Using antibody synergy to engineer a high potency biologic cocktail against *C. difficile*

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ABSTRACT

We applied a mathematical framework originally used to model the effects of multiple inhibitors on enzyme activity to guide the development a therapeutic antibody cocktail, LMN-201, to prevent and treat *C. difficile* infection (CDI). CDI causes hundreds of thousands of cases of severe, often recurrent diarrhea and colitis in the United States annually and is associated with significant morbidity and mortality worldwide. Current therapies for preventing recurrent CDI are only partially successful, and there are no options available to prevent initial bouts of CDI in at-risk populations. Almost all antibody therapies have been developed and administered as monotherapies. Antibody cocktails are relatively rare even though they have the potential to greatly increase efficacy. One reason for this is our limited understanding of how antibody interactions can enhance potency, which makes it difficult to identify and develop antibodies that can be assembled into optimally effective cocktails. In contrast to the view that antibody synergies depend on unusual instances of cooperativity or allostery, we show that synergistic efficacy requires nothing more than that the antibodies bind independently to distinct epitopes on a common target. Therefore, synergy may be achieved much more readily than is generally appreciated. Due to synergy the LMN-201 antibody cocktail, which targets the *C. difficile* exotoxin B (TcdB), is 300- to 3000-fold more potent at neutralizing the most clinically prevalent TcdB toxin types than bezlotoxumab, the only monoclonal antibody currently approved for treatment or prevention of CDI. The efficacy of LMN-201 is further enhanced by inclusion of a phage-derived endolysin that destroys the *C. difficile* bacterium, and which therefore has a complementary mechanism of action to the antibody cocktail. These observations may serve as

42 a paradigm for the development of high potency biologic cocktails against targets that have
43 proven challenging for single-agent therapies.

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INTRODUCTION

Clostridioides difficile is a Gram-positive spore-forming anaerobic bacterium¹. *C. difficile* infection (CDI) causes severe, recurrent diarrhea and colitis and is associated with significant morbidity and mortality world-wide². According to the U.S. Centers for Disease Control and Prevention, *C. difficile* is the most common cause of health care-associated infections in U.S hospitals, where nearly half a million CDI cases occur each year³. Though traditionally considered a nosocomial infection, community-associated CDI is on the rise and now comprises most cases in some regions⁴. Treatment costs are estimated to exceed \$5 billion a year, but the true economic burden of the disease is far higher when lost quality-adjusted life years are considered⁴.

C. difficile virulence is mediated by expression of two homologous, multidomain exotoxins, TcdA and TcdB⁵⁻⁸. The toxins initially engage cell surface receptors on intestine epithelial cells, which results in internalization by endocytosis^{9,10}. Acidification of internalized endosomes triggers a conformational change in the toxin that promotes its translocation into the cytoplasm¹¹. Cytoplasmic toxin undergoes autoproteolysis¹², releasing its N-terminal glucosyltransferase domain that enzymatically inhibits Rho family-GTPases by mono-glucosylation^{13,14}. Inactivation of Rho disaggregates the cellular actin cytoskeleton thereby disrupting the colonic epithelium¹⁵⁻¹⁷, leading to severe inflammation and watery diarrhea characteristic of CDI. The relative importance of TcdA and TcdB for causing *C. difficile* pathology varies by host species, and current balance of opinion is that human pathophysiology is primarily caused by TcdB^{7,18-23}.

Antibiotic therapy is often successful for initial cases of CDI. However, approximately 20%-40% of CDI patients will have a recurrent episode²⁴, and the chance of additional episodes in those patients exceeds 40%^{25,26}. Microbial dysbiosis is a consequence of the antibiotics most often used to treat CDI, which creates a niche favorable to *C. difficile* proliferation²⁷ and promotes recurrent disease. Due to side effects and selection for antimicrobial resistance, antibiotics are no longer used prophylactically.

One alternative to antibiotics for treatment of microbial pathogens is passive immunization by direct infusion of neutralizing antibodies. Intravenous or intraperitoneal infusion of monoclonal and polyclonal antibodies targeting TcdA and TcdB are protective against primary infection and relapse in *C. difficile* challenge models in mice^{8,21,28,29}, hamsters,^{30,31} piglets^{22,32}. It is important that immunotherapies used clinically address the natural antigenic variation displayed by TcdB³³. Bezlotoxumab, a monoclonal antibody against TcdB, has been adopted for clinical use in conjunction with antibiotics for high-risk patients^{23,34}. However, it poorly neutralizes the prevalent TcdB2 variant, and clinically it is only partially effective in reducing recurrent CDI in humans. Oral administration of polyclonal antibodies from cows hyperimmunized with TcdA and TcdB has likewise been evaluated in human trials with reported success but has not progressed to commercial development³⁵⁻³⁷. Most recently, fecal microbiota transplantation (FMT) has also proven effective in reducing recurrence of CDI, presumably by helping to correct the intestinal dysbiosis that predisposes to relapsing disease³⁸. Despite these advances there currently are no clinically approved therapeutics to prevent primary CDI. There are no FDA approved CDI vaccines³⁹, and the most recent Phase 3 clinical trial of an anti-toxin vaccine was terminated due to futility⁴⁰.

We report here the preclinical development of a four-component therapeutic cocktail to both prevent and treat CDI. The cocktail is comprised of homodimeric versions of three toxin-binding proteins (5D, E3, and 7F) derived from camelid single-domain antibody fragments known as VHHs or nanobodies. Each protein was previously shown to inactivate TcdB^{8,28,29,41}. Binding of 5D to TcdB prevents its pH-driven conformational change that enables pore formation and transit into the cytoplasm from internalised endosomes. E3 binds to the glucosyltransferase domain (GTD) of TcdB, interfering with the autoproteolysis step necessary for enzyme activation. 7F also binds to the GTD, inhibiting catalytic glucosylation/inactivation of Rho-family GTPases by the mature N-terminal glucosyltransferase. The fourth cocktail component is a *C. difficile*-specific endolysin⁴². These proteins are highly specific, phage-encoded antimicrobial lytic enzymes that, due their specificity and rapid effect, hold considerable promise for treating bacterial infectious diseases, including CDI^{42–44}. We showed that the selected combination of three dimeric VHHs had a synergistic neutralization potency that vastly exceeded the potency of any single dimeric VHH and had far greater potency and broader strain coverage than bezlotoxumab^{23,34}. We further demonstrated that the therapeutic was enhanced by combining the dimeric VHHs that neutralize TcdB with an endolysin that destroys the *C difficile* bacterium. LMN-201 was manufactured using the edible microorganism spirulina (*Arthrospira platensis*) as the expression platform. It was delivered orally as a powder of whole, unrefined spirulina biomass⁴⁵, and is easily scalable, shelf-stable, simple to administer, and broadly neutralizing against all common TcdB types.

RESULTS

Spirulina strain engineering

The *C. difficile* therapeutic LMN-201 contained four therapeutic proteins: three homodimeric fusion proteins designated pp1005, pp1006, and pp1007, which incorporated homodimeric derivatives of VHs 5D, E3 or 7F, respectively; and fourthly, the catalytic domain of the bacteriophage-derived PlyCD endolysin, designated pp1092.

The primary structures of each monomer subunit in the VHH homodimers were similar. From amino to carboxy terminus this was: the maltose-binding protein (MBP) chaperone (366 amino acids)⁴⁶, a Gly-Gly-Gly-Gly tetrapeptide linker, a 5HVZ dimerization domain (49 amino acids), a Gly-Ser-Gly-Ser-Gly-Gly peptide linker, a TcdB-binding VHH (111-127 amino acids), a Gly-Ser-Gly tripeptide linker and a hexahistidine tag (543-562 amino acids total) (Figure 1A). The 5HVZ dimerization domain was derived from the mammalian cAMP-dependent protein kinase A (PKA) holoenzyme^{47,48}.

The endolysin PlyCD has been previously demonstrated to be *C. difficile* specific⁴²⁻⁴⁴. Full-length PlyCD (pp1093) its catalytic domain alone, PlyCD₁₋₁₇₄ (pp1092), were expressed in spirulina strains sp1286 and SP1287, respectively, with an N-terminal 6x his-tag (Figure 1A).

Genes encoding pp1005, pp1006, pp1007, pp1093 and pp1092 were engineered into the spirulina chromosome by homologous recombination (Figure 1B)⁴⁵. The final strains—SP1308, SP1312, SP1313, SP1286 and SP1287, respectively—contained the gene of interest integrated into the spirulina chromosome at a single, pre-selected location and no other exogenous

genetic information. Clonal spirulina filaments were then isolated by micromanipulation, verified as being homozygous with respect to the transgene, and used to create a frozen strain bank (Figure 1B, C)⁴⁵.

