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Research Article

Development of a novel mucosal multi-epitope subunit vaccine against *Clostridioides difficile*, incorporating recombinant Outer membrane Vesicles and spores

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ABSTRACT

Clostridioides difficile infection (CDI) remains a major global healthcare challenge, driven by antibiotic-induced gut dysbiosis, high recurrence rates, and the absence of an effective vaccine. Current toxoid-based vaccine strategies have failed to prevent intestinal colonization and initial infection, underscoring the need for approaches that induce robust mucosal immunity. Here, we propose a novel mucosal multi-epitope subunit vaccine (MEV) targeting both vegetative cells and spores of *C. difficile* that could possibly block colonization, transmission, and disease. The vaccine combines two complementary delivery platforms: recombinant outer membrane vesicles (OMVs) from *Escherichia coli* expressing the colonization factors FliC and Cwp2 for intranasal administration, and genetically engineered *Bacillus subtilis* spores displaying the spore protein CdeC and the surface lipoprotein CD0873, for oral delivery. This dual-route strategy is designed to stimulate strong mucosal secretory IgA alongside systemic IgG responses. Both OMVs and spores possess intrinsic adjuvant properties, enhancing antigen presentation and immune activation. In a murine CDI model, vaccine efficacy can be assessed by bacterial clearance, histopathology, and cytokine profiling. By targeting surface-expressed antigens rather than toxins alone, this MEV has the potential, we hypothesize, to prevent colonization and recurrence, offering a scalable platform for vaccines against other enteric pathogens.

Introduction

Clostridioides difficile is an anaerobic, spore-forming, toxigenic intestinal bacterium of humans and other animals, transmitted by the faecal-oral route [1]. *C. difficile* infection (CDI), an associated diarrhoeal disease, acquired in hospital and community settings, especially following antibiotic treatment and disruption of normal gut flora, is becoming a re-emerging global health concern.

Infection with *C. difficile* is one of the leading causes of healthcare-associated infections in the UK, adding up to 12 extra days to hospital stays [2]. The annual financial burden to the health care system in the US has been estimated to exceed $5 billion [3]. Unfortunately, the problem with CDI is that systemic antibiotic usage results in recurrent CDI (rCDI) and there is no vaccine available. *C. difficile* infection is a major contributor to morbidity and mortality in health care settings around the world. The global burden of *C. difficile* between 2005 and 2015, measured as the rate of health care facility-associated CDI (HCF-CDI) was 2.2 per 1,000 admissions/yr and 3.5 per 10,000 patient days/yr [4]. CDI had an estimated rate with onset in intensive care and internal medicine wards of 11.1 and 10.8 per 1,000 admission per year, respectively [4].

The main cause for developing dysbiosis of intestinal microbiota is the heavy usage of antibiotics. This renders patients susceptible to *C. difficile* and other intestinal pathogens. In the setting of inflammatory bowel disease, gut microbial composition influences susceptibility to CDI. Recently, Yang et al [5] suggested that intestinal colonization with *Veillonella* spp. in Crohn's disease may facilitate CDI by altering bile acid metabolism and enhancing spore germination. This may highlight a link between dysbiosis and infection risk [5]. Germination of *C. difficile* spores releases vegetative cells. These can then colonise the intestine, producing toxins that damage the epithelium. Furthermore, an impaired immune system brings about rCDI through spore germination, as

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C. difficile spores exhibit intrinsic antibiotic tolerance, surviving treatment without being genetically resistant; thereby allowing for later recurrence. This suggests that attempting to boost the immune response is a valid aim [6].

Microbiome-metabolome interactions are critical to CDI and vaccine development because they define whether the gut is resistant or susceptible to colonization, germination, and toxin production. The metabolites produced by a healthy gut such as bile acids inhibit *C. difficile*, but antibiotic-induced intestinal dysbiosis creates a metabolome that favours the pathogen. Integrated 16S rRNA sequencing and metabolomics studies highlight that susceptibility to CDI is driven as much by functional metabolic disruption as by taxonomic dysbiosis. Using combined microbiota profiling and untargeted metabolomics, Cheng et al. (2022) [7] demonstrated coordinated shifts in gut bacterial communities and metabolites in disease models, identifying strong correlations between expansion of opportunistic pathogens and accumulation of metabolites that promote inflammation and pathogen persistence, alongside depletion of secondary bile acids and short-chain fatty acids that normally suppress *C. difficile*. Similar multi-omics analyses in CDI and recurrence models show that delayed restoration of bile acid metabolism, amino-acid utilisation, and epithelial protective metabolites predicts disease severity and relapse more accurately than 16S data alone [8,9]. These findings support a microbiota-centred vaccine hypothesis in which effective *C. difficile* vaccines indirectly promote recovery of a protective metabolic landscape, by limiting colonisation and inflammation, thereby helping reinforce colonisation resistance and possibly reducing recurrence.

