

**“Bugs For Drugs”: Encapsulation of a Cooperative Bacterial Consortia as a Therapeutic to
Resolve Recurrent *C. difficile* Infection**

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On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

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“Bugs For Drugs”: Encapsulation of a Cooperative Bacterial Consortia as a Therapeutic to Resolve Recurrent *C. difficile* Infection

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Abstract

Clostridioides difficile infection (CDI) poses a significant healthcare challenge, characterized by high recurrence rates and limited treatment options. This study aims to address this issue by proposing the development of an encapsulated biologic of cooperative bacteria as a novel therapeutic approach for CDI. Using computational modeling, a consortium of bacteria comprising *Bifidobacterium longum* ATCC 55813, *Escherichia coli* K12, *Roseburia intestinalis* DSM 14610, and *Streptococcus thermophilus* LMD-99 was previously identified as effective in combating CDI while preserving the gut microbiota. Subsequently, a method for the encapsulation of these bacteria in a biocompatible hydrogel matrix of norbornene-functionalized carboxymethylcellulose (cCMC) was developed and optimized to ensure stability and efficacy under physiologically relevant conditions. Stability studies, including exposure to varying pH levels, temperature, and enzymatic degradation, demonstrated the viability of encapsulated bacteria over time. Notably, the encapsulation method exhibited minimal degradation in acidic conditions mimicking the gastrointestinal tract. Future work will focus on refining encapsulation methods, optimizing the bundling matrix for ease of ingestion, and conducting further degradation studies to confirm the biologic's efficacy of release in the large intestine. Overall, the development of an encapsulated biologic offers a promising advancement in CDI therapeutics, with the potential to introduce new, improved treatment outcomes and alleviate the burden of this persistent healthcare challenge.

Keywords: *Clostridioides difficile* infection (CDI), Carboxymethylcellulose, Encapsulation, Microparticle

Introduction

Medical therapeutics have advanced greatly over the last few decades due to the innovation in research and technology that has been integrated within the biomedical field. The number of new drugs approved each year by the FDA is 60% greater than the yearly average in the previous decade¹. Despite this, treatments for infectious colitis, which causes swelling of the large intestine and numerous gastrointestinal issues², have remained unchanged for the last half-century. One of these diseases, caused by the bacterium *Clostridioides difficile* (*C. diff*), is an infection that is contracted primarily within healthcare-related settings. *Clostridioides difficile* infection (CDI) has an estimated prevalence of 400,000 cases annually, and growing incidence and mortality rates³. High-risk conditions for this infection include prolonged hospital stays, older age, weakened immune system, and, notably,

recent antimicrobial therapy. In most cases, healthy gut bacteria prevent *C. diff* from colonizing, but when broad-spectrum antibiotics are used to treat other health conditions, the normal gut microbiome is disrupted, increasing susceptibility to *C. diff* infection. Those who contract CDI face gastrointestinal symptoms, particularly diarrhea, stomach pain, and fever. Exacerbating its large healthcare burden and severity, CDI poses a unique problem due to its cyclical nature; it has high recurrence rates. Around 60% of CDI patients will contract it again in the two to eight weeks following recovery⁴. This disease also poses a large burden on the healthcare industry and a major risk to geriatric populations: one in 11 people over age 65 diagnosed with a healthcare-associated CDI die within one month⁵.

CDI is transmitted by the oral-fecal route through spores, which, in this case, are dormant cells resistant to many environmental factors, including some disinfectants. After passing through the stomach, spores germinate in the duodenum and continue to grow in the colon, where toxins trigger a complex cascade of cellular responses to cause symptoms and inflammation. The ability of spores to colonize the intestine is greatly influenced by the host microbiota, antimicrobial agents, immune system, and other metabolites⁶.

