, effective, controlled enteric delivery and protection from acid of sensitive complex biologic payload such as live bacteria for vaccine and probiotic applications. Although maximum protection from acid of bacteria dried onto enteric polymer films was achieved when Eudragit was blended with ethylcellulose to reduce film permeability in acid, to achieve release of cells Eudragit alone was required which fully dissolved at intestinal pH. The release kinetics and thereby intestinal release site could be modified by altering the thickness of polymer films used to produce PFL, and by altering the lamination method and adhesive used. The therapeutic LBC dose, timing and location of release depend on the specific LBC application, with vaccines requiring release into the ileum and probiotics benefitting from colonic targeting. Our target

critical product attributes were therefore to achieve complete protection from acid, and variable kinetics of release to allow controlled LBC targeting. This target was achieved, therapeutically relevant dose of 10^8 CFU of a model attenuated vaccine was released within 1 hour after transfer into SIF following 2 hours exposure to SGF. Further studies of PFL are warranted to determine stability, viable cell delivery in conditions that fully simulate gastrointestinal transit, and ultimately in preclinical models, to determine *in vivo* cell delivery performance for therapeutic live bacteria.

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FIGURE LEGENDS

FIGURE 1.Polymer Film Laminate concept. A Diagram of proposed continuous manufacture method to produce live bacterial cells dried directly onto pharmaceutical polymer film web. Cells mixed with lyoprotectants are spread onto a continuous web of enteric polymer film. **B** Alternative methods for laminating enteric formulations from film dried bacterial cells together with enteric polymer film spacers to produce PFL formulations.

FIGURE 2.Ambient temperature drying live *B. breve* onto pharmaceutical polymer films. (A) Individual drops of cells or evenly spread cell suspension were dried onto cast HPMC films at 20ºC in a desiccator and viable cell recovery determined after release into buffered peptone. (**B)** 10µL drops of cells were dried onto films cast from either HPMC, Eudragit or Eudragit:EC blend (50:50 w/w) and live cell recovery determined after release into buffered peptone expressed relative to the cell slurry volume prior to drying. Viable cell density prior to drying was measured from samples of cell slurry by plate counting and is indicated by dotted line. Each bar represents a single sample of cells dried onto film, and error bars indicate standard deviation of 6 replicate cell counts from each sample. Similar recovery after drying was observed in at least 3 independent experiments. *: $p < 0.05$ vs HPMC; ns: not significant. In all groups a significant reduction in cell recovery was observed after drying ($p < 0.05$).

FIGURE 3.Acid permeability of enteric polymer films. Cast films of Eudragit or Eudragit:EC blend (50:50 w/w) were tested for swelling (**A**) and acid permeability in Franz diffusion cells (**B**) in SGF pH 2.0. A control dialysis membrane was used to confirm rapid acid diffusion to the acceptor chamber. (**C**) Dissolution of replicate samples of films was monitored in SIF pH 7.0. Data represent mean values and error bars indicate standard deviation (n=3).

FIGURE 4. Enteric polymer films can protect dried cells from acid. *B. breve* cells were dried at 26ºC in a desiccator onto films of Eudragit or Eudragit:EC blend. Acid resistance was tested by comparing direct release of dried cells into SIF, to direct exposure to SGF, and to exposure of the enteric polymer film to acid on the opposite side to the dried cells, followed by release into SIF. Data are representative of at least 3 repeat experiments testing acid protection provided by enteric polymer films. *****: $p < 0.05$; ns: not significant. In all groups a significant reduction in cell recovery was observed after drying ($p < 0.05$).

FIGURE 5. Prototype enteric Polymer Film Laminate formulations protect and release dried live cells in gastrointestinal conditions. *B. breve* cells were dried at 26ºC in a desiccator onto Eudragit and Eudragit:EC blend films and either kept as film pieces (left "Film") or made into PFL (right "PFL formulation"). Films were released directly into SIF to determine the post-drying viability (left). PFL formulations were exposed to simulated gastrointestinal conditions by immersion in SGF for 2 hours followed by transfer into SIF for 3 hours. Samples were taken at indicated times and viable cell recovery determined by plate counting. Each bar represents a single PFL formulation with the error bar indicating standard deviation of 6 replicate cell counts. Similar viable LBC recovery was observed in 4 different experiments comparing Eudragit with Eudragit:EC blend prototype PFL. Viable cell recovery was significantly reduced by drying compared to cell slurry before ($p < 0.05$), but no significant difference was seen after drying between Eudragit and Eudraget: EC blend films.