Characterization of spirulina-expressed anti-TcdB VHHs: expression and binding kinetics

Expression levels of all 5 candidate therapeutic proteins in spirulina were quantified using a capillary electrophoresis-based immunoassay (Figure 2A). After growth in a bioreactor, dried whole SP1308 biomass was comprised of 0.5% by weight pp1005; SP1312 was comprised of 2.3% pp1006; SP1313 was comprised of 3.4 % pp1007; and SP1287 was comprised of 0.5% pp1092. At least 90% of each of these proteins was soluble following a one-hour extraction in an aqueous buffer at neutral pH. The dimeric state of the VHHs on the 5HVZ scaffold was confirmed by comparing their relative mobilities during size-exclusion chromatography, and by capillary electrophoresis in reducing versus non-reducing conditions (Figure 2B). Intra-scaffold disulfide bonds were present in the extracted proteins (Figure 2B) but were dispensable for stable assembly of the 5HVZ-VHH dimers (Figure 2C).

Binding kinetics were measured by biolayer interferometry using recombinant subdomains of TcdB that corresponded to the specific antigen-binding sites of each VHH homodimer (Figure 3A, B). The apparent K_D 's were in the mid- to low-picomolar range. The dimeric versions of all three proteins displayed substantially greater apparent avidity for TcdB than the corresponding monomeric versions (Figure 3B).

Characterization of TcdB's effect on cell adhesion

Disruption of the actin cytoskeleton by TcdB causes changes in cell shape and cell attachment. *In vitro*, these cytoskeletal changes can be measured in real-time as a change in impedance using cells cultured on a surface embedded with microelectrodes^{49,50}. TcdB1 is the TcdB toxin type most frequently present in worldwide *C. difficile* isolates from infected patients⁵¹. The source of TcdB1 for these analyses was ribotype (RT) 087 (type strain 10463). The concentration range of TcdB1 tested spanned the amounts reported in human patients with *C. difficile* infection⁵². Data describing TcdB1 toxicity were collected and fit to a four-parameter sigmoid using nonlinear regression to minimize the sums of residuals squared:

$$Adhesion = Top - (Top - Bottom) \cdot \frac{T^H}{T^H + IC_{50}^H}$$

where Adhesion indicates the results of the above cell attachment assay; Top and Bottom are the highest (no TcdB1) and lowest (maximal TcdB1) assay values; T is the concentration of TcdB1; IC₅₀ is the concentration of TcdB1 eliciting half maximal inhibition in Adhesion; H is the steepness of the concentration-response relationship.

The concentration of half-maximal inhibition of cell adhesion by TcdB1 was 1.268 fM with a Hill slope coefficient of 1.495 (Table 1; Figure 4).

Synergistic Inhibition of TcdB by anti-TcdB VHH homodimers

Data were collected to evaluate inhibition of TcdB toxicity by each VHH homodimer, and all two- and three-way combinations (Figure 4A; Supplemental Figure 1). Individually, each of the dimeric VHHs had sub-nanomolar EC₅₀ values for TcdB neutralization (Table 1). However,

compared to the single VHHs, the cocktail of the three dimeric VHHs showed remarkable synergy, providing robust neutralization across the very wide range of TcdB concentrations found in patients with CDI⁵² (Figure 4; Supplemental Table 2). To illustrate, a concentration of 1.5 nM of any one of the three dimeric VHHs shifted the IC₅₀ of TcdB1 10- to 60-fold, whereas a 1.5 nM cocktail of all three combined (0.5 nM each) shifted the IC₅₀ of TcdB1 almost 3,000-fold. For TcdB2 the cocktail synergy was similarly striking. At a concentration of 1.5 nM the individual dimeric VHHs shifted the IC₅₀ of TcdB2 10- to 150-fold, while a 1.5 nM cocktail of the three dimeric VHHs (0.5 nM each) shifted the IC₅₀ of TcdB2 more than 16,000-fold.

Surprisingly, the dramatic synergy of antibody combinations requires nothing more than that they bind independently to distinct epitopes on the target. Quantitative modeling using measured K_d's and the effects of dimeric VHH mixtures across a large range of TcdB concentrations indicated that this was likely to be the entire explanation for the synergy we observed (Supplemental Text and Supplemental Figures 1,2,3). This suggested that synergy might be much more easily achieved than is generally appreciated, which has important implications for the development of therapeutic antibody cocktails (see Discussion).

Broad coverage of TcdB toxin-types

Mansfield et al.³³ used *C. difficile* genomic sequences to define 12 distinct TcdB groups that differed from each other by 3% or greater. 96% of the TcdB genes present in that dataset were defined as belonging to groups 1, 2, 3, and 5. Group 1 is the most clinically prevalent group worldwide, and group 2 is expressed by *C. difficile* ribotype 027, the single most prevalent strain type in the United States, and second most prevalent in Europe^{3,51,53,54}.

The high degree of conservation of the pp1005, pp1006, and pp1007 binding sites⁴¹ suggested that each of the dimeric VHs would neutralize most, if not all, of the four prevalent TcdB toxin types, except for neutralization of TcdB group 5 by pp1005. We measured the IC₅₀ values of TcdB types 1.1, 2.1, 3.1, and 5.1 alone and when incubated in 1.5 nM pp1005, pp1006, pp1007, or a cocktail comprised of all three VHH homodimers (0.5 nM of each) and compared these with the neutralizing potency of 3 nM bezlotoxumab (Figure 4). Each VHH homodimer exhibited strong neutralizing activity against all tested toxin types except pp1005, which was only weakly active against toxin type 5. The triple VHH cocktail synergistically neutralized all tested toxin types, with apparent relative potencies of against each toxin type of 2>1>3>5. Compared to the triple VHH cocktail, bezlotoxumab was significantly less potent at neutralizing all tested toxin types, and its neutralizing activity against toxin type 2 (ribotype 027) was notably weak.

Characterization of spirulina-expressed anti-*C. difficile* lysin

PlyCD, a prophage lysin, was found to have a high degree of specificity for degrading *C. difficile* peptidoglycan⁴². The catalytic domain of this protein, PlyCD₁₋₁₇₄, efficiently lysed various strains of *C. difficile* while leaving commensal bacteria unaffected, with rare exceptions that included *B. subtilis* and *C. sordellii*.

Genes encoding pp1093 (full-length lysin PlyCD) and pp1092 (PlyCD₁₋₁₇₄) were engineered into spirulina to produce strains SP1286 and SP1287, respectively. Expression of both proteins in spirulina was quantified using a capillary electrophoresis-based immunoassay. After growth in a bioreactor pp1093 comprised 0.1% of dry biomass and was approximately 50% soluble following aqueous extraction. pp1092 comprised 0.5% of total SP1287 biomass, and after

aqueous extraction greater than 90% of pp1092 was soluble (Figure 5A). Following extraction from SP1286 and SP1287 biomass the pp1093 and pp1092 proteins were found to be monomeric by size-exclusion chromatography and non-reducing PAGE (Figure 5B). However, pp1093 exhibited notable size heterogeneity, with lower molecular size species.

The lytic activities of pp1093 and pp1092 were compared in a turbidity reduction assay using extracts from SP1286 and SP1287, and *B. subtilis* as the target bacterium. In these spirulina cell extracts, the specific activity of pp1092 was 8-fold greater than pp1093 (Figure 5C). The reduced lysin activity of the pp1093 compared to pp1092 was also observed using *C. difficile* strain ATCC 43255 as the target bacterium (Supplemental Figure 4). The lytic activities of pp1093 and pp1092 isolated from spirulina were not significantly different from the same proteins isolated from *E. coli*. (Supplemental Figure 4). SP1287 (expressing pp1092) was selected for inclusion in LMN-201 based upon its superior expression level, homogeneity and bioactivity compared to SP1286. As a control for interference, it was shown that pp1092 did not affect toxin neutralization by the anti-TcdB VHH cocktail (Figure 4).

Stabilities of anti-TcdB VHHs and anti-*C. difficile* lysin

The four therapeutic proteins within LMN-201 all demonstrated resistance to three common intestinal proteases: trypsin, elastase, and chymotrypsin (Figures 6A, 7). When exposed to conditions simulating constitutive levels of these proteases for one hour, pp1005, pp1006, and pp1007 retained nearly 100% ELISA binding activity compared to undigested protein. At a higher concentration of protease that simulated the induced protease amounts following a meal, pp1005 and pp1006 exhibited some sensitivity to digestion with 0.1 mg/mL trypsin but

still retained 63% and 75% of their antigen-binding activities, respectively. SDS-PAGE and western blotting demonstrated that chymotrypsin cleaved the MBP domain of these dimeric toxin-binding proteins within 30 minutes, leaving the VHH-5HVZ dimer intact. This was consistent with the observation that chymotrypsin did not reduce binding affinity as measured by ELISA, and removal of the MBP moiety may explain the paradoxically increased ability to detect bound VHH in an ELISA after chymotrypsin digestion. To confirm that digestion did not affect bioactivity, chymotrypsin-digested pp1005, pp1006, and p1007 were also evaluated in a cell-adhesion assay. No significant changes in toxin neutralizing activity were observed after exposure to chymotrypsin for at least one hour (Figure 6B).