The first line of treatment for CDI is a collection of narrow-spectrum antibiotics, such as vancomycin, metronidazole, and fidaxomicin. Ironically, one of the most significant risk factors for acquiring CDI is recent antibiotic use, because it disrupts healthy gut microbiota communities vital for normal function, particularly of the adaptive immune system. Increased use of antibiotics can have a multitude of negative impacts, such as reduced microbiota species diversity, altered metabolic activity, and antibiotic resistance⁷. Although antimicrobial therapies may provide temporary relief, it is unsustainable, as they continue to create a high-risk environment for *C. diff* colonization. This creates a dangerous cycle of recurrence as well as an increased risk for patients in the hospital for unrelated conditions. After treatment of an initial episode of CDI, the chance of a recurrence within 8 weeks is 15–25%; for a patient with 1–2 previous recurrences, the risk of further recurrences is 40–65%⁷. Many patients receive the same antibiotics for CDI as the ones that were given 50 years ago⁸, which raises the question of why this field of disease has seemingly been left behind in the expanse of medical turnover that has been facilitated by technology. Current alternative therapies, such as fecal microbiota transplantation (FMT), rely on samples from fecal donors, which can be expensive and unappealing to patients. Additionally, there is no reliable, efficient, and systematic way to collect fecal samples at present, creating extra barriers to scaling these therapies to the level that is needed in the U.S. today. Only 2 alternative therapies have currently been approved by the Food and Drug Administration (FDA): Vowst and Rebyota. Both are capsules of fecal microbiota spores, with Vowst taken orally and Rebyota taken as a suppository^{9,10}. Despite research efforts, the scope of treatment for CDI remains limited. Without further innovation, patients, often in hospitals for unrelated health issues, will continue to contract CDI with high rates of recurrence and face intense symptoms without a source of relief.

This project addresses the needs within this field by designing a biologic of encapsulated cooperative bacteria to restore normal gut microbiota and treat recurrent CDI. Unlike current therapies, it does not work to kill a wide range of bacteria, both good and bad, within the gastrointestinal tract. Rather, this method uses a community of bacteria that *cooperate* with *C. diff* and heal patients by reducing its toxicity and replenishing the healthy, vital gut flora that once thrived.

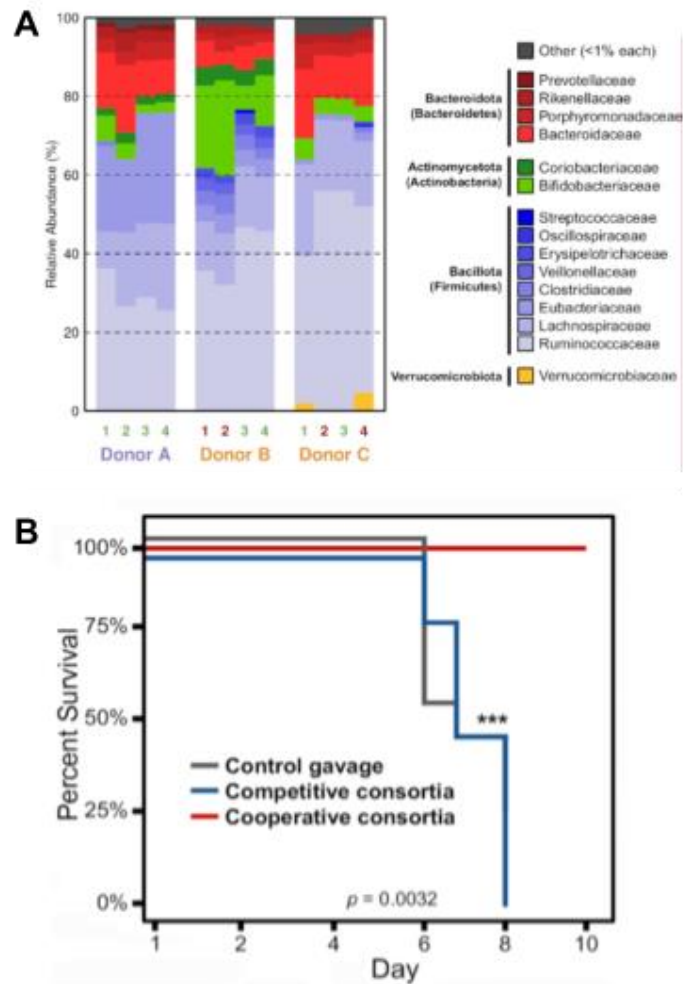


Fig. 1. Results of prior modeling work demonstrating project motivation. (A) Abundance of microbes within stool samples from donors. Purple: super donor, orange: regular donor. (B) Survival of cooperative vs. competitive consortia with *C. diff*

Prior work by the Papin lab at the University of Virginia has identified a cooperative consortium of bacteria that were found to be effective in resolving CDI and lowering toxin levels through initial sequencing studies from UVA fecal microbiota transplant samples (Figure 1a). The specific bacteria in this consortium are *Bifidobacterium longum* ATCC 55813, *Escherichia coli* K12, *Roseburia intestinalis* DSM 14610, and

