

Lactoferrin-loaded Alginate Microparticles to Target *Clostridioides difficile* infection

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ABSTRACT

Some forms of bovine lactoferrin (bLf) are effective in delaying *Clostridioides difficile* growth and preventing toxin production. However, therapeutic use of bLf may be limited by protein stability issues. The objective of this study was to prepare and evaluate colon-targeted, pH-triggered alginate microparticles loaded with bioactive bLf and to evaluate their anti-*C. difficile* defence properties *in vitro*. Different forms of metal-bound bLf were encapsulated in alginate microparticles using an emulsification/internal gelation method. The microparticles were coated with chitosan to control protein release. *In vitro* drug release studies were conducted in pH-simulated gastrointestinal conditions to investigate the release kinetics of encapsulated protein. No significant release of metal-bound bLf was observed at acidic pH; however, on reaching simulated colonic pH, most of the encapsulated lactoferrin was released. The application of bLf (5mg/mL) delivered from alginate microparticles to human intestinal epithelial cells (hIECs) significantly reduced the cytotoxic effects of toxins A and B as well as bacterial supernatant on Caco-2 and Vero cells, respectively. These results are the first to suggest that alginate-bLf microparticles show protective effects against *C. difficile* toxin-mediated epithelial damage and impairment of barrier function in hIECs. The future potential of lactoferrin-loaded alginate microparticles against *C. difficile* deserves further study.

KEYWORDS

Lactoferrin, alginate microparticles, *Clostridioides difficile*, targeted drug delivery, gastrointestinal tract.

1. Introduction

Successful oral delivery of peptides and proteins requires protection of the macromolecules from loss of bioactivity or degradation in the gastrointestinal tract.^{1,2} Targeted delivery of drugs and proteins into the colon is important for local treatment of a range of diseases.³ Different approaches have been developed for colon specific drug delivery including coating with pH- responsive polymers, preparing timed-release formula, and colonic bacteria responsive degradation of formula.^{4,5} Among the aforementioned strategies, the use of pH-responsive polymers holds great promise. Various pH-triggered polymer polysaccharides have been studied for this purpose.⁶

Alginate microparticles possess some unique characteristics that enable these particles to be used for the controlled delivery of proteins. For example, alginate microparticles have excellent biodegradability, biocompatibility, and pH-responsive swelling behaviour. As a consequence, alginates are used in a number of pharmaceutical applications, but alginate particles in particular are used to control release of oral delivery of drugs and proteins.^{7,8,9,10,11,12,13} Alginate-derived polymers can easily be formulated into controllable diameter microparticles with appropriate size distributions for ingestion (~100 µm).⁷ These particles can be prepared by various techniques such as microfluidic fabrication,^{14,15,16,17} air atomization,^{18,19,20} emulsification/internal gelation,²¹ complexation with counterion polymers,²² spray-drying,²³ impinging aerosols,^{24,25} and inkjet/drying processes.²⁶ Among these techniques, emulsification/internal gelation methods have particular advantages for encapsulation of bioactive proteins due to the mild preparation conditions and potential for scale-up. However, a limitation of conventionally-prepared calcium-crosslinked alginate microparticles is the openness of their internal structure which can result in burst drug release profiles. Thus, alginate microparticles are mainly coated with other polycationic polymers to improve their mechanical integrity in the gastrointestinal fluids.^{27,28,29,30,31} Moreover, other studies have found that combination of alginate and other polymers during the gelation process is a promising strategy for gastro-protective delivery of bioactive molecules.^{13,32,33} In particular, chitosan-coated alginate microspheres are effective in protecting probiotic bacteria from gastric acid and bile salts.³⁴