Protease treated pp1092 was analyzed for structural integrity by SDS-PAGE (Figure 7A), and for bioactivity in a turbidity reduction assay using intact *B. subtilis* as the substrate (Figure 7B). pp1092 showed no detectable change in molecular size (25kDa) following digestion with any of the three proteases. In contrast, full length PlyCD (37kDa) was almost 100% cleaved to a smaller 25 kDa product by all three proteases, consistent with release of the smaller, protease-stable catalytic subdomain. Further, pp1092 retained 100% bioactivity following digestion with any of the three proteases at the lower level, and greater than 70% bioactivity following digestion with higher (induced) protease amounts.

***In vivo* efficacy**

Efficacies of cocktail therapeutics containing anti-TcdB VHH homodimers and anti-*C. difficile* lysins were evaluated in mouse (Figure 8) and hamster (Figure 9) pathogen challenge models.

Cohorts of male C57BL/6J mice were infected with *C. difficile* spores 24 hours after administration of one of the following treatments by oral gavage: vancomycin (positive control); wild-type spirulina (negative control); Cocktail 1; or Cocktail 2. Cocktail 1 was comprised of equal proportions of spirulina strains SP1308, SP1312, and SP1313 (expressing pp1005, pp1006, and pp1007, respectively) and SP1286 (expressing pp1093). Cocktail 2 was the same but omitted SP1286. Each treatment was then administered once daily following *C. difficile* challenge.

The largest number of *C. difficile* spores was shed by mice treated with wild-type spirulina (Figure 8A). Treatment with Cocktail 1 significantly reduced fecal spore shedding at 24 hours post infection and at the time of euthanasia ($10^{2.206}$ and $10^{1.867}$ cfu/g lower, respectively), than wild-type spirulina controls and significant by t-test with $p=0.0175$ and $p<0.001$ (Supplemental Table 3). Fewer spores were observed shed by animals treated with only the toxin-neutralizing antibodies (Cocktail 2), but the difference did not reach statistical significance at either timepoint.

The largest maximum weight loss was observed in the negative control group, mice treated with wild-type spirulina, 3.922 g (Figure 8B; Supplemental Table 4). Treatment with either spirulina cocktail or vancomycin all reduced the maximum weight loss significantly. On average, the maximum weight lost by an individual treated with Cocktail 1 was 2.057 g less than the group treated with wild-type spirulina and significant by t-test with $p<0.01$. The maximum weight lost by subjects treated with Cocktail 2 was 2.102 g, a result significant by t-test with $p<0.001$.

Treatment with the spirulina therapeutic Cocktails 1 and 2 also increased survival rates, with borderline significance possibly due to small sample size (Figure 8C). None of the mice treated with Cocktail 1 perished due to *C. difficile*-related causes (4/4, 100% survival) which was equivalent to the vancomycin-treated group (10/10, 100% survival). The increased survival rate was borderline significant relative to the wild-type spirulina-treated group by log-rank test with $p=0.079$. The group treated with Cocktail 2 also exhibited higher survival rates than the negative control (6/7, 87% compared to 2/5, 40%) with borderline significance by log-rank test with $p=0.14$. The mouse efficacy study suggests that a spirulina anti-TcdB cocktail supplemented with the lysin is effective at reducing bacterial load and improving clinical outcomes *in vivo*.

The Golden Syrian Hamster model of CDI has been used previously for evaluating orally delivered antibodies against *C. difficile* toxins³⁰. The experimental groups we used were Cohort 1: no treatment, Cohort 2: wild-type spirulina, Cohort 3: anti-TcdB VHHs (3) + Anti-TcdA VHHs (2) + lysin PlyCD, all delivered within whole spray-dried spirulina biomass. Each treatment was administered twice daily by oral gavage, from days -1 through day 12. It was necessary to include anti-TcdA VHHs in this protocol because TcdA contributes significantly to disease in hamsters (unlike in humans)⁸. We observed that the therapeutic protein cocktail significantly extended survival time compared to the wild-type spirulina control ($p=0.015$) (Figure 9A), and significantly reduced *C. difficile* colonization of the cecum by more than an order of magnitude when compared to untreated or wild-type treated groups ($p=0.046$ and $p=0.0041$, respectively) (Figure 9B).

DISCUSSION

We report here the first use of quantitative analyses of drug synergy for preclinical and clinical development of a therapeutic antibody cocktail. These analyses build on a general framework for evaluating the effects of multiple drugs acting on a single target that was initially developed to model enzyme inhibition⁵⁵. In that context it was shown that mutually exclusive inhibitors (i.e., competitive) have an additive inhibitory effect, whereas mutually nonexclusive inhibitors (i.e., independent) have a multiplicative (i.e., synergistic) effect. This framework can be applied more generally, for example to antibody inhibitors of a protein⁵⁶, and has the potential to inform decisions around drug cocktail formulation, including selection of lead candidates and optimization of their relative concentrations.

Synergy is usually not considered in the development of cocktail therapeutics. To some degree this is due to the costs and complexities associated with developing drug combinations, but it is also because such synergies are often thought to be rare, arising from cooperative or unusual allosteric effects⁵⁷. A small number of anti-viral therapeutics have been intentionally developed as antibody cocktails^{58,59}, but this was done to reduce the likelihood of pathogen escape. Moreover, if drug concentrations are only evaluated within a 2- or 3-fold range of their respective EC₅₀ values, synergy can be difficult to distinguish from simple additivity, and is therefore easily overlooked. Accordingly, almost all clinically approved antibody cocktails were first developed as solo therapeutics, and subsequently only a small subset have been empirically evaluated in combination, often without regard to understanding whether underlying synergies might have contributed to improved efficacy.

In contrast to the prevailing view that synergy is rare and elusive we have demonstrated here, using the specific example of anti-*C. difficile* toxin antibodies, that synergy can simply be the result of the laws of mass action governing the independent interactions of drugs with a common target, and therefore may be more readily achieved than is generally thought. Moreover, the synergistic efficacy of independently acting drugs increases nonlinearly with concentration and can be enormous when concentrations substantially exceed the EC₅₀. We emphasize that this is not a complex function of allostery or cooperativity, but a simple consequence of independent Michaelis-Menten binding. This has general and important implications for therapeutic development. Synergistically acting antibodies will be particularly useful for treating a disease like CDI, where single antibody therapeutics struggle to achieve the very high level of target inhibition required. That the cocktail also offers broader strain coverage and protection against escape variants adds to its appeal.

Beyond infectious diseases, antibody synergy may also be important for treating diseases with complex etiologies where single-agent interventions have only been moderately successful, for example inflammatory diseases of the gastrointestinal tract and cardiometabolic disease. The results here offer antibody drug developers a paradigm for developing high potency cocktail-based interventions against diseases refractory to single-antibody approaches.

CDI is an appealing initial target for clinically evaluating these principles, because current therapies for preventing recurrent CDI are only partially successful, and there are no options available to prevent initial bouts of CDI in at-risk populations. LMN-201 is a spirulina-expressed and orally delivered cocktail of four therapeutic proteins that target both the *C. difficile*

bacterium and the key driver of its virulence, exotoxin TcdB. Orally delivered protein therapeutics may offer significant advantages over their injected counterparts for treatment and prevention of recurrent CDI. Oral administration can deliver high amounts of therapeutic proteins directly to the intestine epithelium, whereas systemically delivered antibodies have poor partitioning coefficients from the vasculature to the gut lumen⁶⁰. Moreover, the risk of off-target toxicity is reduced: the therapeutic proteins within LMN-201 are too large for intact systemic absorption⁴⁵. At least three prior clinical trials have reported safe and efficacious oral administration of bovine antibodies against *C. difficile* toxins derived from milk or colostrum^{35–37}, supporting the feasibility of this approach.