Most pathogens invade through mucous membranes. Lining the respiratory, intestinal and urogenital tracts, these act as the first line of immune defence. In continual contact with potentially pathogenic microorganisms, they are susceptible to infection [10]. Increasing evidence suggests that boosting the mucosal immune response and in particular the production of secretory IgA (sIgA), with its myriad of effector mechanisms, is essential for an effective *C. difficile* vaccine [11]. Vaccination against *C. difficile*, however, is fraught with difficulty; even in one of the latest, advanced phase 3 trials, a chemically modified toxin vaccine, in hospitalised patients aged over 50, despite providing a good immune response and being deemed safe, showed an efficacy of −5.2% [12]. Other trials suggest that inclusion of an adjuvant (which boosts the immune response) is needed [13].

Hypothesis.

We propose the generation of a mucosal multi-epitope subunit vaccine (MEV) targeting *C. difficile* vegetative cells and spores, to block intestinal colonization and infection. Despite high global burden to health, there are few approved vaccines preventing mucosal infections. To develop a successful mucosal vaccine requires: (i) selection of appropriate pathogen-derived antigens (ii) choice of delivery system and (iii) adjuvant to boost the immune response. A two-vaccine delivery system, by administering recombinant, Gram-negative Outer Membrane Vesicles (OMVs) expressing the chosen *C. difficile* vaccine candidate proteins and *B. subtilis* spores expressing *C. difficile* spore protein and colonization factor, will give a strong mucosal immune response. Going forward, such a combined vaccine, delivered intranasally and orally, is proposed, giving strong mucosal immunity as sIgA from salivary/stool samples, as well as strong systemic IgG [14]. As a caveat to that, sIgA titres alone do not reliably correlate with protection against CDI, functional toxin-neutralising antibody activity being more important. Effective immunity is likely multifactorial, requiring coordinated Th17/Treg regulation, robust mucosal innate responses, preservation of epithelial barrier integrity, and neutralization of toxins A and B. Additional correlates of protection include IL-17–driven promotion of neutrophil recruitment, maintenance of tight junctions and epithelial regeneration.

We hypothesise that a multicomponent *C. difficile* vaccine incorporating FliC, Cwp2, CD0873 and CdeC would provide broad, cross-strain protection by targeting conserved, non-redundant colonisation mechanisms. FliC (flagellin) is highly conserved among epidemic ribotypes and induces protective systemic and mucosal antibody responses while engaging innate immunity via TLR5, thereby limiting early motility-dependent colonisation [15]. Cwp2, a conserved S-layer–associated cell wall protein, is immunodominant during natural infection and vaccination induces antibodies that impair epithelial adhesion and reduce intestinal toxin and spore burdens [16]. CD0873 is a conserved surface lipoprotein essential for efficient intestinal colonisation; immunisation limits bacterial adherence, promotes mucosal IgA responses, and protects against lethal challenge in hypervirulent strain models [17]. CdeC will generate neutralising antibodies targeting the spore's outermost layer, preventing adherence to the intestinal lining and bacteria from establishing an infection [18]. Targeting these complementary stages of pathogenesis—motility, stable adherence and nutrient-linked persistence—will we hypothesise possibly reduce compensatory colonisation pathways and enhance long-lasting immunity compared with single-antigen or toxin-focused strategies. However, as antigen conservation and expression may vary across strains/ribotypes it should still be verified experimentally. The complexity of CDI involving spores, vegetative cells and toxins warrants a multicomponent approach to vaccine design. Compared to a simple spore-based or OMV platform alone, our vaccine by simultaneously targeting multiple stages of the infection cycle, should induce both systemic and local immunity, so enhancing the breadth of the immune response.