In a recent study, we reported that some forms of bovine lactoferrin (bLf) are effective in substantially delaying *Clostridioides difficile* growth and preventing production of toxins in a human *in vitro* gut model of *C. difficile* infection (CDI).³⁵ Specifically, iron-saturated, but not iron-depleted bLf at a concentration of 5 mg/mL exerted this inhibitory effect. In separate batch culture experiments, iron-saturated bLf prevented *C. difficile* vegetative cell growth and toxin production, whereas iron-depleted-bLf and iron alone did not.³⁵ Furthermore, in comparison to iron-depleted bLf, iron-saturated bLf was observed to be more resistant to proteolytic degradation. It is well known that the therapeutic use of bLf may be limited by protein stability issues. For example, it has been reported that the absolute oral bioavailability levels of native Lf may be less than 1%, limited mainly by enzymatic degradation in the gastrointestinal tract and by its poor absorption across intestinal epithelium.³⁶ Thus, the objective of this study was to prepare and evaluate colon targeted and pH-triggered alginate microparticles loaded with bioactive lactoferrin and to evaluate subsequently their anti-*C. difficile* defence properties *in vitro*. Previous work has reported a non-toxic profile for various forms of bLf irrespective of their metal saturation status.³⁷ In this study, different forms of lactoferrin (iron-depleted; apo-bLf, iron-saturated; Fe-bLf, and manganese-saturated; Mn-bLf) were encapsulated in chitosan-coated alginate microparticles fabricated under mild conditions using the emulsification/internal gelation method.

2. Materials and Methods

2.1. Materials

Sodium alginate (Fisher Scientific UK, catalogue number 1046-8800, general laboratory grade, molar masses not defined), bovine serum albumin–fluorescein isothiocyanate conjugate (BSA-FITC), high molecular weight chitosan (>75% degree of deacetylation, molecular weight 310-375 kD) were obtained from Sigma-Aldrich UK. iron-saturated (85% Fe³⁺ saturated) bovine holo-lactoferrin (holo-bLf), iron-depleted (1% Fe³⁺ saturated) bovine apo-lactoferrin (apo-bLf), and manganese saturated bovine lactoferrin (Mn-bLf) were prepared according to the published procedure.³⁷⁻³⁸ Highly purified *C. difficile* whole toxins A and B toxinotype 0, strain VPI 10463, ribotype 087, were supplied by Public Health England. All other analytical grade reagents were used without additional purification. Deionized distilled water was used to make all solutions. Calcium chloride dihydrate (CaCl₂·H₂O) was purchased from Sigma-Aldrich UK and used as supplied. Dulbecco's modified eagle media (DMEM), 1% l-glutamine (2 mM), 1% antibiotic/antimycotic solution, 10% fetal calf serum (FCS), and 1 % non-essential amino acids were obtained from Sigma Aldrich (UK). The MTT cell metabolic activity reagent was purchased from Invitrogen. Trypan blue solution was obtained from Sigma Aldrich (UK).

2.2. Synthesis of bLf-loaded alginate microparticles

The emulsification/internal gelation method was used to prepare the protein-loaded alginate microparticles (Figure 1). A solution of sodium alginate (2% w/v) was first prepared in deionized water and after this, a suspension of 5% (w/v) of ultrafine CaCO₃ was added into the alginate solution. Then, a previously prepared lactoferrin solution in deionised water (1% w/v) solution was added to the above mixture and the resultant mixture was homogenised, before being dispersed into a mixture of paraffin oil and surfactant (Span 80 1% v/v) and stirred at 1000 rpm for 15 min using an IKA-Eurostar digital mixer with a Turbine stirrer. Following this step, a mixture of paraffin oil and glacial acetic acid (20 mL) was added to the above water-in-oil emulsion to reduce the pH of the mixture to ~ 5. Continuous stirring resulted in the production of microparticles, which were recovered from the oil phase by using a mixture of Tween 80 (1% v/v): CaCl₂ (0.05 M), distilled water, and acetate buffer at pH 5.5 as washing media. The microparticles were further rinsed until optical microscopy indicated that no more oil was associated with the separated particles.

2.3. Synthesis of chitosan-coated alginate microparticles

A chitosan solution (0.1% w/v) was prepared by dissolving chitosan (0.1g of a 310-375 kD molar mass range) in 100 mL of acetic acid (1% v/v) with continuous stirring for 30 minutes. Next, the pre-prepared alginate microparticles were added to the chitosan solution. The newly formed and surface-coated microparticles were transferred into a CaCl₂ solution (0.5%) and washed with deionized water repeatedly. The resultant microparticles were stored until further use.