The harsh environment of the gastrointestinal tract is considered challenging for orally delivered protein therapeutics. LMN-201 addresses this challenge in several ways. First, the therapeutic proteins in LMN-201 showed significant resistance to the major GI proteases. Second, for human administration the product is delivered orally in polymer-coated capsules designed to release the drug product in the ileum. Third, spirulina-based cGMP manufacturing is highly scalable, making feasible cost-effective administration of the drug product throughout the period of high risk for CDI recurrence, even if the harsh GI environment necessitates daily or twice daily administration to maintain therapeutic levels at the site of action⁴².

Perhaps of greatest significance is the extraordinary potency of LMN-201: each dimeric VHH within LMN-201 had a sub-nanomolar EC₅₀ for TcdB inhibition, and this was substantially enhanced by cocktail formulation. An oral dose of 1,500 mg of spirulina biomass (500 mg of each VHH-spirulina strain) will deliver to the intestine a combined dimeric VHH concentration of

approximately 2.6 μ M, which is more than 1,000-fold higher than minimally required^{61,62}.

Pharmacokinetic data from a Phase 1 trial of LMN-201 confirmed that the amount of bioactive VHH recovered in the distal ileum following oral administration was more than sufficient to neutralize TcdB-levels previously reported in patients at risk for CDI recurrence (manuscript in preparation).

The fourth LMN-201 component—a bacteriophage-derived endolysin—not only broadens *C. difficile* strain coverage but also enhances efficacy. While TcdB is the key virulence factor in CDI, approximately 5% of patients are infected by *C. difficile* strains that secrete TcdA but not TcdB⁵². Other virulence factors are also associated with CDI, including the *C. difficile* binary toxin (CDT), which is associated with increased 30-day mortality⁶³. Inclusion of the lysin may offer protection against these non-canonical *C. difficile* strains, as well as novel strains with new toxin variants that might emerge in the future.

The lysin may also increase therapeutic potency in at least two ways. First, clinical performance is augmented by the lysin's previously demonstrated synergy with standard-of-care vancomycin, which act in concert to disrupt *C. difficile* cell wall structure⁴². Second, these agents independently intervene at two steps in the pathway that controls virulence—*C. difficile* proliferation and toxin action on the gastrointestinal epithelium. The lysin thus complements the VHH homodimers with a potential for orthogonal synergy to enhance LMN-201's clinical real-world effectiveness.

MATERIALS AND METHODS

Engineering of spirulina

Markerless spirulina strains were generated as described⁴⁵. In brief, the natural spirulina gene WP_006618409.1, which confers resistance to kanamycin, was replaced with an exogenous gene encoding resistance to streptomycin to generate spirulina strain SP205. SP205 was then transformed with constructs containing the gene of interest, WP_006618409.1, as well as the homology arms. The transformants were selected with kanamycin. Single filaments were isolated and propagated to generate cell banks. Cells were then thawed from the cell banks and DNA was extracted to be used to confirm complete segregation and verify sequence of the WP_006618409.1 via PCR and Sanger Sequencing.

Illumina sequencing of spirulina

Spirulina genomic DNA was extracted using a Blood and Tissue Kit (Qiagen, 69504) with minor modifications. Spirulina biomass was resuspended in buffer ATL. It was digested with proteinase K (2 mg/mL) overnight at 56 °C and then treated with RNase A (10 mg/mL) for 20 minutes at 56 °C. 400 µL of buffer AL was added and the biomass was pelleted for 10 minutes at 13,000g. The supernatant was then transferred to a fresh tube with 250 µL of 100% ethanol, mixed and applied to DNeasy columns. The samples were washed once with 500 µL buffer AW1 and once with 500 µL AW2 and eluted using molecular grade water. The amount of genomic DNA was quantified by a Qubit dsDNA HS Kit (Thermo, Q33230) and prepared for Illumina analysis using the Illumina DNA prep kit (Illumina, 20018704). Illumina sequencing of

the spirulina genome was performed on a MiSeq instrument using a MiSeq Reagent Kit v2 (Illumina, MS-102-2003).

Illumina dataset analysis for copy number determination

Copy numbers of the genes of interest in SP1287, SP1308, SP1312, and SP1313 were determined by comparing expression of the transgenes to 10 randomly selected genes (referred to as “normalization genes”: *ppaX*, *rpsA*, *chlM*, *degU2*, *radA*, *rbcl*, *sdhA*, *psbC*, *cysA1*, *smc6*) in the genome. A copy number of about 1, within plus/minus 0.15, was expected and set as the benchmark for a full genomic integration of 1 gene per genome copy. As a validation control, we also chose a second set of 10 random spirulina genes (*thrB1*, *hisC1*, *isfD2*, *pilA*, *purB*, *apcE*, *nblA*, *gpmB*, *bicA2*, *cpcBA*) which we compared pairwise to the “normalization genes” set in a manner identical to the transgene. Gene copy number per genome was determined by first using samtools (<http://www.htslib.org/doc/>) to generate an mpileup file with Illumina reads per base pair. Then, the median read per base pair was determined across the 10 “normalization genes” and the transgene. The median read per base pair of the transgene was then divided by the 10 “normalization genes” median read-per-base-pair value.

Preparation of spirulina extracts for analysis of soluble protein

Soluble extracts from spray-dried or paste samples were prepared by bead beating in a Percellys evolution bead beater (Bertin Technologies). Beads for this procedure were purchased in pre-packed tubes from MP-Bio (cat# 6911500). Dried spirulina biomass was resuspended in PBS containing Pierce Protease Inhibitor minitabets and 1 mM PMSF at a biomass concentration of 2-10 mg/mL. Biomass samples were then centrifuged at 15,000g at 4 °C for 30

minutes, and the soluble fraction was transferred to a separate tube for downstream applications.

Expression analysis of soluble recombinant proteins in spirulina

Recombinant protein expression in spirulina was measured by capillary electrophoresis immunoassay (CEIA) using a Jess system (ProteinSimple). The Jess system was run as recommended by the manufacturer. Briefly, dried biomass samples were diluted to a concentration of 0.1-1 mg/mL using water and a 5X master mix prepared from an EZ Standard Pack 1 (Bio-Techne). Purified protein controls used to generate standard curves were typically loaded at a range of concentrations from 0.25-8 µg/mL. A 12-230 kDa Jess/Wes Separation Module (ProteinSimple) was used for separation. A mouse anti-His-Tag antibody (GenScript) was diluted 1:100 and used as the primary antibody. An anti-mouse NIR fluorescence-conjugated secondary antibody (ProteinSimple) was primarily used for detection. Non-reducing denaturing gels were performed in the same manner; however, reductant was withheld from the 5X master mix. Data analysis was performed using the Protein Simple Compass software and Microsoft Excel.

Expression analysis of total recombinant proteins (soluble and insoluble) in spirulina

Spray dried or paste samples were prepared by bead beating as above. The samples were solubilized in PBS buffer mentioned above with a Triton X-100 concentration at 0.02%. Following lysis, samples were diluted to 0.1-1 mg/mL in extraction buffer (60 mM dithiothreitol, 60 mM Na₂CO₃, 6 mM ethylenediamine tetraacetic acid, 2% (w/v) sodium dodecyl sulfate, 12% (w/v) sucrose and 0.02% Triton X-100). After the addition of the lysis buffer, samples were

immediately heated at 100 °C for 120 seconds, then centrifuged at 16,000g for 5 minutes.

Extracted samples were displayed on the Jess system as described above.

Cell lines, bacterial strains, toxins, and antibodies

Vero cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Gibco). The cells were grown in a high-humidity incubator at 37 °C and 5% CO₂. *C. difficile* toxin TcdB-10463 was expressed in *B. megaterium* (a gift from C. Shoemaker, Tufts University) and purified using Ni-NTA affinity and ion exchange chromatography. Purified *C. difficile* toxins TcdB-027, TcdB-1470, and TcdB-078 were purchased from tgcBIOMICS. Bezlotoxumab was sourced from Henry Schein Medical.