FIGURE 6. Dye is rapidly released from PFL with varying enteric polymer composition. PFL were made from Eudragit or Eudragit:EC blend films containing Water Blue dye, and the release kinetics followed in simulated gastrointestinal conditions. Results are representative of 3 repeats.

FIGURE 7. Controlled delivery of probiotic and vaccine live bacterial cells from PFL in simulated gastrointestinal conditions. *B. breve* (A) or *S.* Typhimurium (B) cells were dried onto Eudragit films at 20ºC in a desiccator and PFL were made with varying lamination method and adhesive (A) or film thickness (B). Unformulated films were released directly into SIF to determine post-drying viability. PFL were immersed in SGF for 2 hours followed by transfer into SIF and viable cell recovery determined at indicated timepoints. Each bar represents a single PFL formulation with the error bar indicating the standard deviation of 6 replicate cell counts of each sample. Similar recovery and kinetics were observed in 3 different experiments with *B. breve* and 2 with *S.* Typhimurium.

Figure 1

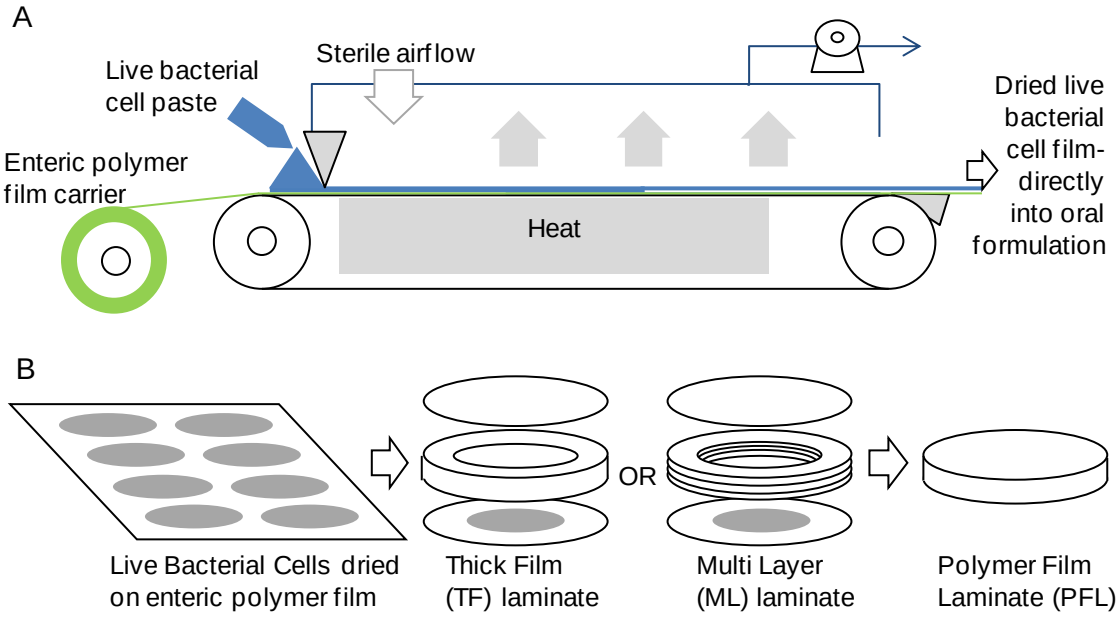


Figure 2

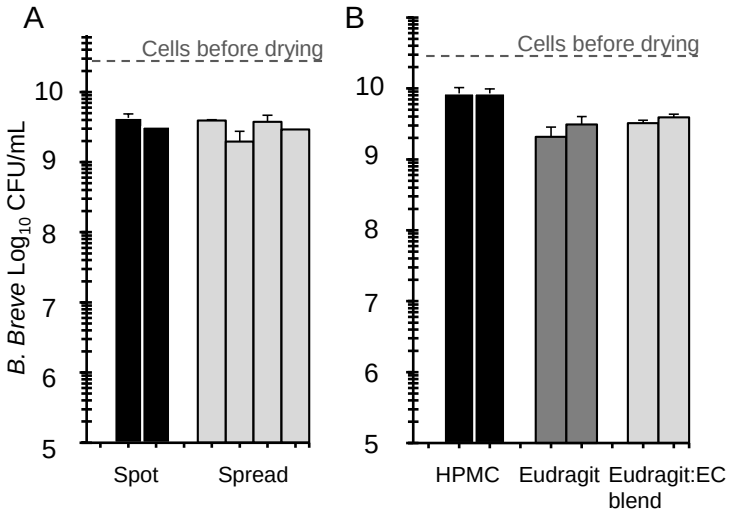


Figure 3

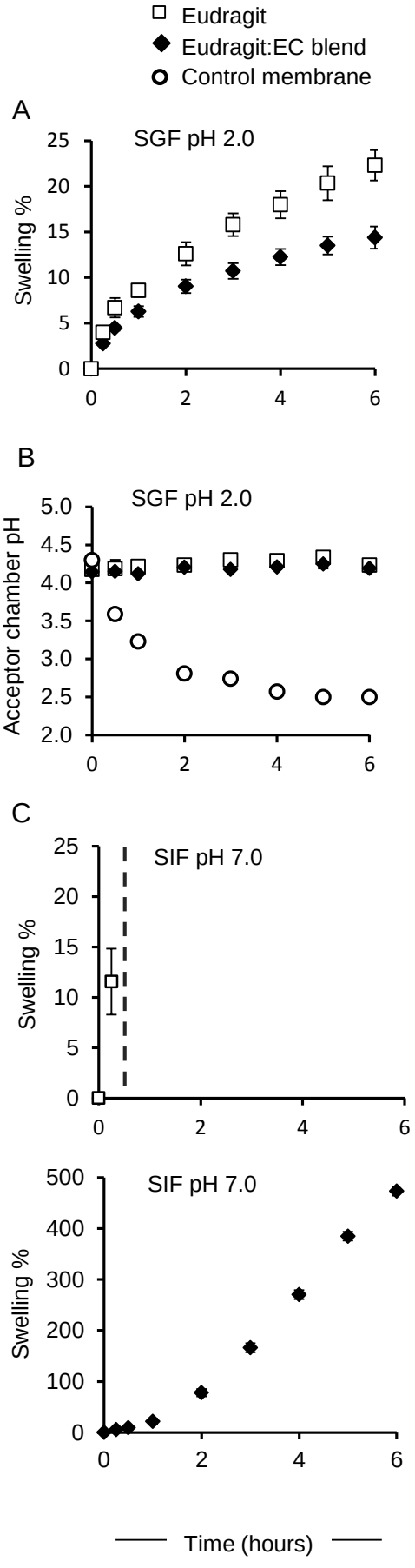

Eudragit
Eudragit:EC blend
Control membrane
A
SGF pH 2.0
Swelling %
25
20
15
10
5
0
0
2
4
6
B
SGF pH 2.0
Acceptor chamber pH
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
2
4
6
C
SIF pH 7.0
Swelling %
25
20
15
10
5
0
0
2
4
6
SIF pH 7.0
Swelling %
500
400
300
200
100
0
0
2
4
6
Time (hours)