2.4. Preparation of Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

A solution of SGF was prepared by dissolving sodium chloride (2g) in 1000 mL of water acidified with HCl to a pH of 1.2. Simulated Intestinal Fluid Test Solution was synthesised by dissolving monobasic potassium phosphate (6.8g) in water (250mL), then adding sodium hydroxide (0.2M, 77mL) and water (500mL). The pH of this solution was adjusted to 7.4 prior to use.

2.5. Determination of protein encapsulation efficiency of the microparticles

Protein-loaded microparticles (10 mg) were dispersed in PBS at pH of 7.4 (100 mL, containing 5% (v/v) ethanol) and stirred continuously for 24 h. UV-Vis spectrophotometry was used to determine the amount of free protein in supernatant via absorption at 450 nm. The protein content and loading efficiency was calculated as described by Bosio *et al* 2014¹² from experimentally determined calibration curves of protein content with UV absorption.

2.6. *In vitro* release studies

Simulated gastrointestinal conditions without enzyme (SGF pH 1.2 and SIF pH 7.4) were used to investigate *in vitro* release of encapsulated protein. Protein-loaded microparticles (10 mg) were added to SGF (pH 1.2, 10 mL) and stirred at 100 rpm for 2 h. Then, the mixture was centrifuged and separated microspheres were added into SIF (pH 7.4, 10 mL) and stirred for another 2 h. Samples were taken at different time intervals from both FGF and SIF solutions to determine the protein concentration spectrophotometrically. All assays were performed in triplicate. Protein release was calculated according to the following equation.

$$\text{Protein release \%} = \text{Mt} / \text{Mt}_0 * 100$$

Where Mt is the amount of protein at time t and Mt₀ is the amount of protein in the microparticles at time t = 0.

2.7. Growth of *C. difficile* *in vitro* and preparation of bacterial supernatants

We used *C. difficile* strain 027 (PCR Ribotype 027/toxinotype III) in this study. This strain was prepared at Maine Medical Center (Portland, ME, USA) and was kindly supplied by Dr Daniela Heeg, CHAIN Biotechnology. Spores were first grown on egg yolk agar/cycloserine/cefoxitin with lysozyme (CCEY/L) for 48 h, then transferred to a Columbia Blood agar plates. To prepare bacterial supernatants, the cultures of *C. difficile* were centrifuged at 10,000 g for 10 min and then filtered using 0.22-µm filter to remove bacterial cells. Both cultures and supernatants were stored at 4°C.

2.8. Cultivation of Vero and Caco-2 cells on culture flasks

Both Vero and Caco-2 cells were maintained in DMEM culture media, in a humidified atmosphere at 37°C and 5% CO₂. The medium was changed every 48 h. When the cells reached 70-80% of their confluence, they were split using trypsin to detach cells.

2.9. Cultivation of Caco-2 cells on transwell plates

Caco-2 cells were maintained in complete DMEM cell culture media. The media was supplemented with 2 mM L-glutamine, 0.1% penicillin-streptomycin solution, 10% fetal bovine serum, and 1% non-essential amino acid solution in a humidified atmosphere at 37°C and 5% CO₂. The cells were allowed to grow under the standard conditions until they reached 60–70% of their confluency. Cells from passages 60-80 were used in all experiments. The cells were seeded on permeable supports (Transwell, Corning, 1.12 cm² area, 12 mm diameter, and 0.4 µ m pore size), and the seeding density was 2×10⁴ cells/cm². The culture medium was added to the upper and lower compartments of the wells and the medium was changed every 48 h.