Expression of TcdB fragments in bacterial systems

The VHH-binding domains on TcdB⁴¹ were designed for bacterial expression with an N-terminal hexahistidine tag followed by MBP and TEV protease recognition site (ENLYFQG) and C-terminal Avi-tag (LNDIFEAQKIEWHE) for enzymatic biotinylation. Based on the structural information, we designed the 5D-binding region, the delivery and receptor-binding domain (DRBD) of TcdB for expression using residues from amino acid position 1092 to position 1433 of TcdB. VHHs 7F and E3 bind regions on the glucosyltransferase domain (GTD). A fragment containing this region was designed to include amino acid positions 2 through amino acid 543 of TcdB. The constructs were subcloned into a modified pET28 b(+) vector where the Kanamycin bacterial resistance gene was replaced with Ampicillin bacterial resistance gene. Constructs were sequence verified by Sanger sequencing (Genewiz). Sequence verified plasmids were transformed into BL21(DE3)

461 *Escherichia coli* cells (New England Biolabs) and plated on LB medium supplemented with 100
462 $\mu\text{g mL}^{-1}$ ampicillin. Protein was expressed using autoinduction protocol⁶⁴. Briefly, 1 L of Super
463 Broth media containing appropriate selection drug was inoculated with overnight cultures from
464 individual transformants, shaken at 37 °C for 8 hours followed by 16 °C for 24 hours. Expression
465 cultures were pelleted by centrifugation and stored at –20 °C until purification. Frozen cell
466 pellets were thawed on ice and resuspended in 100 mL of 50 mM Tris pH 8.0, 300 mM NaCl
467 supplemented with pierce protease inhibitor tablets EDTA free (Thermo Fisher Scientific cat. no.
468 A32965) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Thermo Fisher Scientific cat. no.
469 36978). Cells were lysed by high pressure homogenization (HPH) (Microfluidics LM20) at 12,000
470 psi. The lysates were clarified by centrifugation at 18,500g at 4 °C for 30 minutes using tabletop
471 centrifuge (Eppendorf Centrifuge 5810R). Soluble protein was separated from cell debris and
472 the supernatant was passed through 5 mL HisTrap columns (Cytiva, cat. no. 17524802)
473 equilibrated with 50mM Tris pH 8.0, 300 mM NaCl and 20 mM imidazole (Thermo Fisher
474 Scientific, cat. no. O3196-500). His-tagged protein bound HisTrap columns were washed with 10
475 column volumes (CV) wash buffer (50 mM Tris, pH 8.0, 300 mM NaCl and 20 mM imidazole).
476 Bound his-tagged protein were eluted using gradient flow at slope of 10% - 100% elution buffer
477 (50 mM Tris pH 8.0, 300 mM NaCl and 250 mM imidazole) in 5 CV. Fractions containing the
478 eluted protein were assessed by SDS-PAGE gel. SDS-PAGE analyses were performed by
479 incubating 2 μL of purified protein elution in NuPAGE LDS Sample Buffer (Thermo Fisher
480 Scientific) at 70°C for 10 minutes. Bis-Tris NuPAGE 4 to 12% gels were placed into gel box filled
481 with NuPAGE MES SDS Buffer. Samples were loaded and gels run at constant volt of 170 mV for
482 40 minutes, followed by staining using Coomassie Blue gel stain. Fractions were pooled based

on level of purity, concentrated, and buffer exchanged into PBS. The samples were then filtered through a 0.2 μ m filter and run over a size-exclusion column on AKTA Pure FPLC-based size-exclusion chromatography HiLoad 16/600 Superdex 200 pg column (Cytiva, cat. no. 28989335) equilibrated in PBS. Purity and final analysis were performed using SDS-PAGE gel where 3 μ g purified protein samples were incubated at 70 °C in LDS loading buffer with or without 20 mM dithiothreitol (DTT) reducing agent (Thermo Fisher Scientific, cat. no. R0862). Protein samples were separated by size on NuPAGE Bis-Tris gels as described above. Purified samples were biotinylated using BirA biotin ligase (Avidity Inc.) at 1 μ g of BirA enzyme per 10 nM of protein.

Protein purification from spirulina biomass

The TcdB-binding proteins developed from VHHs 5D (pp1005, batch no. 145), E3 (pp1006, batch no. 147), and 7F (pp1007, batch no. 147), and full-length lysin PlyCD (pp1092, batch no. 025) were expressed in spirulina and purified from spray-dried biomass using Ni-NTA affinity and size-exclusion chromatography 2-step purification protocol. Dried spirulina biomass from strains expressing heterologous proteins were rehydrated at 30 mL/g of biomass in phosphate buffer saline lysis buffer supplemented with protease inhibitor mix of 1 mM phenylmethylsulfonyl fluoride (PMSF), (Thermo Fisher Scientific cat. no. 36978) and Pierce Protease Inhibitor Tablets, EDTA-free, (Thermo Fisher Scientific cat no A32965). Rehydrated and homogenous biomass resuspension were lysed using a high-pressure homogenizer (Microfluidics LM20) at 12,000 psi. The lysates were clarified by centrifugation at 18500g on tabletop Centrifuge (Eppendorf Centrifuge 5810R) for 30 minutes. Supernatant were clarified by filtration using a coffee filter followed by 0.22 μ m membrane filter. Recombinant proteins were purified from the clarified lysate by affinity chromatography as describe above using 5 mL

HisTrap columns (Cytiva, cat. no. 17524802). Hexa-Histidine tagged and HisTrap-purified samples were further refined using AKTA Pure FPLC-based size-exclusion chromatography on HiLoad 16/600 Superdex 200 pg column (Cytiva, cat. no. 28989335). Protein purity and sizing were assayed by size-exclusion chromatography on analytical Superdex 200 increase 10/300 (Cytiva, cat. no. 28990944) where 100 μ L of purified protein at $\sim$ 1 mg/mL was run in PBS. Purified protein samples were also assessed on SDS-PAGE under reducing and non-reducing conditions. 3 μ g protein samples were incubated at 70 $^{\circ}$ C in LDS loading buffer with or without 20 mM dithiothreitol (DTT) reducing agent (Thermo Fisher Scientific, cat. no. R0862). Proteins were determined to be >90% pure and exhibited the expected molecular weight size distribution.

Native gel analysis

To assess the importance of disulfide bonds in pp1005, pp1006, and pp1007 dimer formation, we compared protein migration in native and SDS-PAGE gel under reduced and non-reduced conditions. Native gel analysis was performed following the manufacturer's recommendation (Life Technologies) where 1 μ g of purified protein was mixed with Native Page Sample Buffer supplemented with or without 20 mM DTT. Native page anode buffer was prepared by mixing 50 mL NativePage 20X running buffer and 950 mL single distilled water. Similarly, 1 x NativePage Dark Blue Cathode buffer was prepared by mixing 50 mL NativePage 20 x Running buffer with 50 mL NativePage 20X Cathode Additive and 900 mL single distilled water. Gel boxes were set up on ice and samples were loaded prior to adding running buffer. Inner chamber of gel box was filled with NativePage Dark Blue Cathode buffer while the outer

chamber was filled with NativePage Anode buffer. Gels were run at a constant 150 V for 2.5 hours. Comparable sample preparations were run in SDS-PAGE gel as described above.

Assessment of VHH binding to TcdB fragments with bio-layer interferometry (BLI)

BLI measurements were performed on the Octet RED96 system (ForteBio) using High Precision Streptavidin (SAX) Biosensors (ForteBio). Biosensors were hydrated with phosphate buffer, pH 7.4 at room temperature for 10 minutes on Microplate 96-well flat-bottom (Greiner, 655209). All kinetics experiments were performed at 30 °C temperature with 500 rpm agitation in the kinetics module. Biosensors were dipped into PBS containing wells for 60 seconds prior to antigen loading. Biosensors were loaded with enzymatically biotinylated TcdB antigen fragments at 10 µg/mL in phosphate buffer, pH 7.4 for 200 seconds to achieve ~0.6 – 1 nm response. Loading was quenched by incubating biosensors in 50 µM Biocytin (Sigma Aldrich, 576-19-2) for 60 seconds. Baseline were established by incubating antigen loaded biosensors in kinetics buffer (PBS + 0.02 % Tween 20, 0.1 % BSA, 0.05 % sodium azide) for 300 seconds. After establishing baseline, the rate of association was measured by incubating antigen loaded biosensor tips into 3-fold dilution series of monomeric (pp1239, pp1238, and pp1240 respectively) or dimeric (pp1005, pp1006, and pp1007 respectively) 5D, E3, and 7F constructs. The concentration series used to determine binding kinetics of pp1238 were 93.6 nM, 31.2 nM, 10.4 nM, 3.47 nM, 1.16 nM, and 0.36 nM. Concentrations used to determine binding kinetics of pp1239 were 93.6 nM, 31.2 nM, 10.4 nM, 3.47 nM, 0.38 nM, and 0.38 nM. Concentrations used for pp1240 were 92.2 nM, 30.7 nM, 10.2 nM, 3.41 nM, 1.14 nM and 0.38 nM. pp1005 was assayed at 16.2 nM, 5.39 nM, 1.8 nM, 0.60 nM, 0.20 nM and 0.07 nM. Concentrations used for pp1006 were 16.8 nM, 5.6 nM, 1.87 nM, 0.62 nM, 0.21 nM, and 0.07 nM. pp1007 were assayed

at concentrations of 84.9 nM, 28.3 nM, 9.43 nM, 3.14 nM, 1.05 nM, and 0.35 nM. Analyte bound biosensors were dipped into kinetics buffer for 300 - 600 seconds to measure rate of dissociation. Kinetic analysis was performed using the HT 11.1.1.39 Data Analysis module (ForteBio). Results were double referenced. Both association and dissociation steps were used in 1:1 binding model global data fitting model.