Figure 4

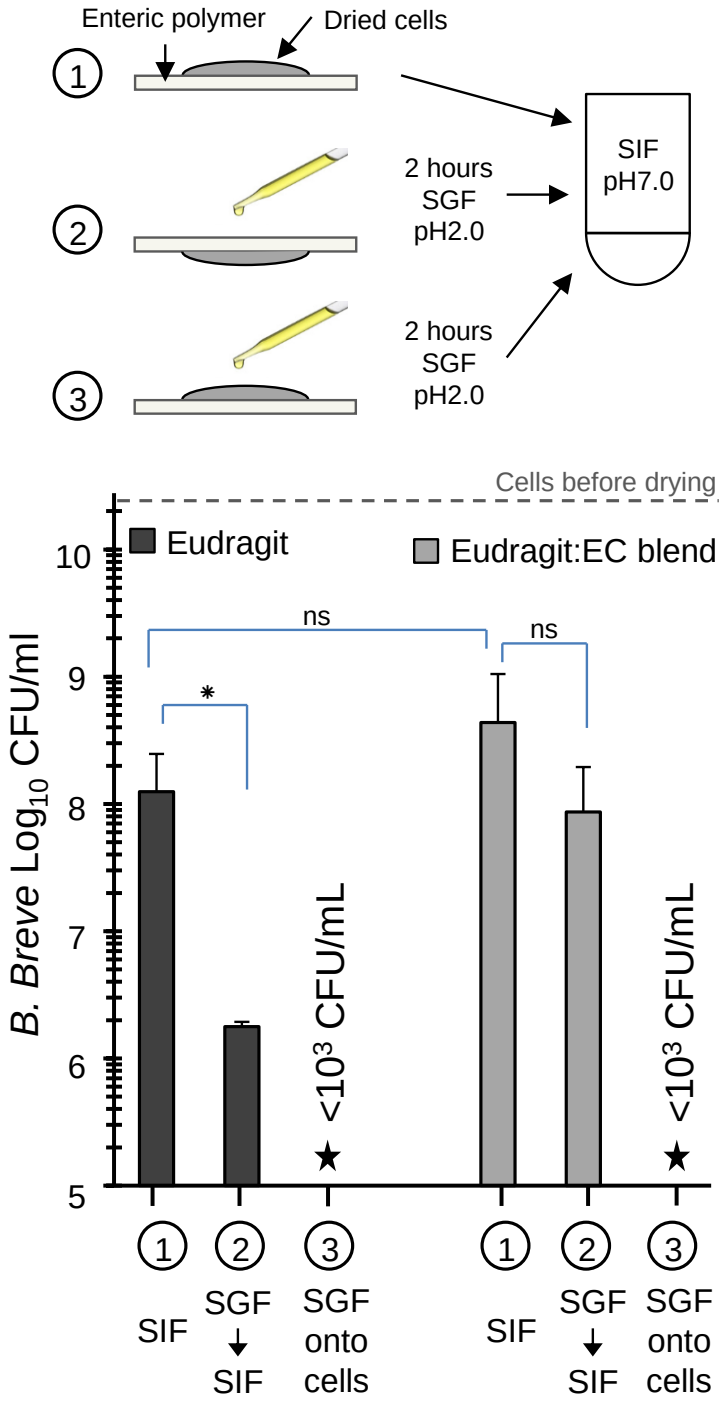

Enteric polymer
Dried cells
1
2
3
SIF
pH7.0
2 hours
SGF
pH2.0
2 hours
SGF
pH2.0
Cells before drying
Eudragit
Eudragit:EC blend
ns
ns
*
B. Breve Log10 CFU/ml
10
9
8
7
6
5
<10^3 CFU/mL
<10^3 CFU/mL
1
2
3
SIF
SGF
SIF
SGF onto cells
1
SIF
2
SGF
SIF
3
SGF onto cells