2.10. Measurement of the Transepithelial Electrical Resistance (TEER)

Barrier function integrity of the Caco-2 cell monolayers was assessed by measurement of TEER, using a portable epithelial Voltohmmeter (EVOM2, World Precision Instruments). Caco-2 cells were grown in the upper compartment of transwell inserts at 37°C, 5% CO₂. Cells were used for TEER measurements between days 14 and 21 post seeding. At the end of the growth period, plates were equilibrated at room temperature for 1 h. TEER measurement was conducted to evaluate possible damage to cell integrity of monolayer cultured cells during the experiments. The TEER values were determined from the potential difference between the upper compartment and lower compartment, connected to a pair of 'chopstick' electrodes. The cells were washed twice with phosphate buffered saline (PBS) and pre-equilibrated for 1 h with Hanks' balanced salt solution (HBSS) before performing TEER measurements. Thereafter, the media was removed and 200 µL of materials (toxins A or B, or/and final chitosan-coated alginate microparticles loaded with 5 mg/mL of bLf) were added to the upper (apical) compartments of the transwell inserts. Subsequently, TEER measurements were conducted at 4 h, 24 h and 48 h and recorded as electrical resistance at time 0. The electrical resistance for blank insert (no cells) and for each well was recorded.

2.11. Trypan Blue Exclusion Assay

Cells were incubated with trypan blue in a humidified atmosphere at 37°C and 5% CO₂ at a final concentration of 0.01% and visualised under light microscopy for determination of the numbers of viable (unlabeled) and damaged (blue) cells. Viability was denoted as the ratio of live cells per total cell number. After culture for 1 h, at least 200 blue cells per well were counted to determine the proportion of damaged cells since uptake of the dye was indicative of loss of membrane integrity.

2.12. MTT Assay

The MTT reagent was dissolved in PBS (pH=7.4) to prepare a 5 mg/mL stock solution, then sterilized through a 0.2 µm filter and stored at 4°C for frequent use, or at -20°C for long term storage. 96 well plates were used to carry out cell viability measurements. The final volume of test compounds and cells was 100 µL/well. The plates were incubated for 24 h in a humidified atmosphere at 37°C and 5% CO₂, after which MTT solution (10 µL/well) was added, such that the final concentration became 0.45 mg/mL. Thereafter, incubation was continued for another 1 to 4 h at 37°C. Finally, 100 µL of solubilisation solution was added to each well and the solution absorbances were measured at 570 nm.

2.13. Statistical analysis

Results are presented as mean ± SD of three separate experiments. GraphPad Prism 7 software was used to determine the significance of differences between the values. Data were analysed by using Analysis of Variance (ANOVA) with Tukey's HSD post hoc test.

3. Results and discussion

3.1. Material preparations

Protein-loaded alginate microparticles with sizes below 100 µm, spherical shape and smooth morphologies were prepared using an emulsification/internal technique. Thereafter, the newly formed particles were coated with high molecular weight (> 310 kDa) chitosan in order to reduce the release rate in simulated gastric conditions.. The mean diameter of uncoated microspheres was 96 µm but this value increased to 113 µm in the case of the chitosan

coated microspheres. Optical microscopy observations of microparticles confirmed that their spherical shapes and smooth topologies were not affected by chitosan coating. A measure of the loading efficiency and the distribution of protein within the microparticles was obtained initially using bovine serum albumin–fluorescein isothiocyanate conjugate (BSA-FITC) as a model therapeutic protein. As apparent from Figure 2 (right-hand fluorescence microscopy images) the protein was clearly present at the surfaces of the microcapsules and in regions extending into the interior. Typical protein encapsulation efficiency of the alginate microparticles ranged from 75-85%.