Toxin-neutralization assay

Toxin-neutralization assays using TcdB1.1, TcdB2.1, TcdB3.1 and TcdB5.1 were performed using the Agilent xCELLigence RTCA MP. This system measures cell adherence via biosensors in the bottom of a 96-well plate by applying a small electric current across microelectrodes. A unitless measurement called cell index (CI) was then calculated based on changes in electrode impedance. Because adherent cells increase electrode impedance, toxin induced cell rounding can be determined from changes in CI.

Agilent 96-well E-plates were seeded with 100 μ L/well of 1×10^5 cells/mL in DMEM culture medium with 10% FBS and 1% Pen/Strep and allowed to rest for 30 minutes before going into an xCELLigence real-time cell analyzer in a Caron CO₂ 37 °C incubator to run overnight. CI was monitored every 15 minutes for 20 to 24 hours. TcdB was serially diluted in culture medium in a 96-well set up plate. The VHH homodimer, lysin, and bezlotoxumab samples were diluted in medium and then added to the serially diluted toxin. The TcdB/protein mixture was incubated at 37 °C for 1 hour. After 1 hour, media was carefully removed from the cells seeded onto the E-plates and 100 μ L of the VHH homodimer and toxin mixture was added to the cells in each well. CI was monitored every 15 minutes for at least 24 hours. Each run included a TcdB only

standard curve as a positive control and cells only as a negative control. VHH homodimer was also tested in the absence of TcdB to assess any effects of VHH alone on CI. At the 24-hour time point after treatment, the CI measurements for each well were normalized by setting the CI to 1 at the last reading taken before treatment was added. The normalized cell index (nCI) was plotted using Prism and a nonlinear regression analysis was performed to calculate the IC₅₀ of TcdB under treatments.

TcdB's effect on cell adhesion and inhibition of TcdB by anti-TcdB VHH homodimers

Cell adhesion assay data were imported, normalized, and analyzed using R (version 4.1.1 The R Foundation for Statistical Computing). Nonlinear regression was performed using the *minpack.lm* routine in the *nlsLM* package, which implements the Levenberg-Marquardt algorithm to perform bounded minimization⁶⁵.

Lysin activity assay

The lytic activity of pp1092 against *Bacillus subtilis* SL4 was assessed based upon previously described methods with modifications to adapt the assay to a 96-well plate format⁴². Mid-log phase grown SL4 were grown in BHI (37 °C, 250 rpm) harvested and washed twice in PBS by centrifugation (room temperature, 6,000g, 10 minutes). Bacteria were then combined with 1ug digested or undigested pp1092 in a final volume of 200 µL (OD = 1.0) and then turbidity reduction was measured over time (37 °C, shaking). Each unknown was fit along with 5 3-fold serial dilutions of undigested standards using a modified 5-parameter nonlinear logistic model. The time of 50% OD₆₀₀, TC₅₀, was estimated as a 4-parameter nonlinear logistic model with lysin concentration as the independent variable. For the standards, this concentration is known. For

the unknown sample, this value is estimated. All results were expressed as the mean $\pm$ standard deviation as calculated by Excel software (v16.48, Renton, WA). Standard curves were determined by linear regression analysis by Excel software (v16.48, Renton, WA). EC₅₀ values were calculated using GraphPad Prism (version number, GraphPad Software Inc, San Diego, CA). For comparison of spirulina-expressed and *E. coli*-expressed PlyCD, *C. difficile* strain ATCC 43255 was grown to mid-log phase (optical density at 600 nm (OD_{600nm}) = 0.5) at 37 °C under anaerobic conditions in reduced Brain Heart Infusion Medium (BD Biosciences) supplemented with 0.5% (wt/vol) yeast extract and 0.1% (wt/vol) L-cysteine. The bacteria were harvested at 4,000 rpm for 15 minutes, washed and resuspended to an OD_{600nm} of 2.0 in 50 mM sodium phosphate, pH 7.0. Using a 96-well nontreated polystyrene microtiter plate (Corning), 100 μ L of buffer with or without (untreated controls) lysin at initial concentrations ranging from 4 to 256 μ g/mL were added to individual wells. Following the addition of 100 μ L bacteria, the OD_{600nm} of each sample was measured every 60 seconds for a total of 15 minutes at 37°C using a SpectraMax M5 microplate reader (Molecular Devices). All data represents the average of triplicate experiments.

VHH homodimer protease digestion for ELISA and neutralization assays

In vitro protease digestions of anti-*C. difficile* VHH homodimers were prepared in a matrix of soluble lysate from wild-type spirulina (SP003) in Bis-Tris buffer pH 6.0 and treated with trypsin, chymotrypsin, or elastase. VHH homodimers in PBS at were diluted in SP3 lysate, Bis-Tris buffer, and protease so that the final concentrations were VHH homodimer, 20 mg/mL of SP3 lysate, and a “fed” or “fasted” amount of protease. This level of SP3 lysate and VHH homodimer

simulates a expression level of 1% in spirulina (by dry weight). For trypsin and chymotrypsin, the “fed” and “fasted” levels of protease in the digestion reactions were 0.1 and 0.01 mg/mL, while for elastase, 5 and 0.5 µg/mL were used respectively. Samples were incubated at 37 °C for 1 hour with shaking at 900 rpm in an Eppendorf ThermoMixer. At the end of the incubation, reactions were quenched with an equal volume of stop buffer containing 2 mM PMSF and 2x Pierce protease inhibitor minitabket (Thermo Fisher) in PBS; elastase digests were additionally neutralized with a 1 mM elastase inhibitor III. Samples were kept on ice until preparation for ELISA or cell rounding assay. The final concentration of each VHH homodimer after protease neutralization was 0.1 mg/mL.

VHH homodimer quantitation by ELISA

Digested VHH homodimers were analyzed for binding activity via ELISA using TcdB. Thermo Scientific Nunc ELISA plates were coated with 100 µL/well of inactive TcdB at 2 µg/mL in CBC binding buffer and incubated overnight at 4 °C. Plates were washed 3 times with PBS supplemented with 0.05% Tween-20 (PBS-tw), then blocked for 1 hour with 300 µL/well of block (PB-tw supplemented with 5% BLOTTO). Dilutions of undigested and digested VHH homodimer were prepared in a low-binding 96-well plate by serially diluting with block. 6 dilutions of each undigested VHH homodimer were prepared for a standard curve. Two dilutions of each protease digested sample were prepared for quantification by interpolation from the standard curve. A 100 µL sample of each VHH homodimer was added to ELISA plate wells and incubated for 1 hour at room temperature on a plate shaker. The plates were washed as before. An HRP-conjugated anti-camelid VHH antibody cocktail (Genscript) was diluted 50,000-fold in block and 100 µL/well was added to the ELISA plates for 50 minutes with shaking.

Plates were washed 2 times with PBS-tw and 1 time with PBS. Finally, 100 μ L/well of TMB Ultra ELISA substrate (Thermo Fisher) was added to each well. The plates were developed in the dark at room temperature on a plate shaker for 10 minutes before being quenched with 50 μ L of 2.5 M sulfuric acid, and the absorbance was read at 450 nm with a Spectra Max M5 plate reader. All digestion reactions were in duplicate, in 3 independent experiments.

Calculation of VHH homodimer protease resistance

The concentration of VHH homodimer in the protease-digested samples was calculated by interpolating the concentration from standard curves generated with undigested VHH homodimer. The percentage of remaining VHH homodimer activity after digestion was calculated by dividing the interpolated value by the amount present if no VHH homodimer were digested. Each replicate was calculated separately, and the average and standard deviation were determined across all replicates.

Toxin neutralization assay of digested VHH homodimer s

Chymotrypsin-digested pp1005, pp1006, and pp1007 were assessed for toxin-neutralizing activity as described. The final concentration of pp1005, pp1006, and pp1007 was 0.05 nM, 5 nM, and 5 nM respectively and the TcdB was 1:5 serially diluted starting at 250 pM.

Lysin digestion conditions

In digestion buffer (20 mM Tris, 150 mM NaCl, 3 mM CaCl_2 at pH 6.4) 0.2 μ g/ μ L protein was mixed with proteases trypsin (Promega sequencing grade), chymotrypsin (BD), and elastase (BD) at 0.1 μ g/ μ L or 0.01 μ g/ μ L and incubated for 1 hour at 37 °C (200 rpm shaking). Reactions were immediately quenched by addition of 1 mM PMSF and Protease Inhibitor Mini Tablets,

EDTA-free (Thermo Scientific, Pierce). For samples with elastase, 1 mM elastase inhibitor III (Milipore Sigma) was added as well. To visualize digestion, 2 µg of digested and undigested proteins were displayed a gradient BOLT-PAGE gel at 200 V for 20 minutes and then stained with Coomassie.