Streptococcus thermophilus LMD-9¹¹. This study used a computational approach to identify an effective consortium. Using 12 samples from 3 donors for deep metagenomic sequencing, a modeling framework was created to predict interactions between microbes. Two types of interactions, cooperative and competitive, were identified. The cooperation was quantified through an iterative addition of metabolites to shared media, with the index represented as a ratio of the growth benefit of the recipient to metabolite export from a donor. The competition was quantified through a stepwise removal of substrates from rich media and indexed using the ratio of the important flux of a competitive metabolite specific to *C. difficile* growth. Through these computational models, it was found that *cooperative* communities protect mice from *C. difficile* symptoms and mortality (Figure 1b)¹¹. After these determinations, the safety of the aforementioned designated microbial community was validated, including an ongoing exploration of FDA Generally Recognized as Safe statuses.

Given that a significant amount of the patient population for CDI is geriatric, ease of ingestion and accessibility must also be considered. Many older patients find it difficult to chew and swallow consumables like pills and other medicines. Given this, our encapsulation will be designed such that it can easily dissolve within the mouth without chewing and will be easily swallowed with water. By creating this encapsulated biologic of cooperative bacteria, our project has the potential to produce significant improvements in the treatment of one of the most devastating healthcare-acquired infections. The ability to treat this viciously recurring infection permanently in an accessible, non-invasive, and affordable way could save a multitude of lives each year and ease the

burden of this disease. Beyond this, the design of an encapsulation method for targeting the delivery of a biologic introduces new research insights that can be used to inform future therapeutics and research technologies. To achieve delivery of the cooperative consortia of bacteria to the intestine, we chose to design an encapsulation and delivery strategy that utilizes hydrogel microparticles to carry and protect bacteria through the gastrointestinal tract. Hydrogels are highly versatile and tunable materials that can easily be made biocompatible and safe for *in vivo* use. They are often used for a broad range of clinical and therapeutic applications, such as wound dressing, injection into surgery sites, drug delivery, etc¹².

Our proposed mechanism of delivery involved encapsulating each species of bacteria in the consortia directly within hydrogel microparticles. These microparticles are then encapsulated in a different, water-soluble material that “bundles” the microparticles together into a hydrogel matrix in a form that resembles a thin “film”. To take the therapeutic, a patient would place the film on their tongue. When placed in the mouth, the water-soluble matrix material dissolves within the saliva, releasing the microparticles to be orally ingested. The size of the microparticles makes this ingestion easy, as the microparticles can be swallowed as one would swallow their own saliva. There is no need for chewing or swallowing a large pill/capsule. During travel through the upper gastrointestinal tract, the hydrogel microparticle material would protect the bacteria from conditions present in the esophagus and stomach. Upon arrival in the intestine, conditions present and specific to the intestine trigger degradation of the microparticles, releasing the bacteria to begin their therapeutic activity in the gut microbiome (Figure 2).