Release studies of bLf were conducted in SGF (pH 1.2, approximate pH of stomach fluid) and subsequently in SIF (pH 7.4, chosen to mimic the pH at the initial entry to the colon rather than in the distal colon where pH can be 7.8-8.5) to mimic the dynamic conditions of physiological gastrointestinal conditions (Figure 3). At pH 1.2 (SGF), a burst release profile of protein was observed during the first 30 min of the assay, with uncoated microparticles releasing 60-67% of encapsulated bLf protein at pH 1.2 with residual protein gradually released at pH 7.4 during the next 90 min. The release of protein was also of a 'burst profile' at pH 7.4 (SIF) during the first 30 min, then of a slower nature throughout the next 90 min. These initial release profiles were likely indicative of protein associated with surface layers of the microparticles rather than entrapped within the cores. The final amount of bLf released was strongly pH-dependent (Figure 3). This we attribute to pH-dependent differences in the electrostatic and hydrogen-bonding interactions of the alginate and chitosan-alginate matrices and bLf molecules. In SGF (pH 1.2), the bLf was well below its pI (~7.9) and thus highly protonated. Accordingly, exposing bLf loaded alginate microparticles to acidic media resulted in neutralization of the negative charge on alginate. This effect led to substantial weakening of the electrostatic interaction between bLf and alginate that in turn resulted in greater release of encapsulated bLf from the uncoated i.e. alginate-only microspheres at pH 1.2 compared to the chitosan-coated microspheres. The latter microparticles were expected to remain strongly protonated at pH 1.2, thereby retaining bLf in the chitosan-coated regions and reducing the burst release at this pH from the coated microspheres, as again apparent from Figure 3. Protein leaching from hydrogel beads due to reduced charge interactions has been widely reported previously.³⁹ At pH 7.4, the lactoferrin protein was close to neutral charge, while the alginate components were more negatively charged, reducing bLf-alginate charge-charge repulsion, but the additional factor of microsphere swelling from alginate-alginate charge-charge repulsion enhanced the release rate of the protein at this higher pH value (up to 100% within 2 h).

As noted above, chitosan-reinforced alginate microparticles showed delayed release of proteins at pH 1.2 and the amount of released bLf in SGF after 2 h was reduced to below 40%. Since chitosan was expected to be positively charged under acidic conditions, the reduced release profile may have been due to the charge effects as described above, but also a function of the hindered diffusion of bLf caused by the additional polymeric layer, which itself was partly charge-related. The release profile at pH 7.4 was similar to that observed for uncoated alginate microparticles, with a difference seen in the starting point. The overall release of the protein at the same time points was lower for the coated microparticles as expected. (Figure 3). These results demonstrated that alginate microparticles that were reinforced with chitosan offered more sustained gastrointestinal release of protein drugs. However, the incomplete bLf release from the chitosan-coated microparticles over the assay period suggested that some of the protein was entrapped in tightly charge-cross-linked-regions of the polyelectrolyte complexes between alginate and chitosan which did not dissociate even at the higher pH values of the SIF medium. Recent studies indicate that this relatively slower release profile can be extended over a time span of 20 h.⁴⁰

3.2. Cell culture studies

Epithelial cell culture integrity was monitored by measuring TEER values ($\Omega \cdot \text{cm}^2$) following treatment with the final chitosan-coated alginate microparticles loaded with bLf; decreasing values reflect increasing epithelial permeability resulting from membrane or tight-junction damage by the protein. The treatment of Caco-2 cells with different forms of encapsulated bLf (5 mg/ml) led to increases in TEER values as compared to treatment with toxin A (Figure 4a) or *C. difficile* strain 027 bacterial supernatant alone (Figure 5). These results suggest that bLf may have protective effects against *C. difficile* bacterial supernatant and toxin-mediated intestinal mucosal damage and impairment of barrier function in intestinal epithelial cells.

The effect of toxin B was more obvious on the metabolic activity of Vero cells. Therefore, an MTT assay was used to test cellular mitochondrial dehydrogenase activity, cell growth, and survival. There was a significant increase in cell viability, as measured by the MTT cytotoxic assay, in Caco-2 monolayers exposed to the higher concentrations (100ng/mL, 1000 ng/mL) of toxin A following treatment with chitosan-coated alginate microparticles loaded with lactoferrin (Figure 6a). Similar protective effects were seen with Caco-2 monolayers exposed to *C. difficile* bacterial supernatant (Figure 6b). Pre-treatment of Vero cell monolayers with all forms of encapsulated bLf followed by exposure to toxin B (Figure 4b) or *C. difficile* strain 027 bacterial supernatant for 24 h induced a dose-dependent fall in mitochondrial enzyme activity (Figure 6c).

Phase contrast microscopy studies demonstrated that *C. difficile* toxin A induced cell rounding in the Caco-2 cell lines. Cells within the detached monolayers initially remained adherent to each other (Figure 7). Subsequently, floating cells, smaller in size than those adherent to each other, appeared in increasing numbers with time. A similar pattern emerged for Vero cells exposed to toxin B or bacterial supernatant. Phase contrast microscopy images showed that detached Vero cells which had become detached from the culture dish seemed to be healthy while adherent to each other (Figure 8).