Densitometry

Pixel intensity of Coomassie stained proteins were measured using ImageJ software, normalizing all bands to the undigested band for each condition after subtracting background pixel intensity of an identically sized bounding box.

Preparation of *C. difficile* spores for mouse study

C. difficile strain M7404, a ribotype 027 clinical isolate from Canada, was cultured on HIS-T agar (3.7 % (w/v) heart infusion (HI) (Oxoid), 0.5 % (w/v) yeast extract, 1.5 % (w/v) agar supplemented with 0.375 % (w/v) glucose, 0.1 % (w/v) L-cysteine and 0.1 % (w/v) sodium taurocholate (New Zealand Pharmaceuticals)) in an anaerobic chamber (Don Whitley Scientific) with an atmosphere of 10 % H₂, 10 % CO₂ and 80 % N₂ at 37 °C. To prepare spores, strains were sub-cultured on HIS-T agar and inoculated into 500 mL tryptone yeast (TY) broth (3.0% tryptone, 2.0% yeast extract, and 0.1% sodium thioglycolate) and grown anaerobically at 37 °C for 10 days. Spores were harvested by centrifugation at 10,000g for 20 minutes at 4 °C and washed 5 times with chilled distilled water prior to heat-shocking at 65 °C for 20 minutes and quantified by plating onto HIS-T agar. Spores were adjusted to 10⁶ CFU/mL in PBS containing 0.05 % Tween-80 in preparation for mouse infections.

Mouse study design

Animal handling and experimentation was performed in accordance with Victorian State Government regulations and approved by the Monash University Animal Ethics Committee (Monash University AEC no. 21731). Male C57BL/6J mice, 6-7 weeks old, were assigned to either a spirulina therapeutic treatment group, positive control (vancomycin-treated), or negative control (wild-type spirulina-treated) group and caged in groups of 5. To induce susceptibility to *C. difficile*, the animals were pre-treated with an antibiotic cocktail in the drinking water containing kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), vancomycin (0.045 mg/mL), and cefaclor (0.3 mg/mL) for 7 days, followed by 4 days of cefaclor (0.3 mg/mL) alone. Starting 1 day prior to infection (day - 1), the animals were orally gavaged with 200 µL of treatment once daily for the first two days (peak of infection) and then 100 µL once daily for the remainder of the study. Mice were infected with 10⁵ spores/mouse by oral gavage.

The spirulina treatments and controls were resuspended in PBS (5% w/v) by mixing up and down using an 18G blunt needle and 1 mL syringe for 2.5 minutes and administered within 10 minutes of preparation. Vancomycin was diluted to 6.25 mg/mL prior to administration. On the day of *C. difficile* infection (day 0), antibiotic pre-treatment was ceased, the mice were switched to plain water, and infection was initiated 3 hours after the treatment dose. Mice were monitored daily for signs of infection, including weight loss, and were euthanized upon losing 15% body weight, if they reached pre-defined clinical endpoints as required by ethics, or at the end of the study (day 4). As a surrogate assessment of colonization, fecal samples collected at 24 hours post-infection and then again at the time of euthanasia were analyzed to enumerate

the shedding of *C. difficile* spores. Subjects treated with a spirulina therapeutic cocktail were compared to the cohort administered wild-type spirulina.

Quantification of spore shedding from mouse feces

Fecal pellets were collected 24 hours post-infection and at time of euthanasia, resuspended in PBS (100 mg/mL), heat shocked (30 minutes, 65 °C) and plated for spore enumeration onto HIS agar supplemented with 0.1% (w/v) L-cysteine, 0.1% (w/v) sodium taurocholate, 0.375% (w/v) glucose, 250 µg/mL D-cycloserine, 8 µg/mL cefoxitin, and 10 µg/mL erythromycin.

Analysis of colonization data was performed by fitting linear models of log₁₀ cfu/g data per landmark time with treatment as a (potential) explanatory factor. A coefficient for wild-type spirulina describes the mean log₁₀ cfu/g for that treatment, and coefficients for each other treatment describes the difference in mean log₁₀ cfu/g for that treatment relative to wild-type spirulina. Standard errors of estimates were reported, along with t-statistics and 2-tailed p-value corresponding to the t-statistic with $\alpha=0.05$.

Analysis of clinical outcomes—mouse study

Analysis of maximum weight loss data was performed by fitting linear models of maximum weight loss data with treatment as a (potential) explanatory factor. A coefficient for wild-type spirulina describes the mean maximum weight loss for that treatment, and coefficients for each other treatment describes the difference in mean loss for that treatment relative to wild-type spirulina. Standard errors of estimates were reported, along with t-statistics and 2-tailed p-value corresponding to the t-statistic with $\alpha=0.05$.

Analysis of survival data was performed on tabulated number at risk and number of events at each time. Data was compared for each treatment to wild-type spirulina results and a P value was computed using the log-rank statistic for each comparison.

Hamster study challenge agent preparation and confirmation

C. difficile strain R20291 (NAP1/BI/027) spore stock was titrated on TCCFA plates after a 30-minute incubation at 65 °C and determined to be 5 x 10⁵ cfu/mL in a volume of 5 mL of PBS. This spore stock was diluted to 15,000 spores/50 µL and further diluted in an equal volume of sterile PBS containing 5% sucrose to facilitate administration of 15,000 spores in 100 µL per animal.

To verify challenge doses, 100 µL of inoculum were serially diluted (10-fold dilutions) and plated onto TCCFA plates. After incubation of the plates under anaerobic conditions at 37 °C for 48 hours, the number of colonies were enumerated, and the doses calculated based on these numbers. The inoculum was measured to be 15,000 cfu.

Hamster study design

Hamsters (Envigo, Indianapolis, IN) were visibly checked upon arrival and allowed to acclimate to their new environment for 2 days. Each animal was given a physical exam and randomly assigned (adjusting only to distribute body weight evenly) to 1 group. They were fed *ad libitum* with sterilized 2018S Teklad Global 18% Rodent Diet (Envigo, Indianapolis, IN) and sterile water provided in standard bottles with sippers during the study. To sensitize the animals to *C. difficile* infection, clindamycin, 15 mg/mL in saline, was administered intraperitoneally at 30 mg/kg, once a day, for 3 consecutive days. During clindamycin treatment, cage bottoms were changed

737 daily for 3 consecutive days beginning on the first day to prevent bacterial recolonization from
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Figure 1. *Spirulina* strain engineering and characterization. A) Diagram of modified anti-*C. difficile* TdcB VHHs 5D, E3, 7F, PlyCD, PlyCD₁₋₁₇₄, and 5HVZ-mediated dimer formation. B) A schematic diagram of the genomic integration locus (WP_006618409.1). PCR was performed with indicated primers to analyze the segregation and specificity of integration for each strain. PCR products with primers MP1/MP2 showed bands of predicted sizes in all strains, and absence of the parental alleles, suggesting the segregation to homozygosity was complete. The primer pairs for amplification of the left homology arm (LHA) and right homology arm (RHA) include one priming site (MP3 and MP6) that is only present in the *Spirulina* genome at the target locus and absent from the donor plasmid. PCR amplification of the LHA and RHA with MP3/MP4 and MP5/MP6, respectively, demonstrated that genomic integration was site-specific. The DNA sequences from all PCR products were verified to match expectations. C) Copy numbers of the genes of interest (GOIs) were determined with next-generation

914 sequencing by comparing reads of the GOIs to ten randomly selected native spirulina genes.
915 Dotted line indicates range in which the relative copy numbers were considered equivalent to
916 one.
917

Figure 2. Characterization of spirulina-expressed anti-TcdB VHs and lysins. A) Chromatograms of pp1005, pp1006, pp1007, pp1092, and pp1093 extracted from spirulina biomass and detected under reducing conditions using a capillary electrophoresis-based immunoassay. Soluble protein, as a percentage of washed dry weight, is indicated on each graph. B) Spirulina expressed and purified pp1005, pp1006, and pp1007 in aqueous solution (PBS, pH 7.4) assayed on a Superdex 200 10/300 GL FPLC column. Grey peaks correspond to molecular size standards. The monomeric and dimeric portions of the VHs are indicated. Disulfide bond formation between monomers was assessed using denaturing gel electrophoresis under non-reducing and reducing (50 mM DTT) conditions. C) Native gel electrophoresis analysis of pp1005 (1, 4), pp1006 (2, 5), and pp1007 (3, 6) under non-reducing or reducing (50 mM DTT) conditions. In the presence of reducing agent the dimeric state of the VHs was mostly preserved.

