Title: Novel Lactoferrin-loaded Alginate Microbeads Display Anti-*Clostridium difficile* Defence Properties

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Introduction: We previously reported that some forms of bovine lactoferrin (bLf) are effective in substantially delaying *C. difficile* growth and preventing production of toxins in a human *in vitro* gut model of *C. difficile* infection (CDI). The aim of the present study was to develop lactoferrin-loaded alginate microbeads coated with high molecular weight chitosan for enhanced protein stability, and to subsequently evaluate their anti-*C. difficile* defence properties *in vitro*.

Methods: Different forms of bLf (iron-depleted; apo-bLf, iron-saturated; holo-bLf, and manganese-saturated; Mn-bLF) and BSA-FITC were encapsulated in calcium-alginate and chitosan-coated alginate particles. Microgel particles were fabricated using the emulsification/internal gelation method. Protein encapsulation efficiency was confirmed by fluorescence microscopy imaging of BSA-FITC-loaded hydrogel particles. *In vitro* release studies conducted in pH-simulated gastrointestinal conditions were employed to investigate encapsulation efficiency and release rate of encapsulated protein. The various encapsulated bLf forms were evaluated for their influence on intestinal epithelial barrier function and cell viability alone, and in combination with purified whole *C. difficile* toxins A and B or bacterial supernatant samples of the epidemic 027 *C. difficile* strain. Enterocyte viability and epithelial permeability were assessed using trypan blue exclusion, MTT cytotoxicity assay and changes in trans-epithelial electrical resistance (TEER) in Caco-2 cells, respectively.

Results: Alginate microparticles are suitable for encapsulation and pH-triggered release of metal-bound bLF proteins (Figure 1). The application of bLf (5 mg/mL) delivered from alginate microparticles to human intestinal epithelial cells (hIECs) significantly reduced the cytotoxic effect of toxin A and bacterial supernatant samples on Caco-2 cells, as illustrated by increased TEER values and enhanced Caco-2 cell viability. Pre-treatment of Vero cell monolayers with all forms of encapsulated bLf followed by exposure to toxin B or bacterial supernatant induced a fall in mitochondrial enzyme activity.

Conclusions: Our results are the first to suggest that alginate-bLf microparticles show protective effects against *C. difficile* toxin-mediated mucosal damage and impairment of

barrier function in hIECs. The future potential of lactoferrin-loaded alginate microparticles in the treatment and prevention of CDI deserves further investigation in preclinical studies.