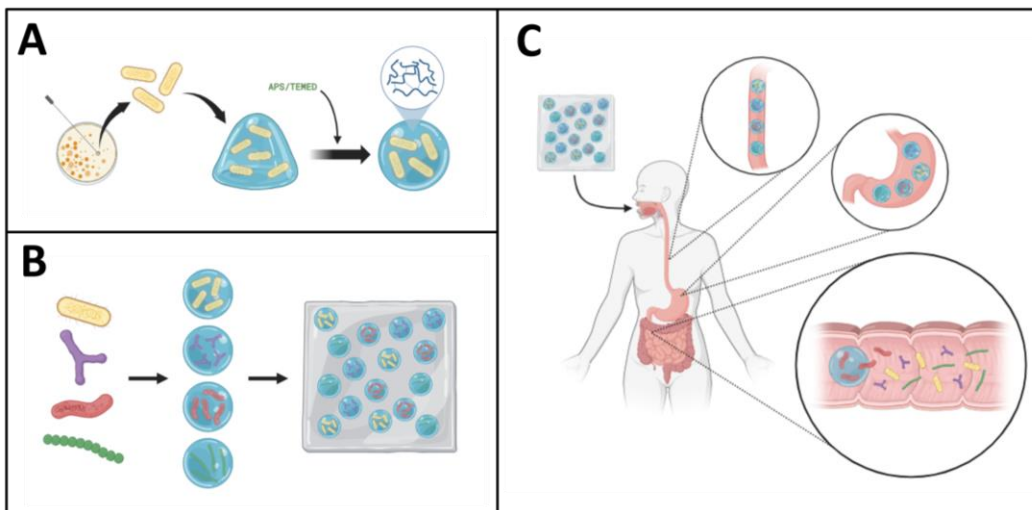


Fig. 2. Schematics depicting proposed method of delivery of the predefined bacterial consortia to the intestine via hydrogel encapsulation (A) Cultured bacteria being added to uncrosslinked hydrogel solution, then being crosslinked into hydrogel microparticles. (B) Encapsulation of the four bacterial species that make up the cooperative consortia into separate hydrogel microparticles, followed by “bundling” of the microparticles together into a singular therapeutic. (C) Oral delivery mechanism for proposed therapeutic that involved dissolution of the bundling matrix in the mouth, ingestion of microparticles, travel through the gastrointestinal tract, and degradation of particles that releases

Results

Bacterial Encapsulation

The success of the encapsulation and microparticle formation methods can be visualized in Figure 3 which depicts GFP tagged *E. coli* localized within carboxymethyl cellulose particles. Particle sizes ranged from 30-100 μm .

In Vitro Stability and Degradation Studies

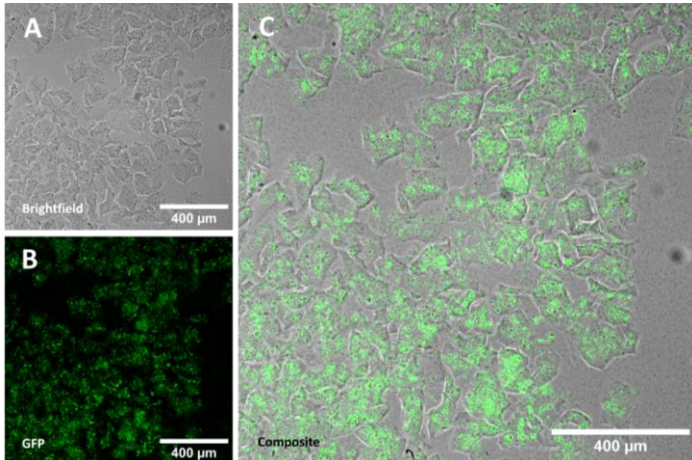
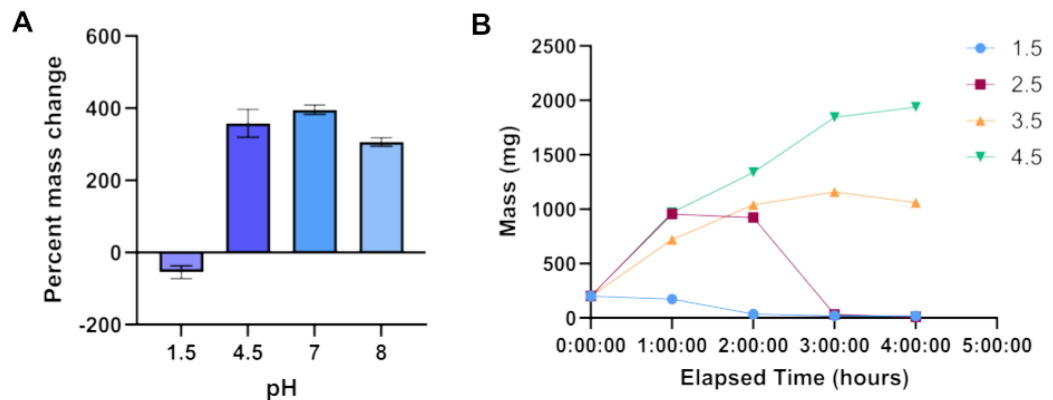


Fig. 3. GFP *E. coli* encapsulated within carboxymethyl cellulose hydrogel microparticles (A) Brightfield image depicting hydrogel microparticles (B) Fluorescent image depicting GFP *E. coli* present (C) Composite brightfield and fluorescent image depicting GFP *E. coli* encapsulated within hydrogel microparticles. All images were taken at 10x objective.