Unlike previous oral vaccines which required whole cell delivery (whether inactivated or attenuated), to be able to reach the small intestine and gut associated lymphoid tissue to induce mucosal immunity, the proposed mucosal multi-epitope subunit vaccine (MEV) targeting cell and spore surface, will provide a combined approach and be delivered orally and intranasally.

The hypothesis will be supported by data demonstrating a dual immune response (high sIgA and systemic IgG), measurable reduction in bacterial colonization and transmission, and functional improvements like toxin neutralization or better epithelial barrier integrity.

The hypothesis will be refuted if significant antigen variation exists between strains, if high antibody levels do not provide actual protection, or if the bacteria bypass the targeted mechanisms using different strategies.

Proposed evaluation

Components of potential *C. difficile* vaccines include bacterial or spore surface proteins (or toxins). The proposed MEV will comprise two *C. difficile* flagellar proteins, both colonization factors involved in adhesion of the bacteria to the host intestinal epithelial cells (FliC [15] and Cwp2 [16]), another colonization factor, a surface lipoprotein (CD0873) [17] and a spore protein (CdeC) [18], all immunogenic proteins being recognised on the surface of *C. difficile* by immune patient sera [19,20]. The vaccine will be exposed to two mucous membranes lining the nasal cavity and intestine. To reach the more challenging intestinal environment the *C. difficile* spore protein (CdeC) and lipoprotein colonization factor (CD0873) will be expressed on resistant spores from the non-pathogenic intestinal bacterium, *Bacillus subtilis*. For the nasal mucous membrane, the two flagellar proteins will be expressed in *E. coli* as recombinant fusion proteins, firmly attached to the surface of the bacteria and then rather than using the whole bacteria, Outer Membrane Vesicles (OMVs) released from the bacteria will be used as part of the vaccine. These OMVs have the added benefit of acting as natural adjuvants, boosting the immune response and ensuring that high levels of antibody (IgA/IgG) are produced [21].

Specifically, the vaccine would involve generating OMVs released from recombinant *E. coli* expressing immunogenic *C. difficile* protein antigens, FliC and Cwp2, as fusion proteins with carrier protein, AIDA-1 (surface-expressed Autotransporter Adhesin Involved in Diffuse

Adherence-1) using expression vector pAIDA1 (which expresses the *aid*A gene encoding AIDA-1) [22] (Fig. 1A). They will offer an intrinsic, enhanced antigen presentation and immune response through stimulation of uptake into Antigen Presenting Cells. There will also be an OMV-derived LPS:Pattern Recognition Receptor-stimulated release of proinflammatory cytokines, DC maturation and directing of an effective T cell response.

The second component of this two-vaccine delivery system, to enable it to reach the more challenging intestinal environment, would involve generating reproductively and metabolically quiescent *B. subtilis* spores, as an inert mucosal vaccine platform [23]. For this, the candidate *C. difficile* genes (*C. difficile* spore protein CdeC and colonization factor CD0873) would be cloned in-frame with the *cotY* gene in the shuttle vector, pDL. Inclusion of a peptide linker (GGGGS) would reduce steric hindrance and improve display, and the fusion proteins would be expressed in *B. subtilis* spores (Fig. 1B). Two days after inducing sporulation, mature recombinant spores, purified by washing can have presence of fusion protein confirmed by western blotting.

In testing the vaccine in a mouse model of infection, the spore version of the vaccine would be delivered orally and the OMV version, intranasally (Fig. 1C). A range of doses would be tested to determine the optimal dose (as number of OMVs or μg antigen) for spores. After primary immunization, one or two booster doses, 2–4 weeks apart will be given. A few weeks after the final booster, a challenge infection will be given by oral gavage with *C. difficile* spores from virulent strain, VPI 10463, the mice having been rendered sensitive to CDI with a cocktail of antibiotics, protection can be monitored. Effective *in vivo* assessment requires a multi-phased approach beginning 7–10 days post-challenge