4. Conclusions

There is still limited information regarding the mode of action of lactoferrin and whether saturation of the protein with iron or manganese, or its encapsulation, effects its function. Currently, most of the *in vitro* digestion studies indicate that lactoferrin gets completely degraded during gastric passage and hence it cannot reach the absorption sites of the intestine.⁴¹ Therefore, encapsulation could be used to modulate the digestion behaviour of lactoferrin and hence achieve sufficient bioavailability through oral administration. The objective of this study was to prepare and evaluate colon-specific and pH-triggered alginate microparticles to carry and protect free and metal-loaded bLf preparations against enzymatic and acidic degradation or loss of bioactivity during gastric passage, to facilitate controlled colon release of protein, and to assess subsequently their anti-*C. difficile* defence properties *in vitro*. In this study, different forms of lactoferrin (iron-depleted; apo-bLf, iron-saturated; holo-bLf, and manganese-saturated; Mn-bLf) were encapsulated in chitosan-coated alginate microparticles fabricated using the emulsification/internal gelation method. We tested one concentration of bLf (5 mg/mL) in line with our previous research which demonstrated that this concentration inhibited *C. difficile* growth and toxin production in a human *in vitro* gut model of *C. difficile* infection.³⁵ Moreover, this same concentration was effective in significantly reducing the occurrence of antibiotic-associated diarrhoea in long-term care patients compared with placebo.⁴²

The biological activities of the various encapsulated bLf forms that differed in their metal saturation levels were evaluated on intestinal epithelial barrier function following interaction with *C. difficile* purified toxins and bacterial supernatants. The overall results suggest that hydrogel microparticles are suitable for capsulation and pH-triggered release of all forms of

lactoferrin, which may be advantageous for controlled delivery of lactoferrin into the colon. Cell culture studies showed that the application of lactoferrin-loaded alginate microparticles significantly reduced the cytotoxic effects of purified whole *C. difficile* toxins A and B as well as bacterial supernatant on hIECs, irrespective of metal saturation status. Although our findings suggest that encapsulated bLf appears to render intestinal epithelia less susceptible to *C. difficile* toxins and bacterial supernatant, it remains to be determined if encapsulated bLf can increase expression of tight junction proteins to enhance epithelial barrier function. Whilst recent research has shown that bLf decreases paracellular permeability and increases TEER through increased expression of claudin-1, occludin, and zonula occludens-1 tight junction proteins,⁴³ others have reported no change in the production of tight junction proteins regardless of lactoferrin metal saturation.³⁷ Moreover, it remains unclear if such effects observed in the present study can be reproduced *in vivo* using a murine model of CDI. Further laboratory studies will also be required to investigate the immunomodulatory, anti-inflammatory and cell-proliferative activities of encapsulated bLf at the mucosal level in animal experiments, and if the antimicrobial activity of bLf exerts its beneficial effects on the structure and function of the intestinal microbiota. Interestingly, Mn-bLf can deliver manganese ions to *Lactobacillus* strains, thereby enhancing their growth, although it is not known if the latter form of bLf can also effect the growth of *C. difficile* or other bacteria in the gut. Moreover, all forms of bLf (apo-, holo, and manganese-saturated) can inhibit secretion of pro-inflammatory cytokines from LPS-activated macrophages.³⁷ In addition, microencapsulation of *L. gasseri* and *B. bifidum* with alginate and chitosan coating can significantly improve bacterial survival in simulated gastrointestinal conditions, and allow viable cells to reach beneficial levels in the colon.³⁴

In conclusion, our results are the first to suggest that the chitosan-coated alginate microparticles loaded with bLf show protective effects against *C. difficile* bacteria and toxin-mediated mucosal damage and impairment of barrier function in hIECs. This may be especially advantageous since lactoferrin would be maintained intact under gastric conditions, thus reaching the lower gastrointestinal tract where it needs to direct its therapeutic effect against this recalcitrant anaerobe and its main virulence factors.