After four hours of incubation of the cCMC microparticles in water at room temperature (22 °C) and internal body temperature (37 °C), no significant difference was observed between the percent mass change of the hydrogel at the differing temperature conditions following repeated studies, with a p-value of 0.08 from Student's T-Test. Similarly, the particles were tested in water and pepsin solution, at a concentration of 0.75 mg/mL, mimicking that of the internal stomach

Fig. 5. pH stability studies. (A) Average percent mass change of encapsulated particles at pH 1.5, 4.5, 7, 8. p-value 0.0001 after conducting ANOVA and Brown-Forsythe test (B) Acidic stability test: mass over time of encapsulated particles at pH 1.5, 2.5, 3.5, 4.5. p-value <0.0001 after conducting two-way ANOVA.



environment¹³. Again, no significant difference was observed, with a p-value of 0.2168 following the Student's T-Test (Figure 4).

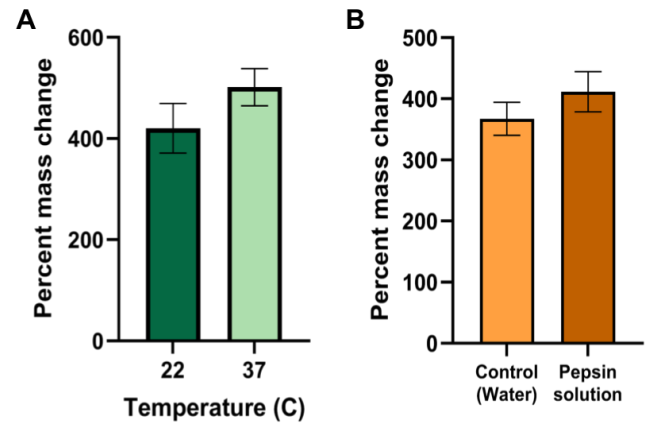


Fig. 4. Average percent change in total encapsulated particle mass at 37 °C, 22 °C, and pepsin (A) Temperature study conducted at 22 °C and 37 °C, p-value 0.08 after Student's t-test (B) Mass change of particles incubated in water and pepsin enzyme, p-value 0.2168 after Student's t-test

Figure 5a demonstrates the first pH study conducted, at pH levels of 1.5, 4.5, 7, and 8 over four hours. Mass loss occurred in the 1.5 pH level, with a percent change of -54.57%, indicating degradation at this pH. The difference between these samples was 0.0001 after conducting ANOVA and Brown-Forsythe test, indicating a clear significant difference. Figure 5b depicts a time-wise stability study conducted solely with acidic pH levels of 1.5, 2.5, 3.5, and 4.5, measuring the mass of particles every hour. Mass loss occurred after the first hour for the samples in the 1.5 pH solutions, and after the second hour for particles within the 2.5 pH solutions, again, indicating degradation at these conditions.

In all conditions except for 1.5 pH, swelling of the microparticles was observed, where the percentage or mass change of the sample is quite large.

Viability Studies

Following the stability studies, viability studies were conducted to confirm the viability of the bacteria within the microparticles. After degrading the cellulose and performing serial dilutions of the bacteria for CFU quantification, the growth of the bacteria can be seen for all temperature and pH levels of 1.5, 4.5, 7, and 8. Growth was observed at all dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) for every experimental group aside for 1.5 pH, in which growth only was visible at the 10^{-1} and 10^{-2} dilutions. The cultures became overgrown, preventing us from counting CFUs and accurately quantifying the recovery of viable bacteria. Still, this assay allowed us to confirm that viable and culturable bacteria can be recovered following the stability studies (Figure 6).

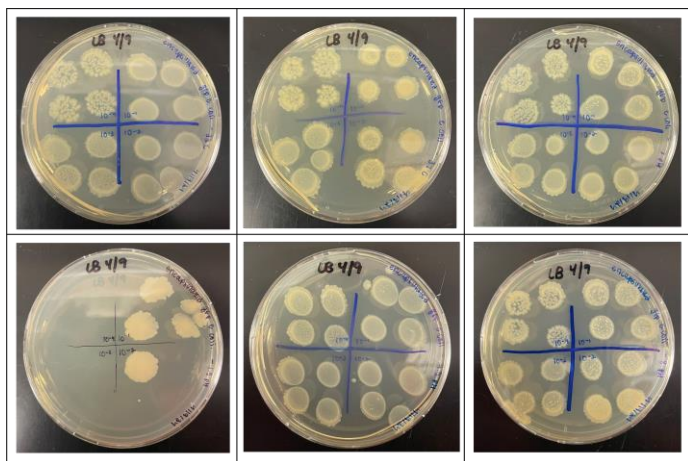


Fig. 6. Reculture of bacteria post-stability study at various conditions. (A) 22 C (B) 37C (C) pH 7 (D) pH 1.5 (E) pH 4.5 (F) pH 8