Figure 5

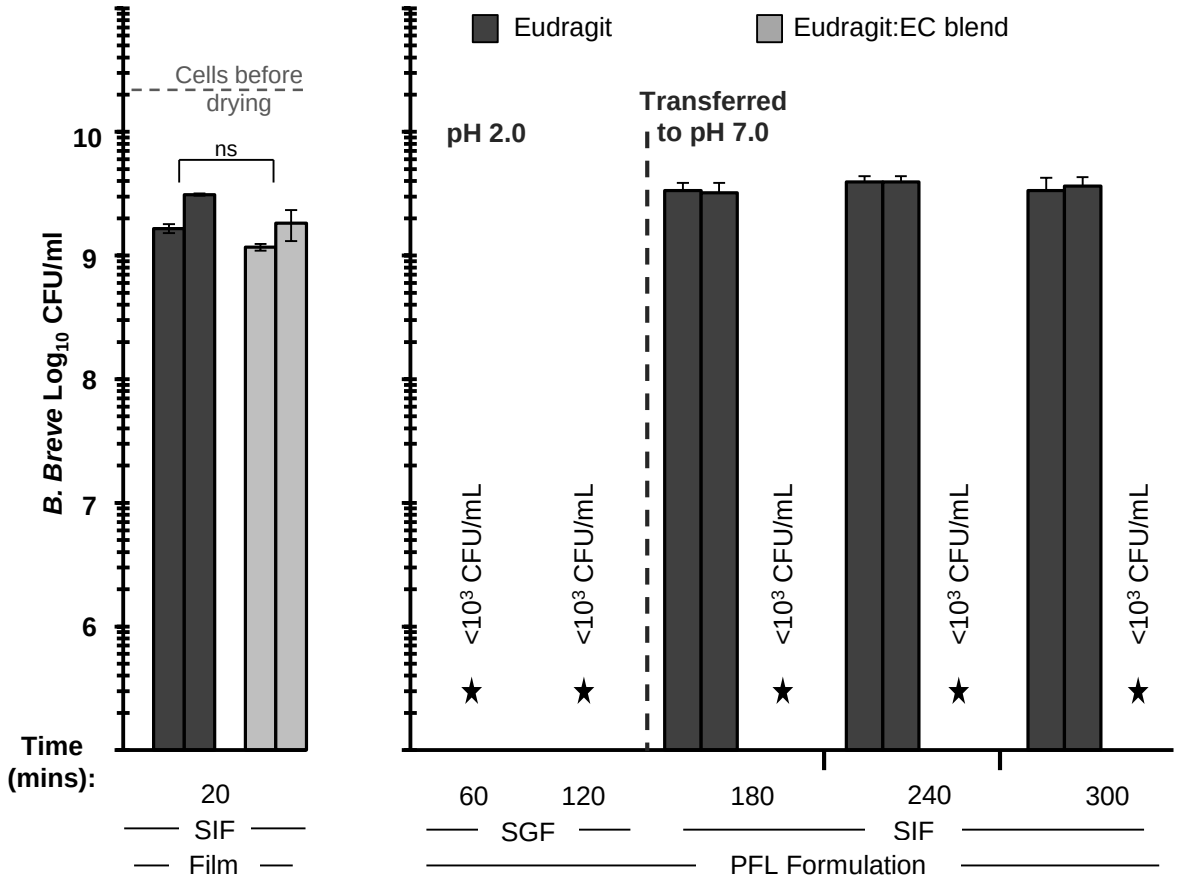


Figure 6

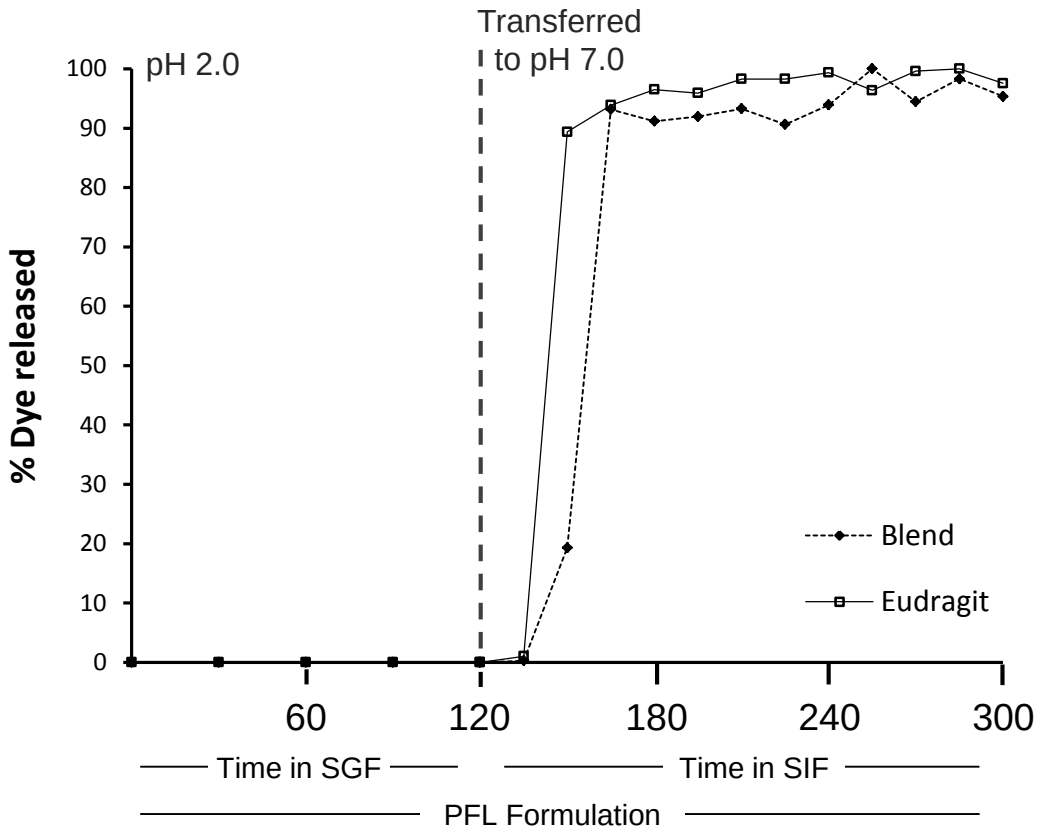
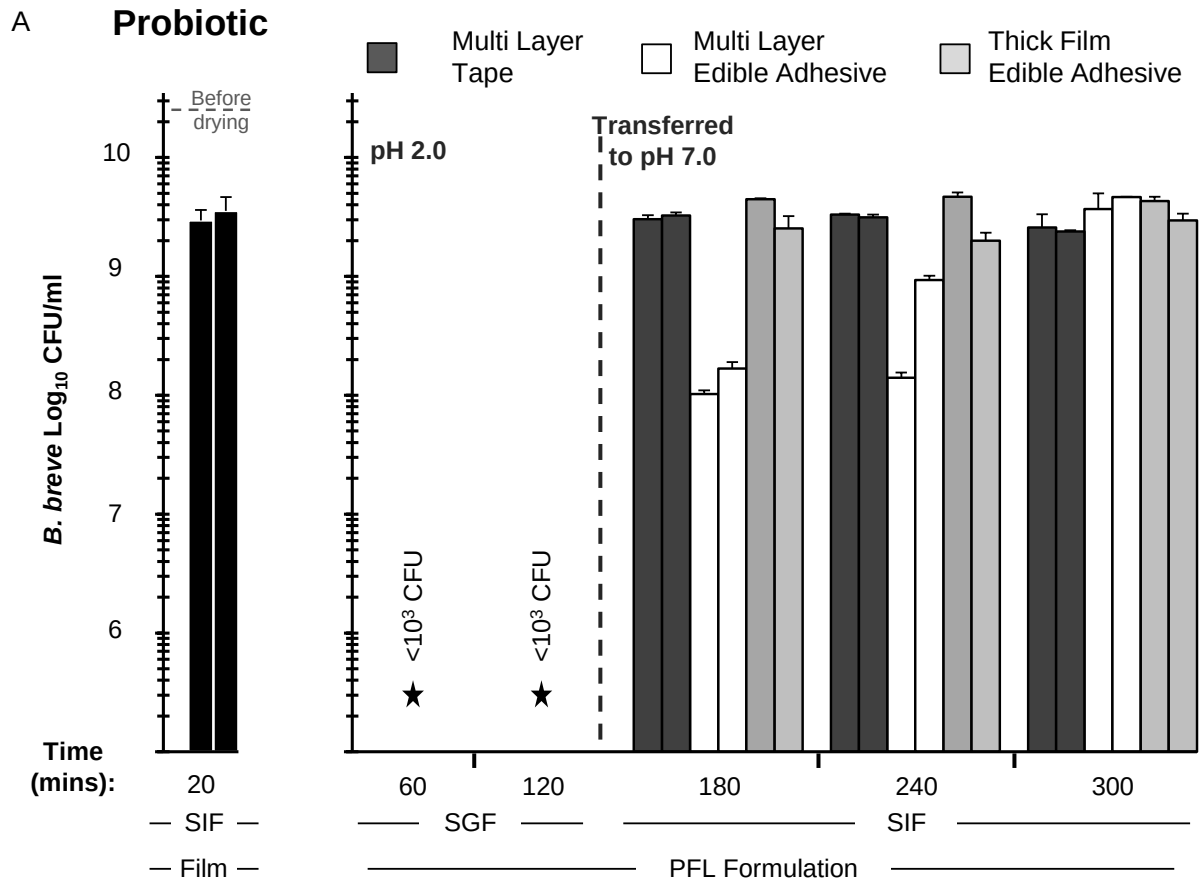
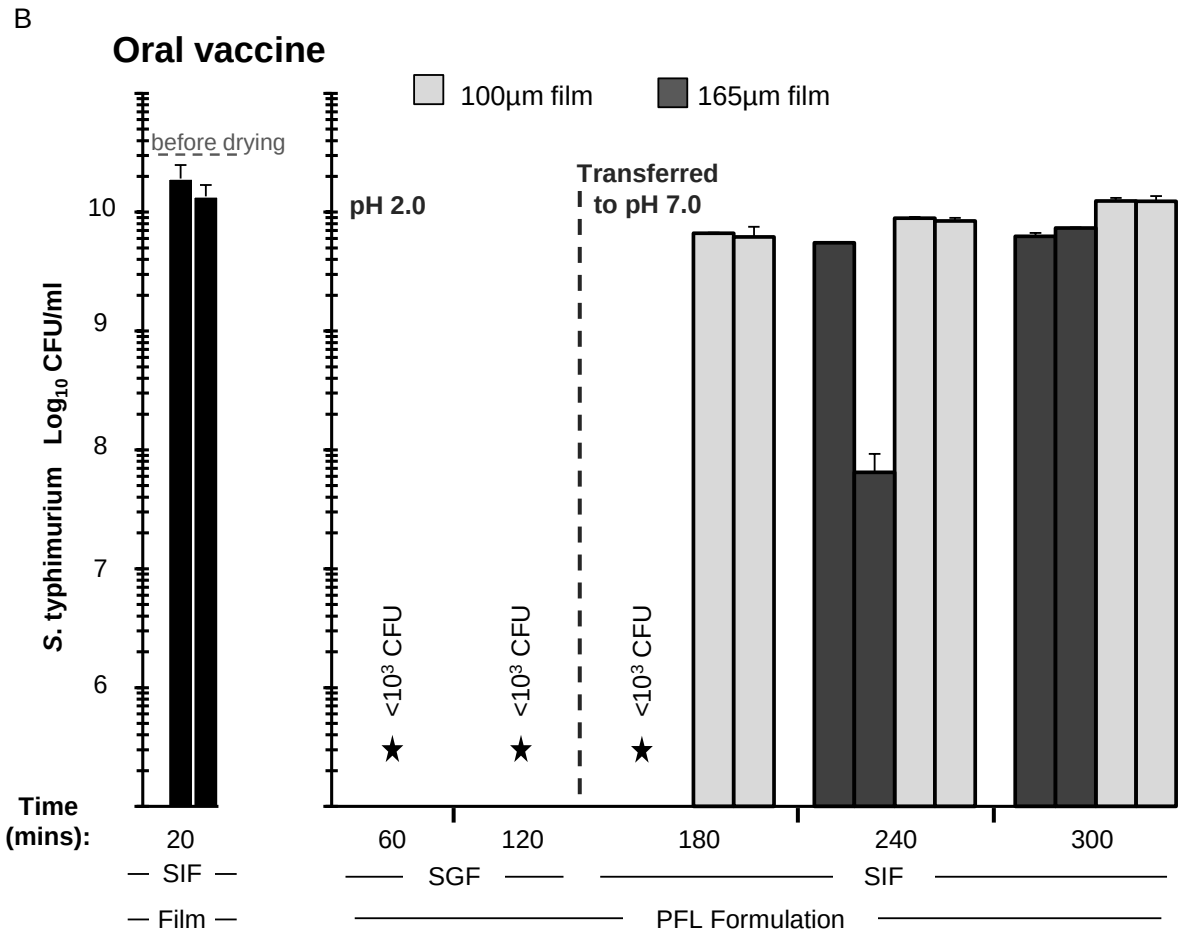


Figure 7


A
Probiotic
Multi Layer Tape
Multi Layer Edible Adhesive
Thick Film Edible Adhesive
Before drying
pH 2.0
Transferred to pH 7.0
B. breve Log10 CFU/ml
10
9
8
7
6
<10³ CFU
<10³ CFU
Time (mins):
20
60
120
180
240
300
SIF
SGF
SIF
Film
PFL Formulation


B
Oral vaccine
100µm film
165µm film
before drying
pH 2.0
Transferred to pH 7.0
S. typhimurium Log10 CFU/ml
10
9
8
7
6
<10³ CFU
<10³ CFU
<10³ CFU
Time (mins):
20
60
120
180
240
300
SIF
SGF
SIF
Film
PFL Formulation