Figure 3. Binding affinities and toxin neutralizing activity of TcdB VHHs. A) BLI binding sensorgrams for analysis of binding kinetic for anti-TcdB VHHs as monomers (pp1239, pp1238, and pp1240) and dimers (pp1005, pp1006, and pp1007). B) The rate constants k_a and k_d were calculated from concentration-dependent responses. The apparent equilibrium binding constant (K_D) for each was determined from the rate of association (k_a), and rate of dissociation (k_d). Values of K_D , k_a and k_d are indicated in the table (M, monomer; D, dimer).

Figure 4. Comparative neutralization of the five major TcdB types by LMN-201 and bezlotoxumab. A) Neutralizing activity of pp1005, pp1006, pp1007, and the triple cocktail on toxin types 1.1, 2.1, 3.1 and 5.1. 1.5 nM of each dimeric VHH or the cocktail of all three dimeric VHHs (0.5 nM of each) was tested against serially diluted TcdB with Vero cells on the RTCA system. As comparison, 3 nM of bezlotoxumab was similarly evaluated. To rule out interference, the effect of 1 nM lysin on VHH cocktail efficacy was assessed. B) IC_{50} of TcdB under treatment was calculated from the data shown in A and compared.

Figure 5. Characterization of spirulina-expressed anti-*C. difficile* lysin. A) A chromatogram of pp1092 after total protein extraction (black) and soluble protein extraction (red). More than 75% of the lysin protein is soluble. B) Spirulina-expressed, purified lysins, pp1092 and pp1093, in aqueous solution (1xPBS, pH 7.4) evaluated by size-exclusion chromatography (Superdex 200 10/300 GL FPLC column). Purified protein samples were also assessed using denaturing gel electrophoresis under non-reducing and reducing (50 mM DTT) conditions. C) Activity of spirulina extracts (wild-type (WT) expressing no lysin, SP1286 expressing pp1093, and SP1287 expressing pp1092) prepared at 2 mg/mL were compared by measuring the time to reduce turbidity of living *B. subtilis* SL4 cultures by 50%.

Figure 6. Stability of anti-TcdB VHHs to gastrointestinal proteases. A) The percentage of TcdB-binding activity of pp1005, pp1006, and pp1007 measured by ELISA after digestion with trypsin (T), chymotrypsin (C), and elastase (E). Two concentrations of each protease were tested to simulate either “fed” (higher concentration) or “fasted” (lower concentration) conditions. Data represent the average of six independent replicates, the error bars representing the standard deviation. B) TcdB-neutralizing activity of protease digested pp1005, pp1006, and pp1007. VHH homodimers treated with chymotrypsin for either 10 or 60 minutes were compared in a cell-rounding assay with the corresponding undigested protein. Each was diluted to a fixed concentration (pp1005 was 0.05 nM, pp1006 was 5 nM, and pp1007 was 5 nM) and incubated with serially diluted TcdB-10463. Each mixture was assayed for its effect on cell adhesion. After digestion all exhibited neutralizing activities similar to undigested controls.

Figure 7. Stability of anti-*C. difficile* lysin to gastrointestinal proteases. A) Full-length lysin (pp1093, 37 kDa), its catalytic subdomain (pp1092, 25 kDa), and a protease sensitive VHH (pp555, 55 kDa) were digested with trypsin (T), chymotrypsin (C), and elastase (E) at pH 6.4 for 1 hour at 37 °C, quenched with protease inhibitors, the products separated by size using PAGE, and peptides visualized by Coomassie staining. Mock digestions without target proteins were performed to visualize the contribution by the proteases and quenching reagents. B) The image in A (representative of six independent digestions) was quantified by densitometry with error expressed as SEM. C) Activities of pp1092 digests were measured by determining the time to reduce the turbidity of a culture of live *B. subtilis* SL4 by 50%. This value was then plotted against a standard curve of undigested reference lysin to determine the activities of the digested samples compared to undigested reference lysin. The dotted line represents the activity of lysin when undigested. Error bars represent the standard error calculated from three technical replicates.

Figure 8. Mouse challenge study. Shown are A) fecal spore counts 24 hours after infection and at the time of euthanasia; B) maximum weight loss for individuals in each group; and C) Kaplan-Meier representation of survival. Individuals were euthanized upon losing $\geq 15\%$ of their initial weight. Individuals in panels A and B are represented as a point and the group median, interquartile range (IQR) and $1.5 \times \text{IQR}$ as boxplots. A) $\log_{10}$ fecal shedding data were fit with a linear model with group as a predictor per time point. Treatment group is a significant predictor ($p < 0.05$) of shedding relative to the wild-type spirulina group (control/intercept) for Cocktail 1 and vancomycin. Cocktail 2 fails significance testing ($p \sim 0.1$ and $p \sim 0.25$). Statistic reports the t ratio based on a Student t reference distribution, and p-value reports the two-tailed p-value corresponding to the t ratio based on the Student t reference distribution. B) Maximum animal

weight loss was fit with a linear model with treatment as predictors of weight loss. Treatment group is a significant predictor ($p < 0.05$) of animal weight loss relative to the wild-type spirulina group (control). C) Kaplan-Meier survival curves for mice infected with 10^5 *C. difficile* spores and treated with vancomycin (orange, $n=10$), wild-type spirulina (black, $n=5$), spirulina therapeutic Cocktail 1 (teal, $n=5$) or Cocktail 2 (blue, $n=7$). Survival rates were compared to treatment with wild-type spirulina by log-rank test. Significance was observed with vancomycin treatment ($p=0.0068$), borderline significance was observed with spirulina Cocktail 1 ($p=0.080$) and Cocktail 2 ($p=0.14$) treatment.

Figure 9. Hamster challenge trial. A) The Lumen therapeutic prolonged survival times when compared to the untreated and group administered wild-type spirulina. Clindamycin-treated Golden Syrian hamsters were administered phosphate-buffered saline, wild-type spirulina, or the Lumen therapeutic two times a day starting one day before infection with *C. difficile* (15,000 spores) and continuing until they reached their humane endpoints or the end of the study (14 days post-infection). The mean survival times were compared using a Mann-Whitney test with continuity correction, 90% confidence level, one-sided test. B) Lumen therapeutic decreased *C. difficile* cecal colonization. Cecal contents were collected after animals reached their humane endpoints or the termination date. *C. difficile* colony counts were quantified by comparing RT-PCR measurements of extracted DNA from these samples as compared to a standard curve generated by DNA extracted from vegetative *C. difficile* cells. Fewer *C. difficile* cells colonized the cecum in hamsters treated with the Lumen therapeutic than in wild-type spirulina-treated or untreated groups. Wilcoxon rank sum test (Mann-Whitney test) with continuity correction, 90% confidence level, one-sided test.

Parameter	Estimate	Std. error	p-value	2.5% CI	97.5% CI
TcdB IC ₅₀ (fM)	1.268	6.57E-05	2.24E-52	0.001138	0.001397
Hill ^{TcdB}	1.495	0.07091	1.35E-58	1.355	1.634
EC ₅₀ ^{pp1005} (pM)	21.44	0.002423	1.28E-16	0.01667	0.02622
Hill ^{pp1005} (-)	1.813	0.1245	1.12E-35	1.567	2.058
E _{max} ^{pp1005} (fold)	45.25	3.076	3.20E-36	39.2	51.31
EC ₅₀ ^{pp1006} (pM)	54.13	0.003761	4.25E-35	0.04673	0.06154
EC ₅₀ ^{pp1007} (pM)	69.26	0.004852	1.11E-34	0.05971	0.07882
Bottom (assay units)	-0.01326	0.006172	0.0326	-0.02541	-0.001107
Top (assay units)	0.9289	0.01145	3.39E-189	0.9064	0.9515

Table 1. EC₅₀ values for pp1005, pp1006, and pp1007. *Independent* model estimates from analysis of the overall data set. TcdB1 acts as an inhibitor of cellular adhesion with femtomolar potency (IC₅₀, H). pp1005, pp1006, and pp1007 act to inhibit TcdB1 activity, shifting TcdB1 potency 2-fold at 10's of picomolar potency (EC₅₀^{pp1005}, EC₅₀^{pp1006}, EC₅₀^{pp1007}). pp1005 activity saturates at around 50-fold (E_{max}^{pp1005}, Hill^{pp1005}) while the somewhat less potent pp1006 and pp1007 do not saturate.