Discussion

The developed method of encapsulation is an efficient and relatively easy process. Synthesizing the gel initially as a bulk gel saves time and effort, and lessens the amount of equipment and material required. It also increases the reliability of crosslinking and homogeneity of microparticle material composition and properties. The fragmenting strategy developed that utilizes a syringe and a cell strainer mesh wastes very little material. When compared to other fragmentation strategies, such as mashing or homogenization, this strategy also limits mechanical pressure on bacteria cells, is very quick (seconds to minutes to create microparticles with one syringe), and does not require much exertion of force. Thus, fragmentation via syringe and cell strainer results in a higher percentage of the initially encapsulated bacteria being retained within the hydrogel microparticles. This allows for reliability in the number of bacteria within each

therapeutic and therefore, reliability in the dosage of the therapeutic.

When studying the behavior of the cCMC hydrogels in physiologically relevant conditions, including stability and degradation of the hydrogels, most of the groups exhibited swelling. The control (water at 22°C), 37 °C, pepsin, and pH 8, 7, and 4.5 groups exhibited percent mass increases of about ~300-500%. This swelling makes it difficult to fully understand the behavior of the hydrogel under varying conditions, and specifically, difficult to quantify any degradation that may have occurred. However, given that the encapsulation is formed from a hydrogel, it is logical that there is some uptake of water within the material. Previous literature has observed similar patterns when hydrogels are placed in aqueous solutions, leading us to believe that swelling can be associated with little to no degradation. Furthermore, there was no significant difference between these experimental groups and the control groups, supporting the conclusion that even if degradation did occur, it was not accelerated by exposure to the tested experimental conditions.

The cCMC hydrogel did exhibit degradation when tested in strongly acidic conditions. At 1.5 pH, no swelling occurred, and some mass loss of the hydrogel did occur within one hour. However, mass loss was not significant until after one hour. After four hours at 1.5 pH and the conclusion of testing, the hydrogel had degraded significantly, but not fully. At 2.5 pH, degradation occurred after two hours. However, in the first two hours, swelling was observed. At the higher pHs tested (3.5 being the lowest of these), only swelling was observed. We believe that this observed degradation at low pH levels, although statistically significant, does not indicate that the cCMC hydrogel is insufficient for the purpose of delivery. When considering the typical pH range of the stomach, 1.5 is the extremely low end¹⁴. Furthermore, food typically remains in the stomach for 40 minutes to 2 hours. 4 hours is near the maximum of what is considered a normal amount of time for food to remain in the stomach before moving on to the small intestine¹⁵. This means that significant degradation requires physiologically extreme conditions for extended amounts of time. In reality, when taken, the microparticles will likely not be exposed to such conditions, and less degradation will be observed.

Viable bacteria were recovered from the cCMC microparticles in every experimental group cultured, including the extreme condition of the 1.5 pH group.

Although the limited testing conducted within the time constraints could not quantify the viability, the existence of living cultures recovered from all experimental groups indicates a degree of success in protecting encapsulated bacteria from physiologically relevant conditions and maintaining the viability of the therapeutic agent.

Further testing is required to quantify the behavior of the cCMC hydrogel more comprehensively across conditions and the level of bacterial viability following encapsulation and exposure to physiologically relevant conditions. However, the data collected in these current stability and viability studies indicate that cCMC hydrogel microparticles can sufficiently withstand physiologically relevant conditions and preserve bacterial viability. Thus, this developed encapsulation material and method are suitable for the proposed delivery method.

Future Work

In future work, degradation studies must be performed to confirm the degradation of the material by trypsin found in the large intestine. In order to do this, a custom peptide crosslinker with the sequence “CGRRRGKC” will be used in place of DEG as a crosslinking molecule. When used as a crosslinker, this peptide has been shown to degrade upon interaction with trypsin. Trypsin is known to cleave at the C-terminal of arginine and lysine residues and each peptide link has four possible cleavage sites, as shown in Supplementary Figure 1¹⁶.