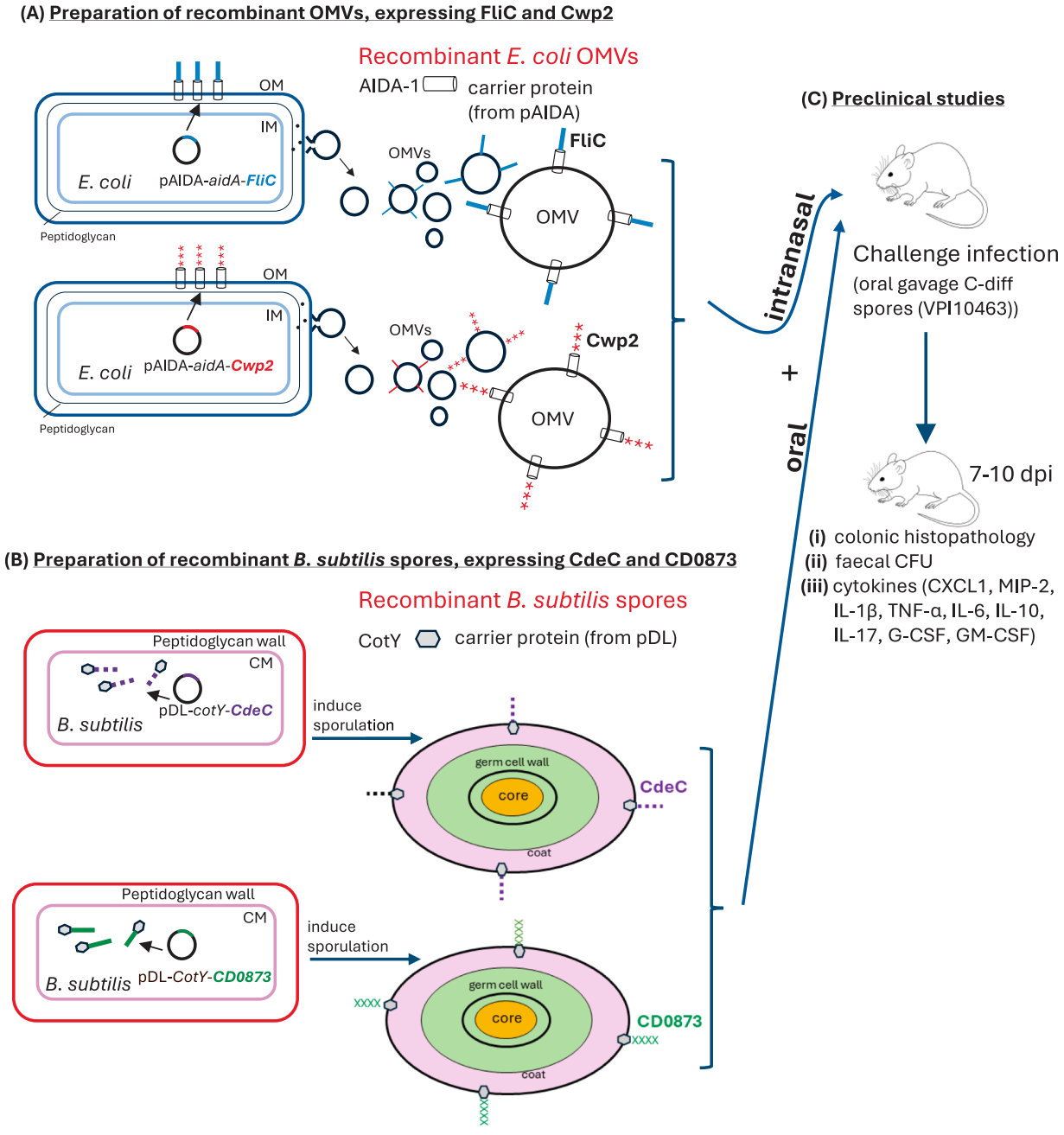

Fig. 1. Preclinical study design for OMV and *B. subtilis* spore-based vaccines. Vaccine Preparation will require generation of recombinant OMVs (FliC/Cwp2) and *B. subtilis* spores (CdeC/CD0873). The infection challenge will be by oral gavage with *C. difficile* spores in a murine model. The outcome analysis will involve evaluation of infection severity 7–10 days post-challenge through faecal bacterial burden (CFU), inflammatory cytokines, and histopathological changes in the colon.

infection, by measuring faecal colony units and colonic histopathology. Weight loss and survival rate will be measured and disease recurrence will be monitored as well as colonization for a further 8 weeks. The aim would be to assess whether the vaccine provides both immediate protection and long-term reduction in transmission. A multiplex analysis of cytokines would also be carried out to monitor for immune function (Fig. 1C). Strong salivary and faecal sIgA, as well as strong, systemic IgG generated would indicate an effective response to infection, promoting clearance of pathogenic *C. difficile*. The MEV, by targeting the cell, and spore surface and delivered to two mucous membranes lining the nasal cavity and intestine, will develop a strong mucosal immunity.