Conflicts of Interests

There are no conflicts of interest to declare.

Acknowledgements

This research was financially supported by an EPSRC Bridging the Gaps grant awarded to TM and CA in 2016.

CRediT author statement

Shwana Braim: conceptualization, methodology, investigation, validation, formal analysis, visualization and writing original draft preparation. **Klaudyna Śpiewak:** investigation, resources, reviewing and editing original draft. **Malgorzata Brindell:** investigation, resources, reviewing and editing original draft. **Daniela Heeg:** resources, reviewing and editing original draft. **Cameron Alexander:** reviewing and editing original draft, supervision and funding acquisition. **Tanya Monaghan:** conceptualization, reviewing and editing original draft, supervision and funding acquisition.

Data access statement

All raw data created during this research are openly available from the corresponding authors (cameron.alexander@nottingham.ac.uk and tanya.monaghan@nottingham.ac.uk) and at the University of Nottingham Research Data Management Repository (<https://rdmc.nottingham.ac.uk/>) and all analysed data supporting this study are provided as supplementary information accompanying this paper.

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Figure Legends

Figure 1

Schematic shows lactoferrin encapsulation in alginate-based microparticles and release in gastric and colonic fluid

Figure 2

Optical and fluorescent microscopy images of protein-loaded alginate microparticles before and after coating with chitosan. Scale bars are 100 μm .

Figure 1

In vitro release profile of different forms of lactoferrin in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF) for uncoated (blue line) and chitosan-coated (red line) alginate microparticles. Results are shown as the means \pm standard deviations derived from three independent experiments.

Figure 2

A. The effects of *C. difficile* toxin A on transepithelial electrical resistance (TEER) of cultured Caco-2 enterocytes at 48 h after treatment are reduced by exposure to bovine lactoferrin. The TEER is presented as percent change from baseline. Values represent mean \pm SE of at least 3 tissue culture wells derived from 3 independent experiments. **B.** Vero cell cytotoxicity assay. Vero cells after treatment with bLf and then subjected to different concentrations of toxin B, as measured by the MTT cytotoxic assay. Results are shown as the means \pm standard deviations derived from three independent experiments. **** = $p < 0.0001$; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$.

Figure 5

A. TEER% vs. time profiles observed for Caco-2 cell monolayers. **B.** Effects of different forms of metal bound and encapsulated bovine lactoferrin on the TEER (% relative to initial value) of polarized Caco-2 cell monolayers in the presence or absence of bacterial supernatant samples of the epidemic 027 *C. difficile* strain at 48 h. Values represent mean \pm SE of at least 3 tissue culture wells derived from 3 independent experiments. **** = $p < 0.0001$

Figure 6

Cell viability in Caco-2 monolayers incubated with different form of metal bound and encapsulated bovine lactoferrin and then exposed to **A.** *C. difficile* toxin A **B.** bacterial supernatant of epidemic 027 *C. difficile* strain. After culture for 24 h, the percentage of trypan blue unstained/viable cells was determined. **C.** Vero cell cytotoxicity assay. Vero cells after treatment with metal bound and alginate encapsulated bovine lactoferrin (Lf-alginate beads) and then subjected to bacterial supernatant of epidemic 027 *C. difficile* strain, as measured by the MTT cytotoxic assay. Results are shown as the means \pm standard deviations derived from three independent experiments. **** = $p < 0.0001$

Figure 7

Phase contrast photomicrograph of Caco-2 monolayer cells exposed to *C. difficile* toxin A and bacterial supernatant from 027 *C. difficile* strain. Toxin-exposed monolayer shows cell rounding and focal loss of cell-cell contacts. A significant proportion of the monolayer has also become detached from the bottom of the culture plate.

Figure 8

Phase contrast photomicrograph of Vero monolayer cells exposed to *C. difficile* toxin B and bacteria supernatant strain 027 201, PCR ribotype 027. Toxin-exposed monolayer shows cell rounding and focal loss of cell-cell contacts. Not evident from the Figure is the fact that a significant proportion of the monolayer has also become detached from the bottom of the culture plate by this time.