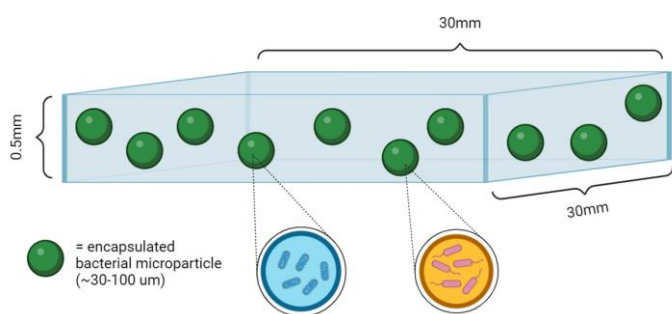


Fig. 7. Proposed final therapeutic design. Future completed therapeutic consists of bacteria encapsulated within particles within the bundling matrix.

Additionally, the encapsulated bacteria must be packaged together in a hydrogel-based bundling matrix that is water soluble and easily ingestible in order to form the completed therapeutic. Chitosan is a commonly used material to create hydrogels with properties that make it a promising candidate for this bundling matrix. Based on the average sizes of a human’s tongue and mouth, dimensions of 30x30x0.5 mm are suitable for the fully assembled

therapeutic (microparticles within a bundling matrix) (Figure 8). These dimensions would result in an overall “film” volume of 450 μL . We propose that each film of this volume contain 200 μL of cCMC microparticles and 250 μL of the bundling matrix material, as we believe this ratio will allow for the integrity of the bundling matrix and help prevent the film from breaking before being taken. With bacteria encapsulated at a concentration of $10^5/\text{mL}$ gel, a dose of 2 films would allow for a bacterial dosage consistent with typical probiotic dosing of 10^{10} viable organisms.¹⁷

Concluding Remarks

Current CDI therapeutic options are lacking in either efficacy, accessibility, or both. This project and proposed therapeutic addresses the deficiency in efficacy by utilizing a cooperative bacterial consortia as the therapeutic agent, and addresses accessibility by utilizing a delivery method of hydrogel encapsulation. Altogether, our work encompasses crucial first steps in the development of an effective therapeutic that addresses a critical need for better therapeutic options for CDI, especially for the largely geriatric patient population.

Materials and Methods

Functionalization and Synthesis of Hydrogel with Encapsulated *E. coli*

Norbornene-functionalized carboxymethyl cellulose (cCMC) was chosen as the hydrogel microparticle material for this encapsulation, as it is a versatile and robust polymer that can be used to create a hydrogel with tunable stiffness and degradation behavior¹⁸. Furthermore, the human gastrointestinal tract cannot naturally digest cellulose. This allows the material to be naturally durable upon exposure to degrading digestive enzymes in the mouth, esophagus, and stomach. cCMC was synthesized according to McOscar et al. cCMC hydrogels with encapsulated bacteria were made at 4 wt% cCMC (30% functionalized). Dry cCMC was dissolved in 90 vol% phosphate-buffered saline (PBS) containing suspended bacterial culture. Cultures of GFP *E. coli* were grown in 4mL of Luria Broth (LB), purchased from BD Biosciences, media overnight until they reached an optical density (OD) of approximately 1.0, which corresponds to $10^{10}/\text{mL}$. These cultures were centrifuged at 8500 rpm for 5 minutes following OD reading, and the supernatant media was aspirated, leaving behind solely the pellet of bacteria. This pellet was resuspended into the volume of PBS that was added to dissolve the cCMC, allowing the

bacteria to become integrated within the uncrosslinked hydrogel solution. An ammonium persulfate (APS) and TEMED system were used as crosslinking initiators. APS was added to the solution at 10 vol%, and a volume of TEMED was added corresponding to a 200:3 APS/TEMED ratio. A typical reaction of 1 ml of gel involved 0.04g cCMC, 0.9ml PBS, 0.1ml APS, and 1.5uL TEMED. 2,2'-(ethylenedioxy)diethanethiol (DEG, molar weight of 182.30 g/mol, specific gravity of 1.12) was subsequently added at a concentration of 1.9 μ L/mL of gel solution to target thiol:norborene ratios of 1:2. All gel solution reagents were purchased from Sigma-Aldrich. Gelation occurred approximately 30 minutes after the addition of the initiators (APS/TEMED) and the crosslinking molecule (DEG). Bacteria was encapsulated within the hydrogel at a concentration of 10¹⁰/800 μ L.