Recent *C. difficile* vaccines reached phase 3 clinical trials and were designed to induce antibody responses against *C. difficile* toxins A and B [24]. Although such toxoid vaccines may prevent life-threating manifestation of the disease such as sepsis, they are likely deficient in preventing *C. difficile* colonization and certainly were unable to prevent initial CDI. The proposed multi-epitope subunit mucosal vaccine, in targeting surface expressed antigens (on both vegetative cells and spores) will, we suggest, prevent carriage and possibly transmission and have a better chance of controlling CDI. These mucosal platforms hold the potential to supersede traditional parenterally administered toxoid-based vaccines, moving beyond their current role as parallel supplements.

Limitations

It is noteworthy, however, that while the multi-antigen, dual-route mucosal approach aims to broaden protective coverage, it introduces potential challenges such as immunodominance, where the response to one antigen overshadows others; antigenic interference, which may diminish overall potency; or the induction of mucosal tolerance, potentially blunting the desired systemic and local immune responses. It must be noted that the use of live, recombinant spores raises safety and regulatory concerns, related to environmental shedding, potential for persistence, and genetic modification. The main strategies to overcome this involve biological containment and spore inactivation [25]. For containment, *B. subtilis* suicide strains can be used that depend on thymine/thymidine, preventing survival outside controlled environments. For spore inactivation, spores can be chemically inactivated to prevent germination and shedding whilst preserving surface antigenicity.

The concept that intranasal immunization enhances intestinal immunity is justified by the Common Mucosal Immune System (CMIS). Intranasal immunization leverages the dual-route immunological rationale by activating the nasopharynx-associated lymphoid tissue (Waldeyer's ring). These activated lymphocytes then traffic via the CMIS to provide protective immunity at both systemic compartments and distal mucosal effector sites, including the gut. However, there may be difficulties also in terms of rapid clearance of particles from the nasal cavity and possible adjustments required such as a prime-boost strategy whereby the "priming" initial vaccine dose is given systemically, resulting in a robust memory T and B cell response, and the nasal administration is administered later as a booster. Intranasal administration of OMVs from *E. coli* will have certain safety implications, due of the presence of lipopolysaccharide (endotoxin) resulting in localised inflammation, and with intranasal administration meaning endotoxin can cross from the mucosa into the systemic circulation resulting in systemic toxicity (the so-called 'cytokine storm') and even neurotoxicity, entering the CNS through the olfactory or trigeminal nerve pathways. To mitigate for these risks we would propose dose control to determine the threshold at which useful mucosal IgA and systemic IgG titres are achieved without severe inflammatory pathology. The OMVs could also be derived from *E. coli* in which Lipid A of LPS has been genetically modified, for example using ΔmsbB or ΔlpxM mutants which produce the less TLR4-stimulating penta-acylated lipid A instead of the native *hexa*-acylated form, thereby reducing its reactogenicity (fever and systemic inflammation). OMVs from less endotoxic bacteria such as *Neisseria meningitidis* or *Acinetobacter* strains could also be used to lower the LPS load Furthermore, repeated mucosal dosing of these potent antigens carries immunological risks including immuno-tolerance ("oral tolerance") where there is hypo responsiveness due to the creation of T regulatory cells, exhaustion where T and B cells respond ineffectively to infection and disruption of the gut microbiome.

Ethics Statement

This article is a theoretical hypothesis and does not involve any experimental research on human participants or animals.

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Declaration of generative AI and AI-assisted technologies

Generative AI and AI-assisted technologies were not used in the preparation of this manuscript.

CRediT authorship contribution statement

Jameel Malhador Inal: Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Erica Muriana Tintor:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data sharing is not applicable to this article as no new data were generated or analysed in this study.

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