Formulation of cCMC Microparticles

To construct cCMC microparticles with encapsulated bacteria, a bulk cCMC gel was formed first with encapsulated bacteria. Then, the cCMC bulk hydrogel was extruded from a syringe through a 40 μ m pluriStrainer cell strainer twice (Figure 10). Forcing the gel through the mesh breaks the cell into micro-sized fragments, thus creating cCMC microparticles with encapsulated bacteria. Repeating this ensures the gel is broken into individual particles, rather than longer strands.

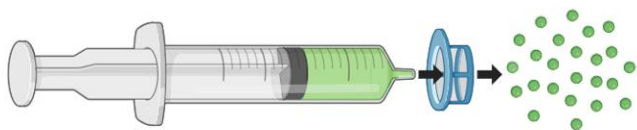


Fig. 8. Microparticle formation method. Fragmentation of a bulk hydrogel into microparticles via extrusion of the bulk gel from a syringe through a cell strainer mesh.

In Vitro Stability Studies

To confirm the stability of the hydrogel microparticles in physiologically relevant conditions, stability studies were performed at various temperatures, pH levels, and using pepsin enzymes. cCMC gel (200 \pm 5 mg) with encapsulated bacteria was used for each trial. For each condition, these particles were placed in 3ml of their respective solution, which was based in deionized water. The pH solutions were made using hydrochloric acid and sodium hydroxide to achieve pH levels of 1.5, 2.5, 3.5, 4.5, 7, and 8. The pepsin solution was created using pepsin powder at a concentration of 0.75 mg/mL, which mimics the pepsin concentration of the stomach. The temperature studies were performed at room temperature (22 $^{\circ}$ C) and internal body temperature (37 $^{\circ}$ C), in ultrapure deionized

water. All samples were incubated for 4 hours at room temperature, except for the 37 $^{\circ}$ C condition, which was maintained at 37 $^{\circ}$ C in an incubator. Following this, the samples were removed from their solution by placing the solution into a 10 μ m pluriScience cell strainer and suctioning using a syringe to remove any excess liquid. They were then weighed again to determine a percent change in mass. Additionally, a time-wise study was performed for a range of acidic pH samples: 1.5, 2.5, 3.5, 4.5. The procedure remained the same, except the sample was weighed and the solution was replaced every hour.

Viability Studies

After conducting the stability studies, the encapsulated bacteria were re-cultured to confirm viability after experimentation. To degrade the hydrogel and release encapsulated bacteria, the particles were incubated in a cellulase solution of \sim 3 mg/mL at room temperature for approximately five hours, which allowed for the deterioration of the carboxymethyl cellulose polymer. Following this, serial dilutions of the degraded particle solution were performed, up to 10⁻⁴, and plated on LB agar plates. They were allowed to grow overnight, and then colony-forming units (CFUs) were counted to quantify the viability of the bacteria post-stability study.

End Matter

Author Contributions and Notes

S.S. and G.H. worked collaboratively on all aspects of the project, such as material identification, method design, biomaterial synthesis, encapsulation strategy, and data acquisition. G.H. was the primary lead for statistical tests and biomaterial design. S.S. was the primary lead for bacterial culture and testing. Both performed research equally. The authors declare no conflict of interest.

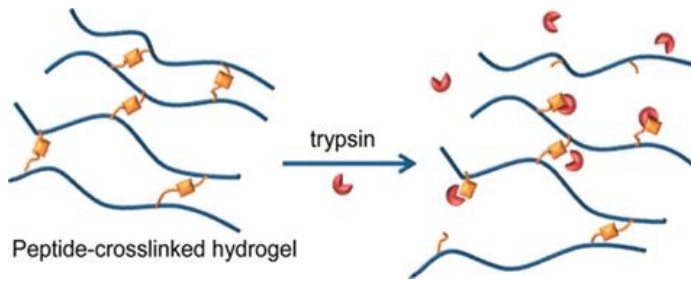
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Supplementary Information



Supplementary Fig. 1. Schematic demonstrating peptide-trypsin interactions resulting in cleavage of crosslinks (taken from Knipe et al¹⁶)